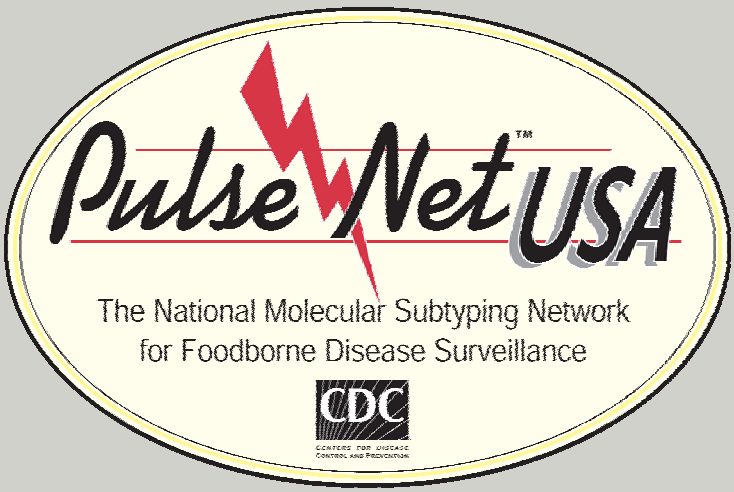
PulseNet Quality Assurance/Quality Control (QA/QC) Manual



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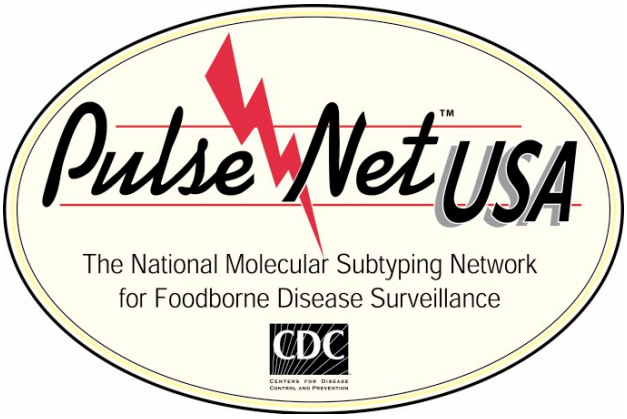

Quality Assurance / Quality Control Manual for the Standardized Pulsed-Field Gel Electrophoresis Technique Used by CDC PulseNet Laboratories Foodborne Disease Surveillance

(Version 2.0 for the Centers for Disease Control and Prevention)

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Send all corrections, comments and questions to
PFGE@CDC.GOV

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Quality Assurance Standards for Pulsed-Field Gel Electrophoresis (PFGE) Testing for the CDC PulseNet Laboratories

I. PREFACE

The objective of this document is to provide CDC laboratories with a set of quality control and quality assurance standards to be followed when subtyping foodborne bacterial pathogens with any of the approved standardized protocols. Strict adherence to standardized procedures is essential to the operation of PulseNet and to ensure that the data submitted to the PFGE National database is of the highest quality possible. All PulseNet CDC laboratories must comply with the following quality assurance standards in order to continue to participate in PulseNet.

The PulseNet USA Task Force is committed to assisting all PulseNet participating laboratories (PulseNet USA and PulseNet International) in achieving the highest quality of data before submitting patterns to the databases in the respective countries or regions. Part of that commitment consists of the development and implementation of standardized laboratory protocols and software analysis parameters that would enable the exchange and comparison of molecular fingerprinting data between laboratories. It is in this context that the PulseNet Task Force presents this document as a template to assist all PulseNet USA and PulseNet International participating laboratories in the development of their own QA/QC manual and program. The standards presented herein were established specifically for PulseNet laboratories at CDC with the understanding that most of the following standards will apply, within varying degrees, to all laboratories participating in PulseNet. We encourage all PulseNet laboratories to adopt these standards whenever possible and to modify them in a manner consistent with the internal policies or guidelines established by their institution, state or country.

Effective Date: These standards shall take effect May 9, 2005.

II. INTRODUCTION

This document consists of definitions and standards as they apply to the protocols and procedures used in PulseNet USA. The standards are quality assurance measures that place specific requirements on CDC laboratories conducting PFGE analysis with the purpose of submitting DNA fingerprint patterns/gel images to the PulseNet USA National database(s). Additional or equivalent measures not outlined in this document may also meet the standard if submitted and approved by the PulseNet USA Task Force of the Foodborne and Diarrheal Diseases Branch at the Centers for Disease Control and Prevention.

This document complies with Good Laboratory Practices (GLPs: 21 CFR Part 58) for conducting nonclinical laboratory studies, which are regulated by the Food and Drug Administration.

This document also satisfies all requirements for the International standard: ISO/DIS 17025 (general requirements for the competence of testing and calibration laboratories).

Molecular subtyping by PFGE is used for epidemiology and surveillance purposes and the results are not used for patient diagnosis or treatment. Thus, these procedures are not subject to the Clinical Laboratories Improvement Amendment (CLIA), 1988. However, every effort has been made to ensure that this document meets CLIA'88 regulations wherever possible. Laboratories that report PFGE data directly to physicians, hospitals, etc. must ensure that they are fully compliant with all CLIA'88 regulations.

The PulseNet USA system encompasses laboratories from the Centers for Disease Control and Prevention (CDC), Food and Drug Administration (FDA), United States Department of Agriculture (USDA), State Public Health Departments, and other County and City Public Health Departments. Certain State Health Departments have been designated as Area Laboratories. The association of these elements within PulseNet USA is indicated in the Organization Chart in Appendix A. A map of the PulseNet USA Area Lab regions can be found in Appendix B.

The standardized protocols developed for use by PulseNet laboratories are available from CDC (E-mail: pfge@cdc.gov) and are provided within the training course manual "Standardized Molecular Subtyping of Foodborne Bacterial Pathogens by Pulsed-Field Gel Electrophoresis." These protocols also are included in the attached document containing the standard operating procedures (SOPs). In addition, the protocols can be found on the PulseNet USA Listserv and on the PulseNet USA website.

III. OBJECTIVE

To institute a quality assurance system to ensure the quality and integrity of the results obtained with the standardized PFGE techniques used to subtype foodborne bacterial pathogens.

IV. SCOPE

The standards describe the quality assurance requirements that all the CDC PulseNet laboratories, defined herein as a facility at CDC in which PFGE analysis is performed for the purpose of identifying clusters of foodborne illness causing bacteria, must follow to ensure the quality and reproducibility of the data.

V. DEFINITIONS

The following terms are defined as used in these standards:

1. **Administrative review** is an evaluation by the PulseNet USA Task Force and/or the PulseNet USA Steering Committee of the report and supporting documentation for consistency with laboratory policies and editorial correctness.

2. **Analyst** is an individual who conducts and/or directs the analysis of all test samples, interprets data, and reaches conclusions.

3. **Calibration** is the set of operations that establishes, under specified conditions, the relationship between values indicated by a measuring instrument, system, or material, and the corresponding known values of that measurement.

4. **Certification set** is a group of well-characterized strains of a specific organism that are part of the PulseNet USA certification process. Individuals wishing to become PulseNet USA-certified must perform PFGE subtyping of these isolates using the appropriate standardized protocol and/or analyze a TIFF image of these isolates. The certification set is available from PulseNet USA Task Force upon request.

5. **Clearance** is the process of review by CDC of all manuscripts, presentations, etc. that have a CDC employee or contractor as an author.

6. **Laboratory** is a facility in which sample testing, including PFGE analysis, is performed.

7. **Laboratory support personnel** are individual(s) who perform laboratory duties but do not analyze PFGE results.

8. **PFGE proficiency test sample** is biological material whose DNA type has been previously characterized by PFGE and which is used to monitor the quality performance of a laboratory or an individual.

9. **Proficiency testing** is a quality assurance measure used to monitor performance and identify areas in which improvement may be needed. Proficiency tests may be classified as:

   (a) Internal proficiency test is one prepared and administered by the **laboratory.**
   (b) External proficiency test (blind) is one that is prepared and administered by the PulseNet USA Task Force.

10. **Pulsed-field gel electrophoresis (PFGE)** is a DNA fingerprinting technique that allows the separation of large DNA fragments (>30 kb) by applying an alternating electric field between spatially distinct pairs of electrodes.

11. **PulseNet International Steering Committee** is made up of coordinating officials of the international PulseNet networks. The committee is chaired by the Chief, Laboratory Unit of the Foodborne and Diarrheal Diseases Branch at CDC, Atlanta. This Steering Committee establishes the principles and procedures by which information exchanges between the PulseNet Networks shall take place. It sets up the rules for sharing of protocols, standards and other material necessary to obtain comparable DNA "fingerprint" patterns; sharing of human resources to perform typing and developing and maintaining methods, protocols, and strain collections; and sharing tasks for the future development of PulseNet International.

12. **PulseNet USA** is a network of public health laboratories in the United States that perform DNA "fingerprinting," using PFGE, on bacteria that may be causative agents of

foodborne disease. The network permits rapid comparison of these fingerprint patterns through an electronic database maintained at the CDC.

13. **PulseNet USA Certification** is the process by which a person demonstrates his or her ability to produce a high-quality gel using the PulseNet USA standardized protocols and/or the ability to analyze a gel using BioNumerics and the PulseNet USA customized scripts. A person must be certified for each pathogen for which he/she wishes to submit PFGE patterns to the PulseNet USA National Database(s).

14. **PulseNet USA Steering Committee** guides the expansion, improvement, and evaluation of the PulseNet USA program. Activities of the PulseNet USA network are coordinated by the PulseNet USA Steering Committee. The PulseNet USA Steering Committee is chaired by the chief of the PulseNet USA unit of the Foodborne and Diarrheal Diseases Branch at CDC and is comprised of participants from state and local PulseNet USA laboratories, FDA, USDA, APHL, and CDC. The Steering Committee meets via conference call several times during the year.

15. **PulseNet USA Task Force** is a group of laboratory and database personnel from CDC. The PulseNet USA Task Force is responsible for the initial review and approval of documents, development and validation of protocols, and other activities related to PulseNet USA.

16. **Quality** refers to freedom from deficiencies.

17. **Quality assurance** refers to all the activities in which the laboratory is engaged to ensure that information generated by the laboratory is correct. These activities are to be implemented in a systematic way to demonstrate that a product or service meets specified requirements for quality assurance.

18. **Quality assurance program** is the organizational structure, responsibilities, procedures, processes, and resources for implementing quality assurance policy.

19. **Quality control** refers to the process used by the laboratory and other personnel as an aid to meeting the product or service goals.

20. **Quality assurance (QA)/quality control (QC) manual** is a document stating the quality assurance policy, quality assurance program, and quality control practices of an organization.

21. **Real-time PFGE** refers to the processing by PFGE, analysis, and submission of PulseNet USA-tracked isolates to the national databases within 48 hours of their receipt by the participating lab. The PulseNet USA Task Force and PulseNet USA Steering Committee recommend that all isolates of *Listeria* and *E. coli* O157:H7 be PFGE subtyped in "real-time."

22. **Restriction fragment length polymorphism (RFLP)** is generated by cleavage of DNA with a specific restriction enzyme, and the resulting variation is due to restriction site polymorphism contained within the DNA used in the analysis.

23. **Review** is an evaluation of documentation for consistency, accuracy, and completeness.

24. **Standard Operating Procedures (SOPs)** are the written procedures that detail all aspects of work in the laboratory or with the database(s). They are written to assure good laboratory practices are followed in the laboratory and in the analysis of data.

25. **Technical supervisor** (or equivalent position or title as designated by the laboratory system) is the individual who is accountable for the technical operations of the laboratory.

26. **Technical review** is an evaluation of reports, notes, data, and other documents to ensure an appropriate and sufficient basis for the scientific conclusions. This review is conducted by a second qualified individual.

27. **Technician** is an individual who performs analytical techniques on test samples under the supervision of a qualified examiner/analyst and/or performs PFGE analysis on samples for inclusion in a database. Technicians do not evaluate or reach conclusions on typing results or prepare final reports unless they are also acting as the technical manager or leader.

28. **Validation** is a process by which a procedure is evaluated to determine its efficacy and reliability, and includes:

    (a) Developmental validation: the acquisition of test data and determination of conditions and limitations of a new or novel DNA methodology for use on suspected outbreak samples.

    (b) Internal validation: an accumulation of test data within the laboratory to demonstrate that established methods and procedures are performed as expected in the laboratory.

    (c) External validation: an accumulation of test data based on the isolates or images (blinded) provided to the PulseNet USA laboratories by CDC. External validation is required in order to demonstrate that established methods and procedures perform as expected.

VI. QUALITY ASSURANCE PROGRAM

Each PulseNet participating laboratory shall establish and maintain a documented quality assurance program that is appropriate to all the testing activities associated with PFGE or other PulseNet-related analyses.

4.1 The quality assurance/quality control manual shall address the following:

(A) Goals and objectives
(B) Organization and management
(C) Policy for performing PFGE
(D) Personnel training
(E) Specimens
(F) Analytical procedures
(G) Documentation
(H) Equipment: calibration and maintenance
(I) Certification
(J) Proficiency Testing (PT)
(K) Review
(L) Changes to standardized PFGE protocol
(M) Validation of new methods
(N) Safety

A. GOALS AND OBJECTIVES

To standardize the performance of PulseNet USA activities in all CDC participating laboratories.

B. ORGANIZATION AND MANAGEMENT

STANDARD B.1

The laboratory shall:

(a) Have a managerial staff with the authority and resources needed to discharge their duties and meet the requirements of the standards in this document.

(b) Have a technical manager or leader who is accountable for the technical operations.

(c) Specify and document the responsibility, authority, and interrelation of all personnel who manage, perform, or verify work affecting the validity of the DNA analysis.

C. POLICY FOR PERFORMING PFGE

PFGE is not used for diagnostic purposes (i.e., ordered by a physician on a specific patient). Rather, it is a powerful tool for monitoring and investigating clusters of illness caused by foodborne bacteria of concern to public health. Each laboratory should formulate its own statement of policy as to when PFGE is to be performed. Such policy must be consistent with the "real-time" subtyping guidelines stated in item 21 of the "Definitions" section (section 3) of this document: laboratories must perform real-time PFGE on all *E. coli* O157:H7 and all *Listeria monocytogenes* isolates received. In situations where real-time subtyping of *Salmonella* serotypes, *Shigella*, and *Campylobacter* is not possible, laboratories

must establish a subtyping policy according to the priorities and resources that still allows for the early detection of clusters of these organisms.

STANDARD C.1

At the Centers for Disease Control and Prevention, Foodborne and Diarrheal Disease Branch, PFGE is performed on all isolates 1) submitted by external public health laboratories or government agencies for that purpose, 2) as requested by CDC epidemiologists investigating a potential outbreak, 3) as requested by the PulseNet USA National Database Administration Team for the clarification of PFGE patterns, and 4) for other research purposes.

C.1.1

PFGE needs to be performed in "real-time" to have a significant impact on public health. All isolates of *Listeria monocytogenes* or *E. coli* O157:H7 shall be PFGE subtyped in "real-time."

D. PERSONNEL TRAINING AND CERTIFICATION

OBJECTIVE: To standardize the performance of PulseNet activities in all CDC participating laboratories.

STANDARD D.1

Laboratory personnel shall have the education, training, and experience to carry out required PulseNet USA activities and responsibilities. The laboratory shall maintain records on the relevant qualifications, training, skills, and experience of all technical personnel.

STANDARD D.2

The technical supervisor is responsible for technical problem solving of analytical methods and for the oversight of training, quality assurance, safety, and proficiency testing in the laboratory.

The laboratory supervisor shall be accessible to provide onsite, telephone, or electronic consultation as needed.

STANDARD D.3

At least one member of each CDC PulseNet participating laboratory must be trained by someone approved/recommended by the PulseNet USA Task Force and shall have successfully completed the certification testing for an organism before being allowed access to the on-line databases in accordance with the "Standard Operating Procedure for Training (PNL17 & PND10)," and "Standard Operating Procedure for Certification of PulseNet USA Personnel (PNQ02)."

This person shall be responsible for training additional staff in their respective laboratory.

STANDARD D.4

At least one person from each CDC PulseNet participating laboratory shall attend annual update meetings and regional meetings when they occur.

E. SPECIMENS

Each laboratory shall formulate its own statement of what is an acceptable specimen for PFGE.

STANDARD E.1

PFGE is performed on pure cultures. The primary consideration is that the organism be viable when received. Mixed cultures do not have to be rejected if it is possible to separate the specific organism in question. Isolates may be from human, animal, produce or animal food product, or environmental sources. An isolate for which identifiers have been lost or are in question shall be rejected.

F. ANALYTICAL PROCEDURES

OBJECTIVE: To assure that all reagents and solutions used in the standardized PFGE protocols are properly prepared and controlled in order to maintain a consistent level of quality.

STANDARD F.1

The laboratory shall have and follow written analytical protocols approved by the PulseNet USA Task Force and the PulseNet USA Steering Committee.

F.1.1

The laboratory shall use the standard PFGE protocols as outlined in the Standardized Molecular Subtyping of Foodborne Bacterial Pathogens by Pulsed-Field Gel Electrophoresis manual (or SOPs PNL03 through PNL06 in this manual), for each organism to be analyzed by PFGE with the purpose of submitting gel images of PFGE patterns to the PulseNet USA National Database(s) at CDC.

F.1.2

The procedures shall include reagent preparation, supplies, sample preparation, extraction protocols, equipment requirements, and appropriate controls that are standard for DNA analysis and data interpretation.

STANDARD F.2

The laboratory shall use reagents that are suitable for the methods employed. Each participating laboratory shall be responsible for assuring that reagents and solutions used for PFGE subtyping are properly prepared, controlled, and stored in order to maintain a consistent level of quality. The following standards shall apply to all reagents and solutions used in the standardized PFGE methods.

STANDARD F.3

Good laboratory practices depend upon the use of appropriate chemicals and reagents. An important aspect of laboratory management is the assurance that materials purchased for

testing meet necessary quality control requirements. Stringent specifications for these materials shall be developed, and adherence to the specifications must be mandatory.

F.3.1
The laboratory shall have written procedures for documenting commercial supplies and for the formulation of reagents (PNL01 & PNL02).

F.3.1.1
All commercially available reagents shall have a quality assurance certificate issued by the supplier, a record of which shall be maintained by the laboratory.

F.3.2
Reagents shall be labeled with the identity of the reagent, storage requirements, titer or concentration, the date of preparation or expiration, and the identity of the individual preparing the reagent. Deteriorated or outdated reagents and solutions shall not be used.

F.3.3
Reagent solutions prepared "in-house" shall have their expiration dates established by the laboratory. A program of periodic testing shall be instituted to determine that reagents/solutions have not deteriorated.

F.3.4
When establishing reagent preparation as a laboratory function, the methods used shall be accurately and completely described in writing. There shall be documentation of each lot prepared, including reagent lots and expiration dates, date prepared, initials of personnel preparing reagent and expiration date of prepared reagent.

F.3.5
Reagents shall remain free of contamination, either chemical or microbiological.

F.3.6
Proper physical conditions of storage shall be carefully maintained. Reagents shall be maintained at the temperatures recommended by the manufacturer. Reagents maintained under refrigerated or freezing temperatures, such as proteinase K and restriction enzymes/buffers, shall not be removed from the storage conditions until ready to use. These reagents shall be maintained at the appropriate temperature(s) following manufacturer's guidelines while in use, and shall be returned to proper storage conditions promptly.

F.3.7
The laboratory shall identify critical reagents and evaluate their acceptability prior to use in sample testing. These critical reagents include but are not limited to:

(a) Restriction enzymes/Proteinase K
(b) In-house/Commercial reagents and buffers

(c) *S.* Braenderup size standard culture/all control plugs

STANDARD F.4

The laboratory shall monitor the analytical procedures using appropriate controls and standards.

F.4.1

The following controls shall be used in PFGE analysis:

F.4.1.1

Quantification standards for adjusting cell suspensions (i.e., McFarland standards, etc.).

F.4.1.2

A procedure to monitor the pH or conductivity of buffers (TE, TBE, etc.).

STANDARD F.5

The laboratory shall check its PFGE procedures annually or whenever substantial changes are made to the protocols against an appropriate and available standard reference material.

F.5.1

Authorized revisions to the Standardized Molecular Subtyping of Foodborne Bacterial Pathogens by Pulsed-Field Gel Electrophoresis manual or SOPs shall be incorporated into current practice in a timely fashion.

F.5.2

Any changes to procedures that may affect the comparability of gels between laboratories shall be sent to the PulseNet USA Task Force for review **before** any gels are submitted to the PulseNet USA National Database.

STANDARD F.6

The laboratory shall have and follow written general guidelines for the interpretation of data. (See PFGE standardization manual, section 13).

G. DOCUMENTATION

OBJECTIVE: To provide all personnel with clear instructions of the goals of the PFGE technique and all the operations needed to fulfill those goals.

Documentation is divided into four levels, as follows:

Level 1. Quality assurance/quality control (QA/QC) manual. This level is covered by the present document, which establishes all objectives and policies necessary for achieving the expected level of quality.

Level 2. Standard operating procedures (SOPs). Standard operating procedures are the tools that provide assurance of the quality and integrity of all data generated during studies utilizing the standard PFGE technique. All PulseNet participating laboratories shall develop and follow approved SOPs.

Level 3. Standard protocol(s). PulseNet USA has already developed "One-Day (24-28 hour) Standardized Laboratory Protocols for Molecular Subtyping of Foodborne Bacterial Pathogens by Pulsed-Field Gel Electrophoresis (PFGE)." The approved protocols specify all the recommended methods for conducting PFGE of foodborne pathogens and submitting images/data to the PulseNet USA National Database(s) (PNL03 – PNL07, and PND02).

Level 4. Records, reports, and images. Includes work documents containing all information related to the receiving and testing of isolates, as well as results and their analysis and interpretation. These documents shall be developed according to the policies of the "SOP for Generation of Records and Reports" (PNL08).

STANDARD G.1

Protocols shall be written according to the "SOP for Writing Standard Operating Procedures" (PNG01), for consistency and ease of use.

STANDARD G.2

The PFGE standardization manual and protocol development are the responsibility of the CDC. The procedures can be written by CDC personnel, PulseNet participating laboratories, or by a collaborative effort between these groups.

STANDARD G.3

CDC PulseNet laboratories shall use the most current version of the standardized protocol. All CDC PulseNet participating laboratories will receive a copy of the most current authorized version of the PFGE manual, protocols, and other procedures electronically (e.g., via the PulseNet USA Listserv). A master of the most recent version is kept at CDC.

STANDARD G.4

Should any CDC PulseNet participating laboratory generate a document considered useful for other participating laboratories, that laboratory shall send a copy of the document to the PulseNet USA Task Force for review (pfge@cdc.gov). After review and approval, the CDC will distribute the document to all members of PulseNet (USA and International) for their knowledge and use.

STANDARD G.5

Reports sent to CDC for analysis shall follow the format included in the "Standard Operating Procedure for the Generation of Records and Reports" (PNL08).

STANDARD G.6

The laboratory shall have and follow written procedures for taking and maintaining notes to support the conclusions drawn in laboratory reports.

STANDARD G.7

The laboratory shall maintain all documentation generated by analysts related to the analysis of samples.

G.7.1

The guidelines for report writing shall include:

(a) Identifier (e.g., outbreak, sporadic case)
(b) Isolate identification number
(c) Source or description of samples examined
(d) Results (including image and pattern number)
(e) Conclusion and/or interpretative statement if appropriate
(f) Date issued
(g) A signature and title, or equivalent identification, of the person(s) accepting responsibility for the content of the report

G.7.2

The laboratory shall have written procedures for the release of case report information.

G.7.3

The laboratory shall follow written local laboratory and PulseNet USA guidelines when dealing with members of the press (PNG03).

G.7.4

The laboratory shall follow written state and federal guidelines for release of information in response to a Freedom of Information Act request (PNG04).

H. EQUIPMENT: CALIBRATION AND MAINTENANCE

OBJECTIVE: To assure that equipment and devices work properly.

STANDARD H.1

The laboratory shall use equipment suitable for the methods employed. A list of necessary equipment is provided in section 5b of the PFGE standardization manual and in SOP PNL01 in this manual.

STANDARD H.2

The laboratory shall have a documented program for calibration of instruments and equipment. Proper instrument operation and maintenance are necessary to assure quality results.

H.2.1

All CDC PulseNet laboratories are responsible for proper handling, supervision, and maintenance of equipment in their respective laboratories.

STANDARD H.3
Equipment requiring calibration shall be calibrated according to the procedures recommended in the respective manufacturer's manual(s). If calibration instructions are not included in the manual, the laboratory shall request written instructions directly from the manufacturer.

H.3.1
Written records documenting the frequency and date of calibration shall be maintained for each instrument requiring calibration.

H.3.2
New instruments and equipment, or instruments and equipment that have undergone repair or maintenance, shall be calibrated before being used in sample analysis.

H.3.3
Written records or logs shall be maintained for maintenance service performed on all equipment.

I. CERTIFICATION

OBJECTIVE: To ensure that all persons submitting patterns to the National Databases have been adequately trained on the PulseNet standardized protocol(s) for PFGE and/or Gel Analysis and are producing good quality gels and analysis.

STANDARD I.1
All members of the CDC PulseNet laboratory (PulseNet Methods Development Laboratory) shall attend CDC-sponsored training, Area Lab training, or customized training (by a certified instructor) and must have successfully completed the certification testing for an organism (in gel preparation, TIFF analysis or both), as described in the "Standard Operating Procedure for Certification of PulseNet USA Personnel" (PNQ02).

I.1.1
This person shall be responsible for training additional staff in their respective laboratory.

I.1.2
No person may submit patterns to any PulseNet USA national database without having been TIFF-certified for that organism.

J. PROFICIENCY TESTING

OBJECTIVE: To participate in the ongoing proficiency testing program, coordinated by the PulseNet USA Task Force and APHL, to ensure that CDC laboratories participating in PulseNet USA maintain a satisfactory level of performance for PFGE or other molecular subtyping methods and gel analysis.

STANDARD J.1

Each certified PulseNet CDC laboratory shall participate in annual external proficiency testing for PFGE as described in the "Standard Operating Procedure for the PulseNet USA Proficiency Testing Program" (PNQ04).

J.1.1

Failure to satisfactorily participate in the external proficiency testing will result in decertification of the laboratory for that specific organism and termination of access to PulseNet USA's on-line database(s). Laboratories may continue to submit .tif and .bdl files via e-mail.

J.1.1.1

Failure to satisfactorily participate means:

J.1.1.1.1

Failure to return proficiency testing results to the PulseNet USA Task Force.

J.1.1.1.2

Two consecutive unsatisfactory proficiency testing results for the same organism over two separate testing events.

J.1.2

Laboratories decertified for failure to satisfactorily participate or complete the external proficiency testing shall have to be recertified (and retrained if necessary) before restoring access to the PulseNet USA on-line database(s).

K. REVIEW

STANDARD K.1

The failure of any component of the standardized protocol (bad reagents, poor standards, equipment failure, and/or inadequately trained lab personnel) may result in gels unacceptable for analysis and inclusion in the PulseNet USA national database(s).

K.1.1

The laboratory shall follow written procedures when a failure is detected in any component of the standardized PFGE protocol.

K.1.2

Failure of a standard shall be treated as a QC failure for purposes of analysis and review. Gels on which the standard strain is missing or on which the standard strain does not match the global standard shall not be analyzed. These gels shall be rerun using a new plug of the standard strain.

STANDARD K.2

The laboratory shall conduct administrative and technical reviews of all reports to ensure that conclusions and supporting data are reasonable and within the constraints of scientific knowledge.

K.2.1

The laboratory shall have a mechanism in place to address unresolved discrepant conclusions between analysts and reviewer(s).

L. CHANGES TO THE STANDARDIZED PFGE PROTOCOL

STANDARD L.1

Any changes made to the standardized PFGE protocol(s) shall be appropriately documented. No changes may be made to the standardized PFGE protocol(s) without approval from the PulseNet USA Taskforce and the PulseNet USA Steering Committee.

L.1.1

Internal validation of such changes shall be performed and documented by the laboratory.

L.1.1.1

Changes to the protocol(s) shall be tested using known samples. The laboratory shall monitor and document the reproducibility and accuracy of the procedure using the appropriate bacterial DNA control(s).

L.1.1.2

The laboratory shall establish and document match criteria based on empirical data in conjunction with the PulseNet USA Task Force.

L.1.1.3

Material modifications made to analytical procedures shall be documented and subject to validation testing by at least three additional PulseNet USA-certified laboratories designated by the PulseNet USA Task Force.

M. VALIDATION OF NEW METHODS

STANDARD M.1

All developmental validation performed shall be appropriately documented.

M.1.1

Internal validation shall be performed and documented by the laboratory.

M.1.1.1

The procedure shall be tested using known samples. The laboratory shall monitor and document the reproducibility and accuracy of the procedure using the appropriate bacterial DNA control(s).

M.1.1.2
The laboratory shall establish and document match criteria based on empirical data in conjunction with the PulseNet USA Task Force.

M.1.3
Where methods are not specified, the laboratory shall, wherever possible, select methods that have been published by reputable technical organizations or in relevant scientific texts or journals, or have been appropriately evaluated for a specific or unique application.

N. SAFETY

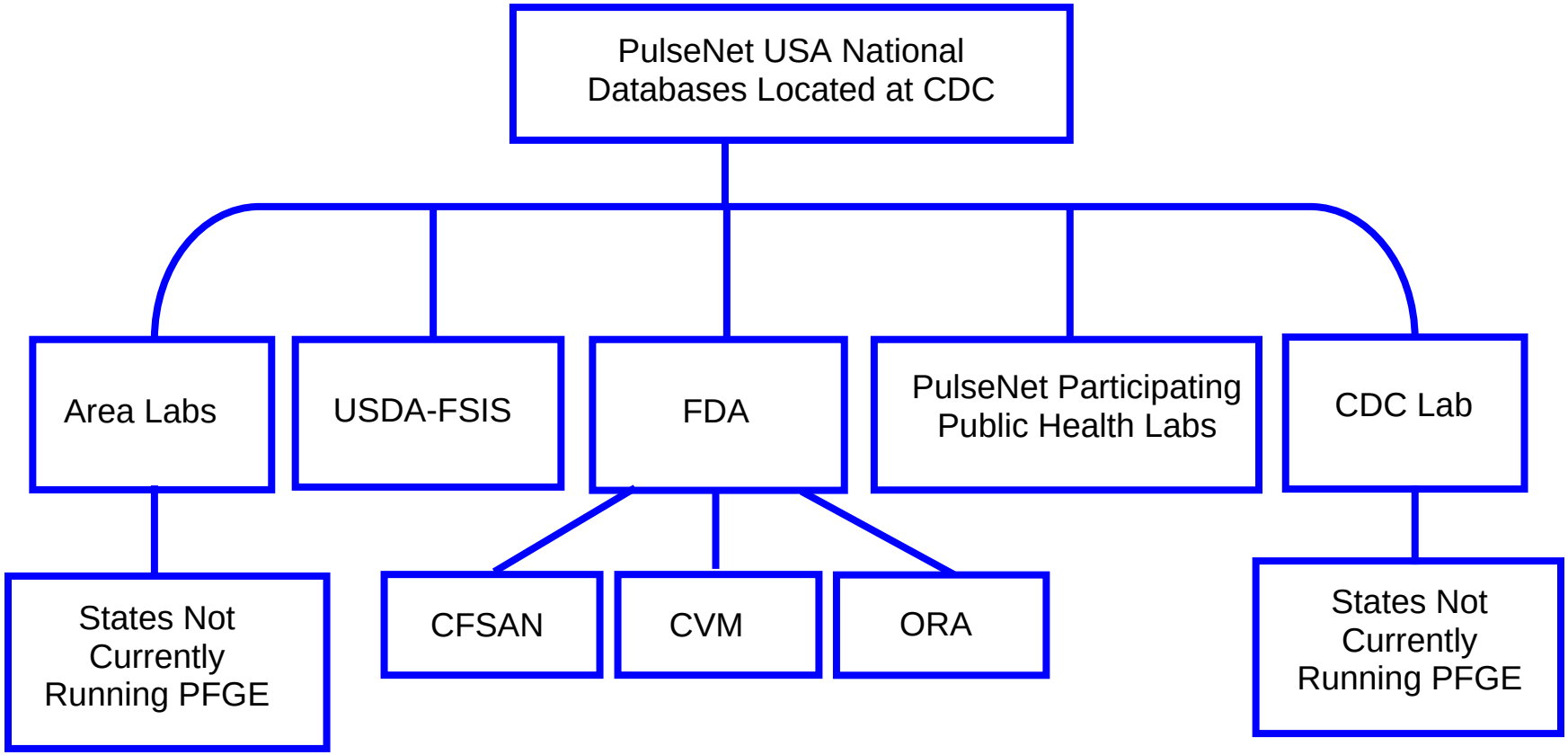
STANDARD N.1
The laboratory shall have and follow a documented environmental health and safety program.

REFERENCES:

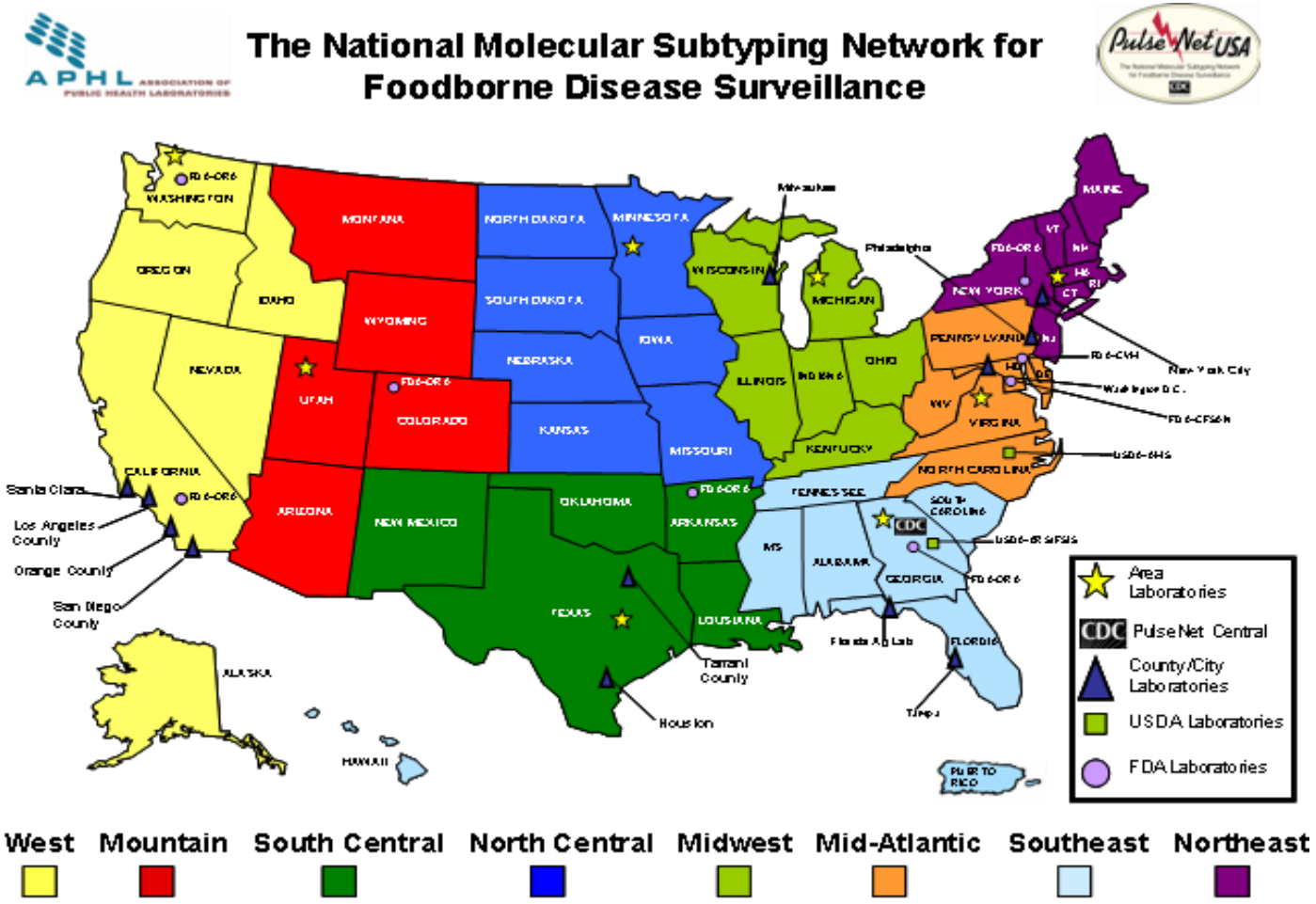
PulseNet USA Manual (last revised on September 2003), Standardized Molecular Subtyping of Foodborne Bacterial Pathogens by Pulsed-Field Gel Electrophoresis. Foodborne and Diarrheal Diseases Branch, Centers for Disease Control and Prevention, Atlanta, Georgia.

PulseNet USA BioNumerics Training Manual (February 2003). Foodborne and Diarrheal Diseases Branch, Centers for Disease Control and Prevention, Atlanta, Georgia.

APPENDIX A
PulseNet USA Organizational Chart



APPENDIX B


The National Molecular Subtyping Network for Foodborne Disease Surveillance
PulseNet USA
APHL
Area Laboratories
CDC PulseNet Central
County/City Laboratories
USDA Laboratories
FDA Laboratories
West
Mountain
South Central
North Central
Midwest
Mid-Atlantic
Southeast
Northeast

APPENDIX C

STANDARD OPERATING PROCEDURES LIST

General Standard Operating Procedures (PNG)

PNG01: Standard Operating Procedure for Writing Standard Operating Procedures

PNG03: Standard Operating Procedure for Handling Inquiries from Members of the Media

PNG04: Standard Operating Procedure for Handling Inquiries from Lawyers and Freedom of Information Act (FOIA) Requests

PNG05: Standard Operating Procedure for Becoming a PulseNet USA Participating Laboratory

PNG06: Standard Operating Procedure for PulseNet USA Area Laboratory Responsibilities

Standard Operating Procedures for the PulseNet USA Laboratory (PNL)

PNL01: Standard Operating Procedure for Laboratory Equipment and Supplies

PNL02: Standard Operating Procedure for Preparation of Reagents Used in the PulseNet USA Standardized PFGE Protocols

PNL03: Standard Operating Procedure for PFGE of *Campylobacter*

PNL04: Standard Operating Procedure for PFGE of *Listeria monocytogenes*

PNL05: Standard Operating Procedure for PFGE of *E. coli* O157:H7, *Shigella*, and *Salmonella* (pending)

PNL06: Standard Operating Procedure for PFGE of *Vibrio cholerae* (pending)

PNL07: Standard Operating Procedure for the Image Acquisition and Production of TIFF Files for Data Analysis

PNL08: Standard Operating Procedure for the Generation of Records and Reports

PNL09: Standard Operating Procedure for Receipt, Shipping, and Storage of Isolates (pending)

PNL10: Standard Operating Procedure for the Use, Inspection, Cleaning, Maintenance, and Calibration of Equipment

PNL11: Standard Operating Procedure for Maintenance of Pulsed-Field Gel Electrophoresis Systems

PNL12: Standard Operating Procedure for Maintenance of Dade Microscan Turbidity Meter

PNL13: Standard Operating Procedure for Waterbath Maintenance and Cleaning

PNL14: Standard Operating Procedure for the Use, Storage, and Disposal of Chemicals and Reagents

PNL15: Standard Operating Procedure for Storage and Use of Expired Reagents

PNL16: Standard Operating Procedure for Evaluation and Correction of Failure of Any Component of Standardized PFGE Protocol

PNL17: Standard Operating Procedure for Laboratory Training

Standard Operating Procedures for the PulseNet USA Databases (PND)

PND01: Standard Operating Procedure for Computer Equipment and Supplies

PND02: Standard Operating Procedure for Image Analysis Using BioNumerics Software

PND03: Standard Operating Procedure for PulseNet USA Listserv Access and Use

PND04: Standard Operating Procedure for Gel Analysis Guidelines

PND05: Standard Operating Procedure for BioNumerics Administrative Duties (pending)

PND06: Standard Operating Procedure for Obtaining and Updating Organism Serotype Codes (pending)

PND07: Standard Operating Procedure for Updating and Maintaining Gel Analysis Software and PulseNet USA Scripts (pending)

PND08: Standard Operating Procedure for Setup and Use of SecurID Key Fob

PND09: Standard Operating Procedure for Authenticating and Connecting to the Server

PND10: Standard Operating Procedure for Database Training

Standard Operating Procedures for the PulseNet USA QA/QC Program (PNQ)

PNQ01: Standard Operating Procedure for TIFF Quality Grading Guidelines

PNQ02: Standard Operating Procedure for Certification of PulseNet USA Personnel

PNQ03: Standard Operating Procedure for Evaluation of Certification Files

PNQ04: Standard Operating Procedure for the PulseNet USA Proficiency Testing Program

PNQ05: Standard Operating Procedure for Laboratory In-House Gel Certification (pending)

PulseNet QA/QC Manual

Standards

	General Standard Operating Procedures (PNG)
PNG01	Standard Operating Procedure for Writing Standard Operating Procedures
PNG02	Standard Operating Procedure for Processing In and Out of PulseNet
PNG03	Standard Operating Procedure for Handling Inquiries from Members of the Media
PNG04	Standard Operating Procedure for Handling Inquiries from Lawyers and Freedom of Information Act (FOIA) Requests
PNG05	Responsibilities for a PulseNet Laboratory and Standard Operating Procedure for Becoming a PulseNet Laboratory
PNG06	Standard Operating Procedure for PulseNet Area Laboratory Responsibilities
PNG07	Standard Operating Procedure for Sharing Database Information between PulseNet USA and PulseNet Canada
PNG08	Standard Operating Procedure for Becoming a PulseNet Participating Laboratory and Handling Requests to Become a PulseNet Laboratory
PNG09	Standard Operating Procedure for Sharing Database Information between PulseNet USA and USDA VetNet (pending)

	Standard Operating Procedures for the PulseNet Laboratory (PNL)
PNL01	Standard Operating Procedure for Laboratory Equipment and Supplies
PNL02	Standard Operating Procedure for Preparation of Reagents Used in the Standardized PFGE Protocols
PNL03	Standard Operating Procedure for PFGE of *Campylobacter*
PNL04	Standard Operating Procedure for PFGE of *Listeria monocytogenes*
PNL05	Standard Operating Procedure for PFGE of *E. coli* O157:H7, *Shigella* , and *Salmonella*
PNL06	Standard Operating Procedure for PFGE of *Vibrio cholerae* and *Vibrio parahaemolyticus*
PNL07	Standard Operating Procedure for Image Acquisition and Production of TIFF Files for Data Analysis
PNL08	Standard Operating Procedure for the Generation of Records and Reports
PNL09	Standard Operating Procedure for Receipt, Shipping, and Storage of Isolates (pending)
PNL10	Standard Operating Procedure for the Use, Inspection, Cleaning, Maintenance, and Calibration of Equipment
PNL11	Standard Operating Procedure for Maintenance of Pulsed-Field Gel Electrophoresis Systems
PNL12	Standard Operating Procedure for Maintenance of Dade Microscan Turbidity Meter
PNL13	Standard Operating Procedure for Waterbath Maintenance and Cleaning
PNL14	Standard Operating Procedure for the Use, Storage, and Disposal of Chemicals and Reagents
PNL15	Standard Operating Procedure for Storage and Use of Expired Reagents
PNL16	Standard Operating Procedure for Evaluation and Correction of Failure of Any Component of Standardized PFGE Protocol
PNL17	Standard Operating Procedure for Laboratory Training
PNL18	Standard Operating Procedure for PFGE of *Yersinia pestis*
PNL19	Laboratory Standard Operating Procedure for PulseNet MLVA of Shiga Toxin-Producing Escherichia coli O157 (STEC O157) and *Salmonella* Enterica Serotypes Typhimurium and Enteritidis – Beckman Coulter CEQ 8000/8800/GeXP Platform

PNL20	Standard Operating Procedure for adding Thiourea to 0.5X TBE Buffer for Strains of *E. coli* O157:H7, *Salmonella* , *Vibrio* and other Species or Genera that are "Untypeable" by PFGE
PNL23	Laboratory Standard Operating Procedure for PulseNet MLVA of Shiga Toxin-Producing *Escherichia coli* O157 (STEC O157) and *Salmonella* Enterica Serotypes Typhimurium and Enteritidis-Applied Biosystems Genetic Analyzer 3130 Platform
PNL25	Standard Operating Procedure for PFGE of *Clostridium botulinum*
PNL28	Laboratory Standard Operating Procedure for PulseNet MLVA of Shiga Toxin-Producing *Escherichia coli* O157 (STEC O157) and *Salmonella* Enterica Serotypes Typhimurium and Enteritidis – Applied Biosystems Genetic Analyzer 3500 Platform
PNL31	Laboratory Standard Operating Procedure for PulseNet PFGE of *Cronobacter Species*
	Standard Operating Procedures for the PulseNet Databases (PND)
PND01	Standard Operating Procedure for Computer Equipment and Supplies
PND02	Standard Operating Procedure for Image Analysis Using BioNumerics Software
PND03	Standard Operating Procedure for PulseNet Listserv Access and Use
PND04	Standard Operating Procedure for PFGE Gel Analysis
PND05	Standard Operating Procedure for BioNumerics Administrative Duties (pending)
PND06	Standard Operating Procedure for Obtaining and Updating Organism Serotype Codes (pending)
PND07	Standard Operating Procedure for Updating and Maintaining Gel Analysis Software and PulseNet Scripts (pending)
PND08	Standard Operating Procedure for Setup and Use of SecurID Key Fob
PND09	Standard Operating Procedure for Authenticating and Connecting to the Server
PND10	Standard Operating Procedure for Database Training
PND11	Standard Operating Procedure for Beta Testing BioNumerics Scripts for New PulseNet Databases (pending)
PND12	Standard Operating Procedure for Naming PulseNet Outbreaks and Clusters
PND13	Standard Operating Procedure for Naming PulseNet PFGE Patterns (pending)
PND14	PulseNet Standard Operating Procedure for Analysis of MLVA Data of Shiga Toxin-Producing *Escherichia coli* O157 (STEC O157) and *Salmonella* Enterica Serotypes Typhimurium and Enteritidis in BioNumerics-Beckman Coulter CEQ 8000/8800/GeXP Data
PND16	PulseNet Standard Operating Procedure for Analysis of MLVA Data of Shiga Toxin-Producing *Escherichia coli* O157 (STEC O157) and *Salmonella* Enterica Serotpyes Typhimurium and Enteritidis in BioNumerics-Applied Biosystems Genetic Analyzer 3130/3500 Data

	Standard Operating Procedures for the PulseNet QA/QC Program (PNQ)
PNQ01	Standard Operating Procedure for TIFF Quality Grading Guidelines
PNQ02	Standard Operating Procedure for Certification of PulseNet USA Personnel
PNQ03	Standard Operating Procedure for Evaluation of Certification Files
PNQ04	Standard Operating Procedure for the PulseNet USA Proficiency Testing Program
PNQ05	Standard Operating Procedure for MLVA Certification of PulseNet Personnel for the Beckman Coulter CEQ 8000 Platform
PNQ06	Standard Operating Procedure for MLVA Certification of PulseNet Personnel for the Applied Biosystems Genetic Analyzer 3130XL Platform

1. PURPOSE: To describe the guidelines for the creation, review, and approval of procedures regulating PFGE activities used by PulseNet.

2. SCOPE: This procedure applies to all PulseNet procedures written in relation to the standardized PFGE protocols and analysis.

3. DEFINITIONS/TERMS:

3.1 SOP: Standard Operating Procedure
3.2 PFGE: Pulsed-field Gel Electrophoresis
3.3 Effective Date: the day the procedure will be in operation and from which any revisions, corrections, and distribution times may be calculated.
3.4 QA/QC: Quality Assurance/Quality Control

4. RESPONSIBILITIES:

4.1 The correct format for an SOP can be found in Appendix PNG01-1.
4.2 Assign a concise title to the procedure that gives a general idea of the procedure's contents.
4.3 Assign a code to the procedure, containing five digits, assigned as follows:
  4.3.1 Acronym *PN*, which refers to PulseNet, and either a *G* for General SOPs, *L* for Laboratory SOPs, *D* for Database SOPs, or *Q* for QA/QC SOPs.
  4.3.2 Two digit consecutive number of the procedure. For example: The "*STANDARD OPERATING PROCEDURE FOR WRITING STANDARD OPERATING PROCEDURES*" is the first *General* procedure for *PulseNet*, so it has the code "PNG01."
4.4 SOPs should be detailed enough to provide meaningful direction to personnel who conduct routine laboratory activities.
4.5 SOPs will be reviewed every two years after the effective date, although reviews may be done sooner if significant changes in the procedure occur.
  4.5.1 SOPs will be reviewed by the original author or laboratory supervisor.
    4.5.1.1 If changes in the procedure will represent a new standard protocol, the SOP should be reviewed and the revision approved.
    4.5.1.2 If an exception to an SOP is to be made for an individual study, that exception must be authorized in writing by the laboratory chief or supervisor.
  4.5.2 Any changes made must be documented in section 8 of the procedure *Amendments.*
  4.5.3 Author and laboratory supervisor will sign and date in the appropriate areas.
  4.5.4 Authorization will be signed and dated by the laboratory chief or director.
4.6 The page number will be written in the lower right corner of each page.
4.7 All personnel following the SOP must read, sign, and date the corresponding *Reading Control Sheet* (Appendix PNG01-2).
4.8 Personnel performing activities must have access to the related procedure.
  4.8.1 The master of an SOP must be authorized, signed, and dated by the laboratory chief or supervisor, and an inventory should be kept of when, where, and to whom copies of the SOP have been distributed.
  4.8.2 Electronic and hard copy versions of SOPs must be readily available to personnel.
  4.8.3 No unauthorized copies should be made and/or distributed. This will provide better control over the distribution of SOPs, ensuring that all outdated versions of SOPs are retired.
  4.8.4 All previous versions shall be retired when new revisions are distributed.
    4.8.4.1 Retired SOPs must have the Out-of-Service date listed in red across the top of each page and be retained in the laboratory for two years from the retirement date. The master copy must be archived indefinitely.
4.9 The procedure must include:
  4.9.1 **PURPOSE:** Describes in a clear and simple way the mission of the SOP.
  4.9.2 **SCOPE:** Indicates the persons and/or activities concerned with the procedure.

4.9.3 **DEFINITIONS/TERMS:** Provides meanings of abbreviations or important words contained in the procedure. Appropriate terms and their definitions are listed in Appendix PNG01-2.
4.9.4 **RESPONSIBILITIES:** Provides obligations that should be met (i.e., Safety). Will sometimes be combined with Procedure.
4.9.5 **PROCEDURE:** Describes each activity in a clear and orderly fashion. Will sometimes be combined with Responsibilities.
4.9.6 **FLOW CHART:** Simplifies understanding of the procedure.
4.9.7 **BIBLIOGRAPHY:** Includes all literature consulted for the procedure.
4.9.8 **CONTACTS:** Provides contact information for persons mentioned within the SOP.
4.9.9 **AMENDMENTS:** Tracks all changes, updates, and corrections to a procedure.

5. PROCEDURE:

6. FLOW CHART:

7. BIBLIOGRAPHY:

Weinberg, Sandy. **GOOD LABORATORY PRACTICE REGULATIONS**. Second edition. Marcel Dekker, Inc. USA (1995).

8. CONTACTS:

9. AMENDMENTS:

Appendix PNG01-1

HEADER SIMILAR TO ABOVE

1. PURPOSE:

2. SCOPE:

3. DEFINITIONS/TERMS:

4. RESPONSIBILITIES:

5. PROCEDURE:

6. FLOW CHART:

7. BIBLIOGRAPHY:

8. CONTACTS:

9. AMENDMENTS:

FOOTER SIMILAR TO BELOW

Appendix PNG01-2

Terms and Definitions:

1. **SOP:** Standard Operating Procedure
2. **PFGE:** Pulsed-field Gel Electrophoresis
3. **TIFF:** Tagged Image File Format. A file of a gel image that can be analyzed in BioNumerics
4. **CDC:** Centers for Disease Control and Prevention
5. **BioNumerics:** Gel analysis software used by PulseNet, developed by Applied Maths, Belgium
6. **EIS:** Epidemic Intelligence Service. EIS Officers are epidemiologists who are given two-year appointments to help in the investigation of foodborne outbreaks
7. **PulseNet liaison:** Contact for Foodborne and Diarrheal Diseases Branch epidemiologists and PulseNet Database Administration Team
8. **MSDS:** Material Safety Data Sheets
9. **PPE:** Personal Protective Equipment (goggles, gloves, lab coat, etc.)
10. **PFGE inbox:** An e-mail account that is maintained and checked by all database managers at CDC. The address is: PFGE@cdc.gov
11. **SecureID key fob:** Token that displays a six-digit passcode. When used in combination with a four-digit pin number, allows access through the CDC firewall
12. **Effective date:** The day the procedure will be in operation and from which any revisions, corrections, and distribution times may be calculated
13. **N/A:** Not Applicable
14. **QA/QC:** Quality Assurance/Quality Control
15. **NCID:** National Center for Infectious Diseases
16. **BMD:** Bacterial and Mycotic Diseases
17. **FOIA:** Freedom of Information Act
18. **OCOO:** Office of the Chief Operating Officer
19. **PulseNet laboratory:** A laboratory that performs PFGE using the approved CDC protocols and receives support funding via the Epidemiology and Laboratory Capacity (ELC) or Emerging Infections Program (EIP) federal grants
20. **PulseNet Certification Program:** Program that tests a individual's ability to perform PFGE, perform analysis, create a bundle, and upload a specific organism to the National Database using the PulseNet standardized protocols.
21. **PulseNet Proficiency Testing Program:** Program that regularly tests the ability of an individual or laboratory to perform PFGE and pattern analysis
22. **Support zone:** A group of state and local health departments that can utilize laboratory services provided by their Area Laboratory
23. **Area Laboratory:** Laboratory, designated by CDC, which has agreed to assume responsibility for additional PulseNet duties for laboratories within their support zone. The current Area Laboratories include MA, MN, WA, TX, VA, UT, MI and CDC
24. **Cluster:** A group of isolates with the same serotype determined to possess indistinguishable PFGE DNA patterns using one or more enzyme restrictions
25. **DNA:** Deoxyribonucleic acid

26. **PulseNet laboratory protocols:** Protocols or standard operating procedures followed by all laboratories participating in the PulseNet program in order to submit DNA fingerprint patterns for inclusion in the National Database
27. **Troubleshoot:** To investigate a problem and come up with solutions
28. **Surge capacity:** Ability to provide additional testing when workload exceeds existing capacity
29. **Bundle file:** A file with a .bdl extension that is produced in BioNumerics and contains the analysis of at least one lane of a gel image and may include specified isolate demographic information
30. **Foodborne disease epidemiologist:** An individual who studies and/or investigates the transmission and control of foodborne diseases
31. **Outbreak:** A rise in disease rates as a result of an association or exposure to a specific vehicle
32. **Proficiency testing:** An annual assessment of the quality of the work being performed in PulseNet participating laboratories. For each pathogen, proficiency testing includes two parts – a "TIFF sent by CDC" and a "TIFF generated by the participating laboratory (i.e., in-house TIFF)"
33. **Proficiency testing packet:** A package sent to the PulseNet participant with the results of his/her PT evaluation. Includes a hard copy of the cover letter, report, in-house TIFF, and submission e-mail
34. **TIFF sent by CDC:** This is a part of the proficiency testing program where all laboratories analyze the same TIFF sent to them by CDC
35. **TIFF generated by the participating laboratory, also called the "in-house TIFF":** A part of the proficiency testing program where laboratories run a gel and produce a TIFF of the gel that contains the *Salmonella* Braenderup H9812 standards and the proficiency testing strain restricted with the primary and secondary enzymes. The TIFF is analyzed and submitted to the on-line database
36. **Proficiency Testing survey:** An annual survey consists of two rounds of testing, a fall round and a spring round
37. **Comparison list:** A list of analyzed lanes from comparison TIFFs that is saved in BioNumerics and used to compare to the analysis of the submitted TIFF by the certification file evaluator and the analysis of the submitter in the certification bundle file
38. **Gel certified:** Formerly "TIFF certified." An individual or laboratory that is certified in laboratory methods for PFGE and image acquisition
39. **Analysis certified:** An individual who is certified in BioNumerics gel analysis
40. **Certification files:** TIFF and/or bundle files submitted by PulseNet participants for certification evaluation
41. **Certification file evaluator:** An individual who evaluates certification files
42. **TIFF quality:** The grading of the appearance and ease of analysis of a TIFF according to the PulseNet TIFF Grading Guidelines. This is a main component of the evaluation of a TIFF submitted for certification
43. **Gel analysis assessment:** The grading of the whole analysis of a TIFF, including gel and lane definition, normalization, and band marking, according to the PulseNet Gel Analysis Guidelines. This is a main component of the evaluation of a bundle file submitted for certification
44. **Certification file reviewer:** An individual who reviews and signs off on the certification reports submitted by the certification file evaluator
45. **Comparison TIFFs:** One or more TIFFs run by CDC for a specific pathogen for use in comparing PFGE patterns and band resolution against submitted certification TIFFs. Comparison TIFFs can also be a group of certification TIFFs submitted by several laboratories to monitor PFGE patterns and band resolution over several submitting laboratories. The latter is most easily accomplished through a saved list in BioNumerics
46. **APHL:** Association of Public Health Laboratories
47. **Host lab:** Term used to describe laboratory hosting a training course
48. **OHS:** Office of Health and Safety

49. Training Personnel: Term used to describe PulseNet participants who have been approved by CDC to train other PulseNet participants

1. **PURPOSE**: To describe the responsibilities and procedure associated with processing in and out of PulseNet.

2. **SCOPE:** This procedure applies to all PulseNet participating laboratories and personnel. If you are a member of a laboratory that would like to become a PulseNet participant, please refer to PNG05, Responsibilities for a PulseNet Laboratory and Standard Operating Procedure for Becoming a PulseNet Laboratory.

3. DEFINITIONS/TERMS:

3.1 SOP: Standard Operating Procedure
3.2 PFGE: Pulsed-field Gel Electrophoresis
3.3 QA/QC: Quality Assurance/Quality Control
3.4 APHL: Association of Public Health Laboratories
3.5 CDC: Centers for Disease Control and Prevention
3.6 PulseNet Listserv: A closed, electronic web conference used for communication among PulseNet participants. The PulseNet Listserv is open to all laboratory staff at PulseNet participating laboratories. Epidemiologists working in collaboration with these laboratories, when approved by the PulseNet laboratory contact, U.S. food regulatory staff, and PulseNet International representatives may also have access. The PulseNet Listserv is not open to university or private industry personnel unless their inclusion is deemed to be in the interest of public health. The current program being used is WebBoard.
3.7 SecurID key fob: A secure authentication device used to allow access to resources on the CDC network.
3.8 *PulseNet News*: The newsletter distributed by PulseNet on a tri-annual basis.

4. RESPONSIBILITIES:

4.1 PulseNet laboratories are expected to notify the PulseNet Database Team and/or APHL (see Contacts section 8 below) when new laboratory staff begin performing PulseNet-related duties and/or when existing staff will no longer be performing PulseNet-related duties.
4.2 Personnel listed within section 5.1.3.1, "The Distribution List" are responsible for updating the contact information and any associated documents as follows (see Appendix PNG02-1 for Steps to be Taken at CDC):
- 4.2.1 PulseNet Database Unit Chief sends the "New Contact Information Sheet" (appendix PNG02-3) to new PulseNet laboratory staff to complete and return to PFGE@cdc.gov
- 4.2.2 PulseNet Database Team
  - 4.2.2.1 The PulseNet Database Team keeps the PFGE contacts list up-to-date, including but not limited to:
    - 4.2.2.1.1 Adding new participants and their contact information
    - 4.2.2.1.2 Updating existing contacts with new information and tracking any changes within the information box with the date the change was made
    - 4.2.2.1.3 Removing any contacts that no longer perform PulseNet-related duties
  - 4.2.2.2 The PulseNet Database Team also sends an email (see appendix PNG02-2 for template) to a participant who is either departing or will no longer be performing PulseNet-related duties to make sure to return their SecurID key fob to CDC and provide CDC and/or APHL with any other pertinent information that might be available (i.e. plans to hire a new person to take over PulseNet-related duties). The email will be cc'ed to the main contact of the participant's Area Laboratory.
- 4.2.3 IT Support
  - 4.2.3.1 PulseNet Listserv Access
    - 4.2.3.1.1 Adding new participants as their access is approved
    - 4.2.3.1.2 Removing participants who no longer perform PulseNet-related duties
  - 4.2.3.2 Access to National Database(s)
    - 4.2.3.2.1 As participants are certified, assigning SecurID key fobs and access to national database(s)
    - 4.2.3.2.2 Collecting SecurID key fobs and removing access to national database(s) once notified that participant is no longer performing PulseNet-related duties
  - 4.2.3.3 Information Update
    - 4.2.3.3.1 Emails participants with access to the National Database(s) to verify contact information on an annual basis

4.2.4 QA/QC
4.2.4.1 Updating all certification and proficiency testing documents as participants are granted access to the national database(s) and as participants are no longer performing PulseNet-related duties
4.2.5 Newsletter
4.2.5.1 Updating newsletter distribution documents with any new or changed contact information and removing participants who no longer perform PulseNet-related duties
4.2.5.2 Including welcome or farewell announcement in the next issue of the newsletter

5. PROCEDURE:

5.1. New PulseNet personnel
5.1.1. When a laboratory staff member begins performing PulseNet-related duties, the PulseNet Database Team and APHL must be notified.
5.1.2. The laboratory must contact the PulseNet Database Team and/or APHL with the new person's contact information including name, title, position within the lab, mailing address, email address and phone number. Other information such as fax number and time spent performing PulseNet-related duties would also be useful.
5.1.3. Once contact information is received, the CDC or APHL person who received this information is responsible for sending it to everyone on "The Distribution List."
5.1.3.1. The Distribution List:
PulseNet Database Unit: PFGE Inbox PFGE@cdc.gov
PulseNet Database Unit Chief: Kelley Hise KHise@cdc.gov
PulseNet Methods Development and Reference Unit Chief: Efrain Ribot ERibot@cdc.gov
Listeria Identification and Subtyping Unit Chief: Lewis Graves LGraves@cdc.gov
IT Support: Brenda Brown BLBrown1@cdc.gov
QA/QC: Jennifer Kincaid JKincaid@cdc.gov and Deborah Sheehan Deborah.shea@cox.net
Newsletter: Nehal Patel NJPatel@cdc.gov
APHL: Sharon Rolando Sharon.Rolando@aphl.org
PulseNet International: Ahmed ElSedawy AElsedawy@cdc.gov (only include on distribution list if information is regarding a PulseNet International participant)
5.1.4. Information is added to the PFGE contact list
5.1.5. Once the participant is analysis-certified, access to the national database(s) may be granted and participant will receive a SecurID key fob (please refer to SOPs PNQ02 and PND08 for information on PulseNet certification and SecurID key fobs)
5.1.6. PulseNet Listserv access may be requested (please refer to SOP PND03 for information on requesting access and use of the PulseNet Listserv)
5.1.7. Welcome announcement will be printed in *PulseNet News* and contact information will be added to the newsletter distribution list

5.2. Change in contact Information
5.2.1. If contact information should change, participants must notify the PulseNet Database Team and/or APHL.
5.2.2. CDC will email participants who have access to the National Database(s) to verify contact information on an annual basis.
5.2.3. Once updated contact information is received, the CDC or APHL person who received this information is responsible for sending it to everyone on "The Distribution List" (see section 5.1.3.1 above) so that the appropriate people are notified and information may be updated promptly.

5.3. Participant departs from a PulseNet Laboratory (this includes changing positions or leaving the laboratory)
5.3.1. When a laboratory staff member is no longer performing PulseNet-related duties
5.3.1.1. The PulseNet Database Team and APHL must be notified
5.3.1.1.1. Once the PulseNet Database Team and/or APHL has been notified that a participant has left their current position and is no longer performing PulseNet-related duties, the CDC or APHL person who received this information is responsible for sending it to everyone on "The Distribution List" (see section 5.1.3.1 above).

5.3.1.2. If the laboratory staff member was the main PulseNet contact, contact information for the person who will serve as the replacement/interim contact person should be provided.

5.3.1.3. If the participant was analysis-certified, access to the national database(s) will be removed and the SecurID key fob must be returned to CDC.

5.3.1.3.1. Please return key fob to:
Centers for Disease Control
Attn: Mike Korth
1600 Clifton Rd. NE
MS-C03
Atlanta, GA 30333
(404) 639-3334

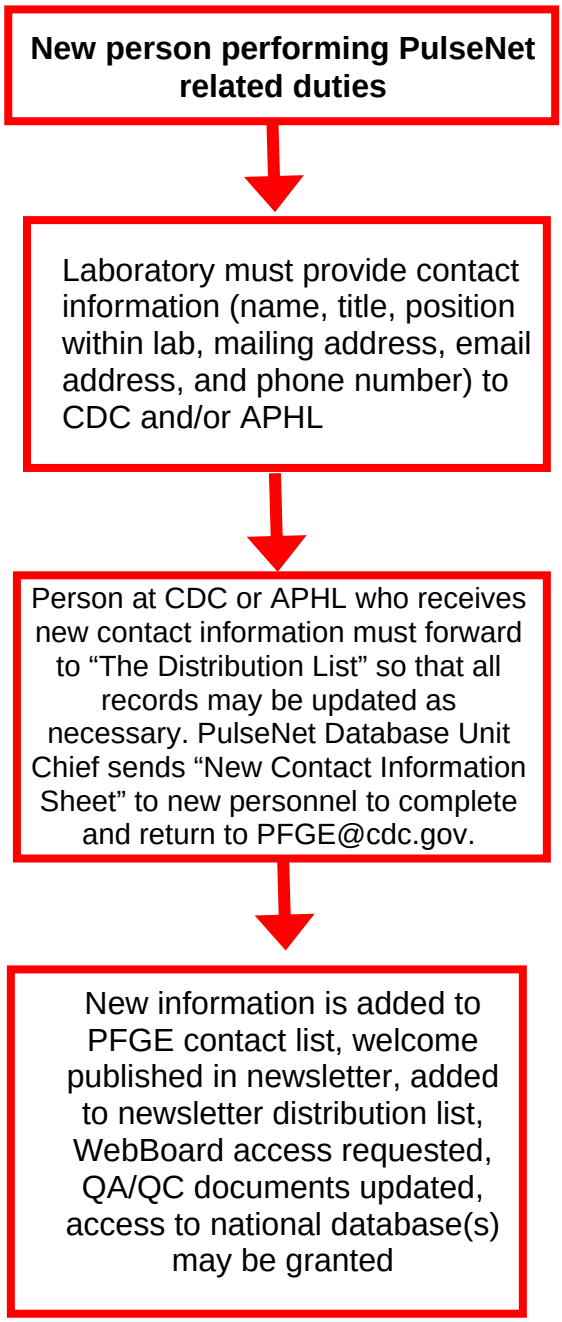
5.3.1.4. Access to the PulseNet Listserv will be removed

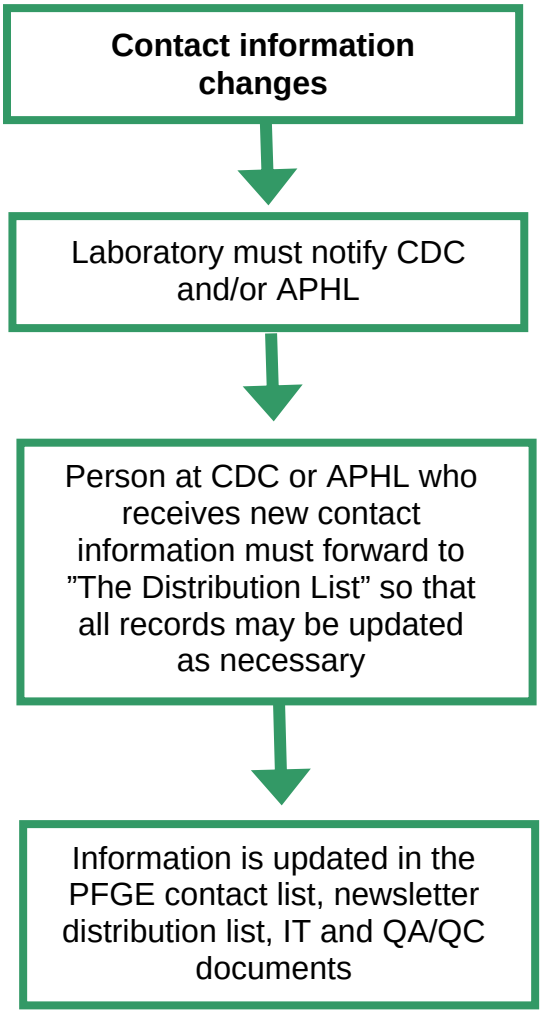
5.3.1.5. Information will be removed from the PFGE contact list

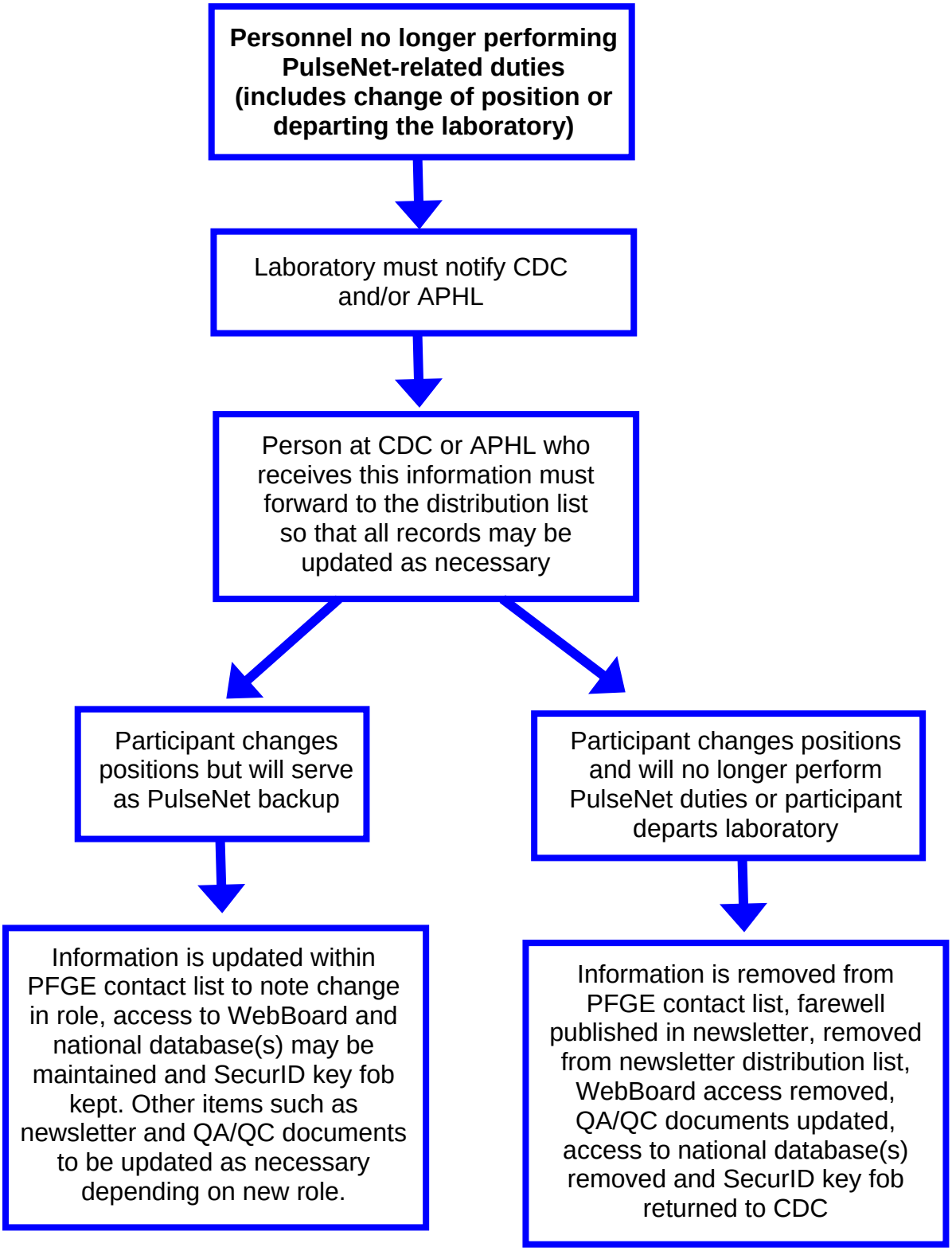
5.3.1.6. Farewell and/or change in duties announcement will be printed in *PulseNet News* and contact information will be removed from the newsletter distribution list

5.3.1.7. If the laboratory staff member was promoted or reassigned to a different position within their current laboratory and will no longer be performing routine PulseNet duties, but will serve as a PulseNet backup, access to the PulseNet Listserv and the national database(s) (if participant was analysis-certified) may be retained; therefore the staff member should keep their assigned SecurID key fob. They will not be removed from the PFGE contact list, but their information should be updated and a note must be written in the information box to indicate this participant's new role.

6. FLOW CHARTS:




Contact information changes
Laboratory must notify CDC and/or APHL
Person at CDC or APHL who receives new contact information must forward to ”The Distribution List” so that all records may be updated as necessary
Information is updated in the PFGE contact list, newsletter distribution list, IT and QA/QC documents


Personnel no longer performing PulseNet-related duties (includes change of position or departing the laboratory)
Laboratory must notify CDC and/or APHL
Person at CDC or APHL who receives this information must forward to the distribution list so that all records may be updated as necessary
Participant changes positions but will serve as PulseNet backup
Participant changes positions and will no longer perform PulseNet duties or participant departs laboratory
Information is updated within PFGE contact list to note change in role, access to WebBoard and national database(s) may be maintained and SecurID key fob kept. Other items such as newsletter and QA/QC documents to be updated as necessary depending on new role.
Information is removed from PFGE contact list, farewell published in newsletter, removed from newsletter distribution list, WebBoard access removed, QA/QC documents updated, access to national database(s) removed and SecurID key fob returned to CDC

7. BIBLIOGRAPHY:

8. CONTACTS:

8.1. **APHL**

Sharon Rolando
Senior Manager for Food Safety
8515 Georgia Avenue
Suite 700
Silver Spring, MD 20910
Sharon.Rolando@aphl.org
(240) 485-2777

8.2. **CDC PulseNet Database Team**

PFGE@cdc.gov
(404) 639-4558

9. AMENDMENTS:

Appendix PNG02-1

Steps to be Taken at CDC

CDC PulseNet personnel are responsible for sending information regarding new PulseNet participants, updated contact information, and participants no longer performing PulseNet-related duties to "The Distribution List" (listed in PFGE contacts as "1. The Distribution List," refer to section 5.1.3.1 of SOP PNG02 for more information). This appendix may serve as a "what happens next" document for internal use. Names of the person(s) responsible for each task may be inserted in the blanks for reference. The following may take place simultaneously. Please refer to SOP PNG02 for more specific information on responsibilities and procedure.

- **PulseNet Database Team Member** moves email to "Contacts to Update" folder within the PFGE inbox.

  _____

  √ **Personnel responsible** for checking the "Contacts to Update" folder will add, update, or remove information within the PFGE contacts as necessary.

  _____

- If the PulseNet participant has notified CDC that they will no longer be performing PulseNet-related duties, the **PulseNet Database Unit Chief** sends them an email (see appendix PNG02-2 for template) to make sure they return their SecurID key fob (if analysis-certified) and to let them know that access to WebBoard and the National Database(s) will be removed. The email will be cc'ed to the main contact of the participant's Area Laboratory. If there is a new PulseNet participant, the PulseNet Database Unit Chief will send them the "New Contact Information Sheet" (appendix PNG02-3) to complete and return to PFGE@cdc.gov.

  _____

- **PulseNet IT Support** will add, update or remove access information for WebBoard and the National Database(s) and collect SecurID key fobs as they are returned to CDC. On an annual basis, will verify contact information for those with access to the National Database(s).

  _____

- **PulseNet QA/QC Manager(s)** will add, update or remove certification and proficiency testing information as necessary.

  _____

- **PulseNet News Editor(s)** will update newsletter distribution documents and ensure any welcomes or farewells are printed in the next issue as appropriate.

  _____

- **PulseNet Methods and Development Reference Unit Chief** and the **Listeria Identification and Subtyping Unit Chief** will ensure all laboratory personnel and documents are updated as necessary.

  _____

Appendix PNG02-2

Template: Email to a participant who is either changing positions (no longer performing PulseNet-related duties) or departing the laboratory and cc the main contact at the participant's Area Laboratory

Dear *PulseNet Participant,*

We are sorry to hear that you will be leaving PulseNet. It has been a pleasure working with you.

If you were analysis-certified, please make sure to return your SecurID key fob to CDC at the address provided below.

Attn: Michael Korth
Centers for Disease Control
1600 Clifton Rd, NE
MS-C03
Atlanta, GA 30333
(404) 639-3334

Your access to the PulseNet WebBoard and the national database(s) will be removed as of *mm/dd/yyyy (date leaving).*

The main contact for your Area Laboratory has been cc'ed on this email in order to maintain communication flow.

If you have any questions or additional information (i.e. who will be taking over your PulseNet-related duties), please let us know.

We wish you the best of luck with all future endeavors.

Thank you for your hard work and dedication,

PulseNet Database Team

Appendix PNG02-3

New contact Information Sheet

Prefix: Mr./Ms./Mrs./Dr.

First Name:_____

Last Name: _____

Suffix: Jr./Sr./PhD/MS/MPH/other _____

Position Title: _____

Hire Date: ___/___/_____

% of time spent on PN-related activities: _____%

Public Health Lab: _____

Branch: _____

Unit: _____

Business: ( ) _____-_____ Ext. _____

Fax: ( ) _____-_____

E-mail1:_____

E-mail2:_____

Address:_____

_____

City: _____ State: _____ Country: _____ Zip: _____

Would you like to receive the PulseNet Newsletter? Yes ____ No ____

STANDARD OPERATING PROCEDURE FOR HANDLING INQUIRIES FROM MEMBERS OF THE MEDIA	CODE: PNG03 Effective Date: 5 9 05

1. PURPOSE: To describe guidelines to handle inquiries from members of the media.

2. SCOPE: This procedure applies to all PulseNet, NCID, CDC media-related inquiries.

3. DEFINITIONS/TERMS:

3.1 SOP: Standard Operating Procedure
3.2 NCID: National Center for Infectious Diseases
3.3 CDC: Centers for Disease Control and Prevention

4. RESPONSIBILITIES:

4.1 If approached by the media in the office, at home, or on the road, NCID staff should notify NCID media relations.

5. PROCEDURE:

5.1 Take down the reporter's name and affiliation and contact NCID media relations.
  5.1.1 Contact the NCID Senior Press Officer or Press Officer.
5.2 A press officer will evaluate the request, provide communications guidance, and ensure appropriate coordination or clearance is achieved.
5.3 Please express to the reporter our willingness to help, but that all requests must come through the press office.
  5.3.1 Press officers are available 24 hours a day, seven days a week, 365 days a year.
5.4 In addition, the CDC Division of Media Relations press officers can be reached during work hours at 404-639-3286, and after hours daily at 404-639-2888. Ask for the on-call press officer.
5.5 For additional information regarding CDC media relations, visit: http://www.cdc.gov/od/oc/media/index.htm

6. FLOW CHART:

7. BIBLIOGRAPHY:

8. CONTACTS:
8.1 NCID Senior Press Officer Dave Daigle, W: (404) 639-1143; C: (404) 353-7449; E-mail: drd4@cdc.gov
8.2 NCID Press Officer, Jennifer Morcone, W: (404) 639-1690; C: (404) 867-7493; E-mail: zgy5@cdc.gov

9. AMENDMENTS:

1. PURPOSE: To describe the steps necessary to address inquiries from lawyers, or Freedom of Information Act (FOIA) requests.

2. SCOPE: All CDC laboratory personnel must follow this procedure regardless of the source or scope of the request. All non-CDC labs MUST contact their State Attorney General's Office for all regulations specific to their state.

3. DEFINITIONS/TERMS:

3.1 SOP: Standard Operating Procedure
3.2 FOIA: Freedom of Information Act
3.3 BMD: Bacterial and Mycotic Diseases
3.4 OCOO: Office of the Chief Operating Officer
3.5 EIS: Epidemic Intelligence Service: EIS officers are epidemiologists who are given two-year appointments to help in the investigation of foodborne outbreaks.

4. RESPONSIBILITIES/PROCEDURE:

4.1 All requests for FOIA information must come through CDC's FOIA office.
4.1.1 FOIA office will distribute requests to the branch secretary.
a. The branch secretary will deliver to the appropriate laboratory, OR
b. The branch secretary will deliver to the supervising epidemiologist who determines who needs to work on it and in what order.
(1) EIS officer
(2) Laboratory
4.1.2 Upon receipt in the laboratory section, administrative support will log the request into the FOIA tracking logbook.
a. Administrative support will deliver the FOIA request to the laboratory Section Chief with the tracking form attached.
b. Administrative support will deliver the FOIA request to appropriate laboratory personnel as indicted on the tracking form.
(1) In the laboratory, FOIAs are received and distributed to appropriate personnel by the Unit Chief. Once completed, a summary of findings is attached. The Unit Chief forwards the FOIA request to the next person listed on the routing slip.
(2) Once the last person has seen the FOIA request, it is then returned to administrative support.
c. After all information has been compiled and FOIA is completed:
(1) Administrative support will return the FOIA request to the laboratory Section Chief for review and signatures.
(2) Administrative support will perform cost analysis and make copies.
4.1.3 The branch secretary will return the FOIA request to CDC's FOIA office.
4.2 Freedom of Information Act requests
4.2.1 CDC/FDDB Policy on FOIA Requests
4.2.1.1 Determine if request is reasonable:
a. If yes, provide copies of records after redacting information that may infringe on another individual's rights.
b. If no, suggest that requester narrow the scope of the request.
4.2.1.2 Provide the requested information **after** an ongoing investigation is completed and a final report is prepared.
4.2.1.3 Provide only existing records; **do not** create new records.
4.2.1.4 May charge for records (fees for copying, faxing and mailing records).
4.2.2 How to handle requests for food samples
4.2.2.1 No legal basis exists to force compliance with request (CA Supreme Court decision)

4.2.2.2 General strategy

a. Keep whatever is needed for public health purposes.
b. Give whatever you can spare to a designated laboratory.
c. Direct all sample requests to the person/organization that originally provided the sample.
d. Send back any unused samples.

4.2.3 How to handle requests for patient isolates

4.2.3.1 No clear directive on sharing isolates.

4.2.3.2 Discretionary, but if you provide isolates to one, you may have to provide to others.

4.2.3.3 CDC/FDDB policy is to wait until an investigation is completed before providing isolates to outsiders.

4.2.3.4 CDC/FDDB often charges for expenses incurred in providing isolates.

4.2.3.5 If requester provides a public-health-related justification, it is difficult to deny access to isolates.

4.3 Requests from lawyers

4.3.1 Attorneys may request information from federal establishments under the Freedom of Information Act.

4.3.1.1 Federal statutes apply:

a. Only to records, not samples or isolates.
b. Only to documents or records that were previously produced (not required to create a document for a FOIA).
c. Only to those records that do not infringe on a person's privacy.

4.3.1.2 States may have different requirements

a. Some states have a FOIA equivalent:
(1) Open Records Act
(2) Public Records Act

4.3.2 Refer questions to the laboratory Section Chief or to the attorney's office in OCOO.

5. FLOW CHART:

6. BIBLIOGRAPHY:

7. CONTACTS:

8. AMENDMENTS:

1. PURPOSE: To describe the expectations for and responsibilities of a PulseNet participating laboratory.

2. SCOPE: This procedure applies to all PulseNet participating laboratories.

3. DEFINITIONS/TERMS:

3.1 APHL: Association of Public Health Laboratories
3.2 PFGE: Pulsed-field Gel Electrophoresis
3.3 CDC: Centers for Disease Control and Prevention
3.4 PulseNet Laboratory: A laboratory that performs PFGE using the approved CDC protocols and receives support funding via the Epidemiology and Laboratory Capacity (ELC) or Emerging Infections Program (EIP) federal grants.
3.5 QA/QC: Quality Assurance/Quality Control
3.6 SOP: Standard Operating Procedure
3.7 PulseNet-related Duties: Duties related to PulseNet work, e.g., preparing PFGE gels, analyzing TIFFs of PFGE gels, posting to the PulseNet workspace on CDC Team.
3.8 BioNumerics: Gel analysis software used by PulseNet, developed by Applied Maths, Belgium
3.9 TIFF: Tagged Image File Format. A file of a gel image that can be analyzed in BioNumerics
3.10 PulseNet Workspace on CDC Team: A closed, web-based collaboration application used for communication among PulseNet participants. The PulseNet Workspace on CDC Team is open to all laboratory staff at PulseNet participating laboratories. Epidemiologists working in collaboration with these laboratories, when approved by the PulseNet laboratory contact, U.S. food regulatory staff, and PulseNet International representatives may also have access. The PulseNet Workspace on CDC Team is not open to university or private industry personnel unless their inclusion is deemed to be in the interest of public health. (http://team.cdc.gov). For additional information on CDC Team please refer to SOP PND03.
3.11 PulseNet Certification Program: Program that tests an individual's ability to perform PFGE on a specific organism using the standard protocols, perform analysis, create bundles, and upload patterns to the National Database.
3.12 PulseNet Proficiency testing program: Program that regularly tests the ability of an individual or laboratory to perform PFGE and pattern analysis
3.13 Support zone: A group of state and local health departments that can utilize laboratory services provided by their Area Laboratory
3.14 PulseNet Area Laboratory: Laboratory, designated by CDC, which has agreed to assume responsibility for additional PulseNet duties for laboratories within their support zone. The current Area laboratories include MA, MN, WA, TX, VA, UT, MI and CDC.

4. RESPONSIBILITIES:

4.1 PulseNet laboratories must, at a minimum, perform PFGE on PulseNet-tracked organisms as requested by CDC or state epidemiologists.
  4.1.1 The organisms currently tracked by PulseNet are *E. coli* O157:H7, Non O157 (STEC), *Listeria monocytogenes*, *Salmonella*, *Shigella*, *Campylobacter jejuni*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *and Clostridium botulinum*.
  4.1.2 If unable to complete this testing in-house in real time, the laboratory must forward the isolates immediately to CDC or to the appropriate Area Laboratory for subtyping.
4.2 PulseNet laboratories must adhere to the protocols and requirements of the PulseNet QA/QC manual available on the PulseNet Workspace on CDC Team.
4.3 Individuals performing PulseNet-related duties must submit certification file(s) and have them reviewed before being able to submit TIFF images to the PulseNet National Databases (PNQ02).
4.4 Certified laboratories performing PulseNet-related duties must participate in the annual CDC- sponsored proficiency-testing program (PNQ04).

4.5 PulseNet laboratories must submit *all* PFGE patterns and corresponding information to the PulseNet national database (if certified for pattern upload) or to the PulseNet database team at CDC within 24 hours of the day results are generated.
- 4.5.1 Real-time surveillance should be the goal of all PulseNet laboratories and should involve real-time subtyping of PulseNet tracked organisms that are received by the laboratory.
- 4.5.2 CDC does not require subtype information of isolates generated by PFGE or another PulseNet subtyping method, with the same serotype and same PFGE pattern, from the same patient, collected within 6 months of each other to be uploaded to the PulseNet National Databases unless the subtypes of the isolates are different.
  - 4.5.2.1 If a duplicate isolate (one from the same patient, with the same serotype and PFGE pattern within 6 months) is run by PFGE, please use the best representation of the pattern produced to submit to the national database.

4.6 PulseNet laboratories will agree to store isolates that have been subtyped by PFGE for as long as space will allow and for a minimum of 1 year. These isolates will be submitted to CDC when requested for isolate collections or epidemiologic investigations.

4.7 PulseNet laboratories must work with their epidemiology department on a routine basis for the investigation of clusters and outbreaks.

4.8 PulseNet staff must sign and submit the "PulseNet CDC Team Non-disclosure Agreement" to receive access to the PulseNet workspace on CDC Team (see SOP PND03).

4.9 PulseNet laboratories must post information to the PulseNet Workspace on CDC Team on foodborne disease clusters and outbreaks occurring in their state in a timely manner and must respond to postings within 48 hours (PND03).

4.10 PulseNet laboratories must send at least one representative to the Annual PulseNet Update Meeting.

4.11 PulseNet laboratories must send at least one representative to Regional PulseNet Meetings hosted by their assigned Area Laboratory.

4.12 PulseNet laboratories are expected to notify APHL, their Area Laboratory, and CDC when new laboratory staff begins performing PulseNet-related duties and/or when existing laboratory staff will no longer be performing PulseNet-related duties (see SOP PNG02).

4.13 PulseNet laboratories are expected to write articles for the PulseNet News newsletter when requested by APHL and/or CDC.

4.14 PulseNet Laboratories will participate in national and regional conference calls organized by APHL, their Area Laboratory, or CDC.

5. PROCEDURES:

5.1 Labs that are interested in becoming a PulseNet participant must contact the PulseNet Program Manager at APHL and the PulseNet Database Team at CDC (see SOP PNG08 for information on becoming a PulseNet Laboratory).

5.2 Prior to being considered a PulseNet laboratory, the laboratory must acquire the necessary equipment, reagents and supplies, communication tools, and software (PNL01, PNL02, PND01).

5.3 PulseNet participating laboratories must complete a PulseNet-sponsored training (PNL17, PND10), subscribe to the PulseNet Workspace on CDC Team (PND03), and request the *Salmonella* ser. Braenderup H9812 standard strain, the latest version of the PulseNet MasterScripts for the PulseNet customized version of BioNumerics from the PulseNet Database Team, and a copy of the PulseNet QA/QC Manual. All laboratories are expected to maintain knowledge of the current laboratory methods and software configurations utilized by the network.

5.4 PulseNet laboratories must perform PFGE digested with two enzymes on all *E. coli* and *Listeria monocytogenes* isolates immediately after receipt in their laboratory.

5.5 When PulseNet participating labs submit data to the National Database(s) (either directly or to the PulseNet Database Team), as much information as possible should be included. At a minimum, the required information includes (using pick lists whenever applicable):
- 5.5.1 City and/or County (if known)
- 5.5.2 State

5.5.3 Species/Serotype (even if this is undetermined, pending, untypable)
5.5.4 Source Type (even if unknown)
5.5.5 Source Site (even if unknown)
5.5.6 Other State Isolate Number (when applicable)
5.5.7 Isolation Date
5.5.8 Received Date
5.5.9 Age and Sex

6. FLOW CHART:

7. BIBLIOGRAPHY:

8. CONTACTS:

8.1 APHL:
Kristy Kubota MPH
Association. of Public Health Laboratories
Phone (240) 485-2720
Fax (240) 485-2700
Kristy. Kubota@aphl.org

8.2 Centers for Disease Control and Prevention:
Kelley Hise MPH
Phone: (404) 639-0704
PulseNet: (404) 639-4558
Fax: (404) 639-3333
pfge@cdc.gov

9. AMENDMENTS:

9.1 2011-09-13 Section 4.11 was added: PulseNet laboratories must send at least one representative to Regional PulseNet Meetings hosted by their assigned Area Laboratory.

1. PURPOSE: To describe the responsibilities and duties of a PulseNet Area Laboratory.

2. SCOPE: This procedure applies to all PulseNet Area Laboratories.

3. DEFINITIONS/TERMS:

3.1 PFGE: Pulsed-field Gel Electrophoresis
3.2 CDC: Centers for Disease Control and Prevention
3.3 SOP: Standard Operating Procedure
3.4 Area laboratory: Laboratory, designated by CDC, which has agreed to assume responsibility for additional PulseNet duties for laboratories within their support zone. The current Area Laboratories include MA, MN, WA, TX, VA, UT, MI, and CDC
3.5 Support zone: A group of state and local health departments that is served by a particular Area laboratory
3.6 BioNumerics: Gel analysis software used by PulseNet developed by Applied Maths, Belgium
3.7 PulseNet Listserv: Secure, closed unmoderated Listserv used by registered PulseNet participants to post possible clusters or outbreaks, PulseNet-related questions, technical information, and responses to postings
3.8 Cluster: A group of isolates with the same serotype with indistinguishable PFGE DNA patterns by one or more restriction enzyme
3.9 DNA: Deoxyribonucleic acid
3.10 PulseNet Standardized PFGE 24 hour Protocols: Protocols or standard operating procedures followed by all laboratories participating in the PulseNet program in order to submit DNA fingerprint patterns for inclusion in the National Database
3.11 Troubleshoot: To investigate a problem and come up with solutions
3.12 Surge capacity: Ability to provide additional testing when workload exceeds existing capacity
3.13 Bundle file: A file with a .bdl extension that is produced in BioNumerics and contains the analysis of at least one lane of a gel image and may include specified isolate demographic information
3.14 Foodborne disease epidemiologist: A trained individual who studies and/or investigates the transmission and control of foodborne diseases
3.15 Outbreak: A cluster of cases of infections with a common epidemiological exposure, e.g. to a specific food product.
3.16 QA/QC: Quality Assurance and Quality Control

4. RESPONSIBILITIES/ PROCEDURES:

4.1 PulseNet Area Laboratories must fulfill the responsibilities of all PulseNet laboratories (PNG05).
4.2 Train laboratory personnel in their support zone to perform PFGE, image analysis, and data analysis using BioNumerics software when requested.
  4.2.1 Train laboratory personnel on all aspects of the PulseNet Standardized PFGE protocols and the safety requirements associated with performing the testing. Upon completion of training, the trainees should be competent to perform all steps of the protocol, including preparing plugs, performing cell lysis, digesting DNA with a restriction endonuclease, preparing and loading an agarose gel, and preparing all required reagents.
  4.2.2 Train laboratory personnel on image acquisition and the use of BioNumerics software (version 3.5 or greater). Upon completion of training, laboratory personnel will be competent to visually evaluate gel and fingerprint quality using the PulseNet TIFF Quality Grading Guidelines released in May 2004, recognize reasons for poor banding or image quality (i.e. incomplete restriction, ghost bands, sub par resolution, etc.), provide technical suggestions for quality improvements, and electronically save acceptable gel images as TIFF files. Laboratory personnel will be competent to use BioNumerics software to define a gel strip lane, normalize a gel, correctly assign DNA fingerprint band positions according to the PulseNet Gel Analysis Guidelines (PND04), compare PFGE DNA fingerprint patterns to a locally established database, and generate a bundle file for submission of images to the PulseNet mailbox (pfge@cdc.gov).

4.2.3 Once a lab is certified for analysis and receives a SecurID fob, they should be trained on how to compare patterns in their local database(s) to patterns on the national database. Advanced BioNumerics training is encouraged for all personnel.

4.2.4 Train laboratory personnel how to access the PulseNet Listserv, respond to postings, post a new cluster (PND03), download a bundle from the Listserv, and compare the downloaded pattern to a local database to identify possible matching fingerprint patterns.

4.3 Provide PFGE troubleshooting assistance to states in their support zone when requested.

4.3.1 Work with laboratory personnel to critically troubleshoot and identify possible solutions to technical problems with gel quality, image analysis or fingerprint pattern analysis using BioNumerics software.

4.3.2 Provide laboratory personnel with appropriate contact person(s) at the CDC to address problems the area laboratory is not able to resolve.

4.4 Provide PulseNet surge capacity for laboratories in an area laboratory support zone when requested.

4.4.1 In instances where a laboratory cannot perform PFGE on all mandatory isolates (*E. coli* 0157:H7, *Listeria monocytogenes*, and designated *Salmonella* serotypes or other organisms involved in an outbreak), the area laboratory will perform PFGE on these isolates as requested by the support zone laboratory. Situations requiring area laboratory surge capacity testing support may include outbreaks, response to enhanced pathogen surveillance, loss of support zone laboratory certification, or staffing shortages at the support zone laboratory.

4.4.2 Upon completion of PFGE, the area laboratory will upload the PFGE DNA fingerprint patterns to the appropriate national database.

4.4.3 The area laboratory will email a TIFF and bundle file containing the completed PFGE DNA fingerprint patterns to the support zone laboratory for inclusion in the state's local database.

4.5 Coordinate multi-state outbreak investigations in their support zone at CDC's request.

4.5.1 Provide regular updates to the foodborne disease laboratorians in affected states regarding new isolates posted to the national database that are indistinguishable from the outbreak pattern.

4.5.2 Communicate with affected states to verify that all isolates associated with an outbreak are being subtyped by PFGE in real-time, and if not, coordinate transport of isolates to area laboratory for testing.

4.5.3 Provide surge capacity testing for support zone states as necessary.

4.5.4 Update PulseNet Listserv when relevant demographic data, epidemiologic or laboratory information is available.

4.6 Perform advanced surveillance of non-routine pathogens, including but not limited to *Vibrio* sp., *Clostridium* sp., *Campylobacter* sp., and rare *Salmonella* serotypes in their support zone when requested.

4.6.1 Become proficient in the protocols required to perform PFGE on non-routine pathogens.

4.6.1.1 Perform PFGE DNA fingerprinting of non-routine isolates as requested by states within the support zone.

4.6.1.2 Create and maintain databases for non-routine organisms under surveillance by the PulseNet program.

4.7 Evaluate new software and laboratory procedures or procedural modifications of existing procedures when requested by CDC.

4.7.1 Assist with the evaluation and beta-testing of new software scripts and programs or laboratory protocols from CDC prior to general release.

4.7.2 Provide technical recommendations to the CDC regarding software changes that may improve its use at the state level.

4.8 Participate in research and development projects independently and with CDC.

4.8.1 Project or grant announcements will be distributed via the PulseNet Listserv and by APHL.

4.8.2 Data should be collected according to the protocol or grant and shared with CDC upon request.

4.8.3 States should be willing to work with CDC and other involved states on joint publications and presentations, where applicable.

4.9 Provide guidance for program issues and make recommendations for development of the PulseNet network.

4.9.1 Participate on the PulseNet Steering Committee, upon invitation.

4.9.2 Participate in QA/QC meetings, upon request.

4.9.3 Participate in Area Laboratory conference calls.

4.9.4 Review PulseNet documents.

4.9.5 Participate in Annual Update Meeting (speaker, moderator, etc.).

4.10 Host regional and national PulseNet update meetings and training conferences.

4.10.1 Designate a laboratory point of contact to coordinate training conference planning activities.

4.10.2 Provide conference resources as defined in the planning meetings.

4.10.3 Work with designated agencies to provide state and laboratory resources for the conference.

4.10.4 Provide assistance to other Area Lab states or non-Area Lab states that may offer to host the meeting following location approval by the CDC.

5. FLOW CHART:

6. BIBLIOGRAPHY:

7. CONTACTS:

8. AMENDMENTS:

1. **PURPOSE**: To describe the guidelines for the access and use of information within the PulseNet USA and PulseNet Canada national databases.

2. **SCOPE:** This SOP applies to PulseNet USA and PulseNet Canada personnel who will have read-only access to each other's national databases. Personnel at PulseNet Canada must comply to be granted access to PulseNet USA's national databases and personnel at PulseNet USA must comply to be granted access to PulseNet Canada's national databases. This SOP is a "living" document, subject to modifications after discussions between PulseNet USA and PulseNet Canada personnel.

3. DEFINITIONS/TERMS:

3.1 SOP: Standard Operating Procedure
3.2 PFGE: Pulsed-field Gel Electrophoresis
3.3 QA/QC: Quality Assurance/Quality Control
3.4 CDC: Centers for Disease Control and Prevention
3.5 DNA: Deoxyribonucleic acid
3.6 MOU: Memorandum of Understanding
3.7 BioNumerics: Gel analysis software used by PulseNet, developed by Applied Maths, Belgium
3.8 National database: Database that houses all isolate and image data for a particular organism at the national level
3.9 TIFFs: Tagged Image File Format, a file of a gel image that can be analyzed in BioNumerics
3.10 Bundle File: A file with a .bdl extension that is produced in BioNumerics and contains the analysis of at least one lane of a gel image and may include specified isolate demographic information
3.11 Cluster: A group of isolates, identified within the past 30 days for *Yersinia*; 60 days for *Salmonella*, *E. coli*, *Shigella*, *Campylobacter*, and *Vibrio*; and 120 days for *Listeria*, with the same serotype with indistinguishable PFGE DNA patterns by one or more restriction enzymes.
3.12 Outbreak: A cluster of cases of infections, identified within the past 30 days for *Yersinia*; 60 days for *Salmonella*, *E. coli*, *Shigella*, *Campylobacter*, and *Vibrio*; and 120 days for *Listeria*, with a common epidemiological exposure, e.g. to a specific food product.
3.13 PulseNet Listserv (CDC PulseNet WebBoard): A closed, moderated, electronic web conference used for communication among PulseNet participants. The PulseNet Listserv is open to all laboratory staff at PulseNet participating laboratories. Epidemiologists working in collaboration with these laboratories, when approved by the PulseNet laboratory contact, U.S. food regulatory staff, and PulseNet International representatives may also have access. The PulseNet Listserv is not open to university or private industry personnel unless their inclusion is deemed to be in the interest of public health.
3.14 SecurID key fob: Token that displays a six-digit pass code. When used in combination with a four-digit pin number, allows access through the CDC firewall.
3.15 VPN: Virtual Private Network
3.16 PulseNet certification program: Program that tests an individual's ability to perform PFGE on a specific organism using the standard protocols, perform analysis, create bundles, and upload patterns to the PulseNet USA national database.
3.17 PulseNet proficiency testing program: Program that regularly tests the ability of an individual or a laboratory to perform PFGE and pattern analysis.

4. RESPONSIBILITIES AND PROCUDURE:

4.1. Accessing the national databases

4.1.1. Prior to accessing either country's national database(s), an individual must:

4.1.1.1. Have received approval from their supervisor to request access

4.1.1.2. Be analysis-certified. The individual must submit certification file(s) to CDC and become analysis-certified according to PulseNet USA's SOP PNQ02 for the database for which they are requesting access (e.g. to be granted access to the *Salmonella* database, one must be analysis-certified for *Salmonella*). Within the certification submission email, the individual must state that he or she is requesting access to the Canadian and/or USA national database.

4.1.1.2.1. If an individual in PulseNet Canada is already analysis-certified and needs access to the USA national database, the individual will receive approval from the PulseNet Canada Section Head, who will send a request via email to PFGE@cdc.gov.

4.1.1.2.2. If an individual in PulseNet USA is analysis-certified and needs access to a PulseNet Canada national database, the individual will receive approval from the PulseNet USA Database Team Leader, who will send a request via email to PN_Canada@phac-aspc.gc.ca.

4.1.2. Individuals accessing the PulseNet USA national database(s) will receive a SecurID keyfob, instructions on how to set up the PIN (see SOP PND08), database login(s) and password(s).

4.1.2.1. Once analysis-certification is successfully completed, database access will be requested by the PulseNet USA QA/QC Manager(s)

4.1.2.2. SecurID keyfob, database login(s), and password(s) will be mailed to the individual

4.1.3. Individuals accessing the PulseNet Canada national database(s) will receive a VPN, instructions on how to set it up, database login(s) and password(s).

4.1.3.1. Once analysis-certification is successfully completed, database access will be requested by the PulseNet Canada Section Head.

4.1.4. To maintain access to the national database(s), laboratories must successfully complete annual proficiency testing (for each organism certified) sponsored and conducted by CDC as described in SOP PNQ04.

4.1.5. Instead of emailing questions about possible matches to ongoing clusters or outbreaks and/or historical data, each country will be able to access this information directly to perform their own comparisons on an "as needed basis."

4.2. Reporting significant findings

4.2.1. If PulseNet USA finds a potential match (or matches) to a cluster or an outbreak within the PulseNet Canada national database, PulseNet Canada **must** be notified by email, PN_Canada@phac-aspc.gc.ca or phone (204) 789-5067. PulseNet Canada is then responsible for deciding if there is something significant to report and if so, how to proceed (i.e. reporting to epidemiologists, posting to a listserv, and/or taking any additional action).

4.2.2. If PulseNet Canada finds a potential match (or matches) to a cluster or outbreak within the PulseNet USA national database, PulseNet USA **must** be notified by email, PFGE@cdc.gov or phone (404) 639-4558. PulseNet USA is then responsible for deciding if there is something significant to report and if so, how to proceed (i.e. reporting to epidemiologists, posting to a listserv, and/or taking any additional action).

4.2.3. If one country wishes to use data found in the other's database for a project, report, and/or publication, this **must** be discussed and approved by both laboratories before work commences.

5. FLOW CHART:

6. BIBLIOGRAPHY:

7. CONTACTS:

7.1 PulseNet USA
Kelley Hise
Database Team Leader
Centers for Disease Control and Prevention
1600 Clifton Road, NE
MS-C03
Atlanta, GA 30333
PFGE@cdc.gov
(404) 639-4558

7.2 PulseNet Canada
Celine Nadon
Research Scientist and Section Head, PulseNet Canada
National Microbiology Laboratory
Public Health Agency of Canada
1015 Arlington Street
Winnipeg MB R3E 3R2
Canada
celine_nadon@phac-aspc.gc.ca, PN_Canada@phac-aspc.gc.ca
(204) 784-7507

8. AMENDMENTS:

1. **PURPOSE:** To describe the application process for becoming a PulseNet participating laboratory and to describe the steps to process this request through CDC and APHL.

2. **SCOPE:** This procedure applies to CDC, APHL and all laboratories that are interested in becoming PulseNet participants.

3. **DEFINITIONS/TERMS:**

3.1. APHL: Association of Public Health Laboratories
3.2. PFGE: Pulsed-field Gel Electrophoresis
3.3. CDC: Centers for Disease Control and Prevention
3.4. USDA: United States Department of Agriculture
3.5. FDA: Food and Drug Administration
3.6. EDLB: Enteric Diseases Laboratory Branch
3.7. QA/QC: Quality Assurance/Quality Control
3.8. SOP: Standard Operating Procedure
3.9. EISO: Epidemic Intelligence Service Officer
3.10. PulseNet-related Duties: Duties related to PulseNet work, e.g., preparing PFGE gels, analyzing TIFFs of PFGE gels, participation on the PulseNet SharePoint site (duties and responsibilities detailed in SOP PNG05)
3.11. BioNumerics: Gel analysis software used by PulseNet developed by Applied Maths, Belgium
3.12. TIFF: Tagged Image File Format. A file of a gel image that can be analyzed in BioNumerics
3.13. PulseNet SharePoint Site: A closed, web-based discussion forum used for communication among PulseNet and OutBreakNet participants. SharePoint is open to all laboratory staff at PulseNet participating laboratories and Epidemiologists working in collaboration with these laboratories, U.S. food regulatory staff, and some PulseNet International representatives may also have access. SharePoint is not open to university or private industry personnel unless their inclusion is deemed to be in the interest of public health. Throughout this document, the PulseNet SharePoint site will be referred to as "SharePoint."
3.14. Certification: Program that tests an individual's ability to perform PFGE on a specific organism using the PulseNet standardized protocols, perform analysis, create bundle files, and upload patterns to the national database.
3.15. Proficiency Testing: Program that annually tests the ability of a certified PulseNet-participating laboratory to perform PFGE, pattern analysis, and upload results to the proficiency testing database.
3.16. Area Laboratory: Laboratory, designated by CDC and APHL, which has agreed to assume responsibility for additional PulseNet duties for laboratories within their support region. The current Area Laboratories include MA, MN, WA, TX, VA, UT, MI and CDC.
3.17. Contacts Database**:** Access database where all PulseNet contact information, including but not limited to name, address, phone, fax, email, laboratory information, certification status, mailing lists, and any additional comments is housed. The database is located \\cdc\project\CCID_NCZVED_DFBMD_PulseNet\Admin\Contacts.
3.18. PulseNet Steering Committee: A committee chaired by the chief of the EDLB at CDC and comprised of participants from state, local and agriculture PulseNet USA laboratories, USDA, FDA, APHL, and CDC. The steering committee meets via conference call several times a year to guide the expansion, improvement, and evaluation of the PulseNet USA program.

4. **RESPONSIBILITIES:**

4.1. PulseNet-participating laboratories currently include state, city, county, food regulatory (USDA and FDA), agriculture and veterinary labs. PulseNet is not open to university or private industry laboratories unless their inclusion is deemed to be in the interest of public health.
4.2. Prior to being considered a PulseNet laboratory, the lab must acquire the necessary equipment, reagents and supplies, communication tools, and software (refer to SOPs PNL01, PNL02, PND01, and PND03 for detailed information).
4.3. Laboratories must agree to follow the responsibilities of a PulseNet-participating laboratory as described in SOP PND05.

5. PROCEDURE:

5.1. Laboratories interested in becoming a PulseNet participant must contact CDC (see section 8 for contact information).

5.1.1. All requests sent to PFGE@cdc.gov will be moved to the folder "Prospects" in the "Participants" folder within the PFGE inbox.

5.1.2. Once the email has been forwarded to CDC's PulseNet USA leadership and copied to APHL, the email should be marked with the forwarder's initials and flagged with what action was taken (Ex: KH: fwd to PGS and APHL).

5.2. The PulseNet USA Steering Committee will review and approve or disapprove the laboratory's request to become a PulseNet participating laboratory.

5.2.1. If the PulseNet USA Steering Committee does not approve of the laboratory's participation in PulseNet, the PulseNet Database Unit Chief must email the laboratory and copy APHL.

5.2.2. If the PulseNet USA Steering Committee approves of the lab's participation in PulseNet, the PulseNet Database Unit Chief will determine, in collaboration with APHL, level of participation expected from the laboratory. For example, some laboratories may only become gel-certified and may not receive access to the national databases.

5.3. APHL will verify the laboratory's APHL membership status and determine any costs associated with PulseNet participation. For example, if a lab is not an APHL member, there are fees associated with PulseNet MasterScripts, certification, proficiency testing, and training courses held by CDC (see appendix PNG08-1 for more information).

5.4. If the laboratory is located in a state or facility where there already are PulseNet-participating laboratories, as a courtesy, the pre-existing PulseNet lab will be notified by APHL. For example, if a state's agricultural laboratory would like to participate, the state laboratory must be notified of the agricultural lab's request to participate.

5.5. Once CDC and APHL have determined the laboratory's level of participation, this information is sent via email to "The Distribution List" (see PNG02 for more information regarding "The Distribution List" and the In-Processing procedure for new participants) to notify others at CDC and APHL. Contact information (name, laboratory, address, phone, fax, and email) must also be provided. This information will be entered into the Contacts Database and the new lab/person will be listed as a "Prospect."

5.5.1. The Distribution List:

PFGE inbox	PFGE@cdc.gov
Kelley Hise	Kpb6@cdc.gov
Molly Freeman	Evy7@cdc.gov
Jennifer Adams	Izk9@cdc.gov
Efrain Ribot	Eyr4@cdc.gov
Deborah Sheehan	Zde9@cdc.gov
Eija Trees	Eih9@cdc.gov
Kristy Kubota	Kristy.kubota@aphl.org

5.6. CDC will email the PulseNet Memorandum of Understanding (appendix PNG08-2) and the Terms of Reference (appendix PNG08-3) to the prospective laboratory. The laboratory director must agree to abide by the terms set forth in these documents and return the Memorandum of Understanding signed and dated to CDC. Address and contact information provided in section 8 below.

5.7. The PulseNet Database Unit Chief will assign a PulseNet LabID. This information is entered in the spreadsheet "LabIDs.xls" which is saved under \\Cdc\project\CCID_NCZVED_DFBMD_PulseNet\Admin\PN Participants.

5.7.1. PulseNet LabIDs consist of two to four characters as follows:

5.7.1.1. For state laboratories the LabID is the two letter state postal code

5.7.1.2. For city or county laboratories the LabID is the two letter state postal code and two appropriate characters designated by the PulseNet Database Unit Chief

5.7.1.3. For government laboratories the PulseNet Database Unit Chief will determine the most appropriate LabID

5.7.1.4. For agricultural laboratories the LabID is the two letter state postal code and "AG" for agricultural

5.7.1.5. For veterinary laboratories the LabID is the two letter state postal code and "VT" for veterinary

5.7.1.6. For university laboratories the PulseNet Database Unit Chief will determine the most appropriate LabID

5.7.1.7. For international laboratories the PulseNet Database Unit Chief will determine the most appropriate LabID (in some cases the international hubs will assign their own LabIDs).

5.8. The PulseNet Database Unit Chief will send an email welcoming the laboratory to PulseNet and list any associated costs for non-APHL members. This email will be copied to APHL, the appropriate Area Laboratory (see appendix PNG08-4 for email template), the person who initiated contact with CDC and/or APHL, and any associated state laboratories.

5.8.1. Laboratories that must purchase the PulseNet MasterScripts will send payment to APHL as described in appendix PNG08-1.

5.8.2. The personnel evaluating certifications and proficiency tests will notify APHL by email of participating laboratories that are not APHL members so they may be billed for this testing according to PNG08-1.

5.9. The PulseNet Database Unit Chief will send a starter packet to the participating laboratory. The packet will include:

5.9.1. Welcome letter containing their PulseNet LabID, contact information (including websites) for CDC, APHL and their Area Laboratory (see appendix PNG08-5 for template)

5.9.2. Responsibilities of a PulseNet Laboratory SOP PNG05

5.9.3. Access and Use of the PulseNet SharePoint site SOP PND03

5.9.4. Certification SOP PNQ02

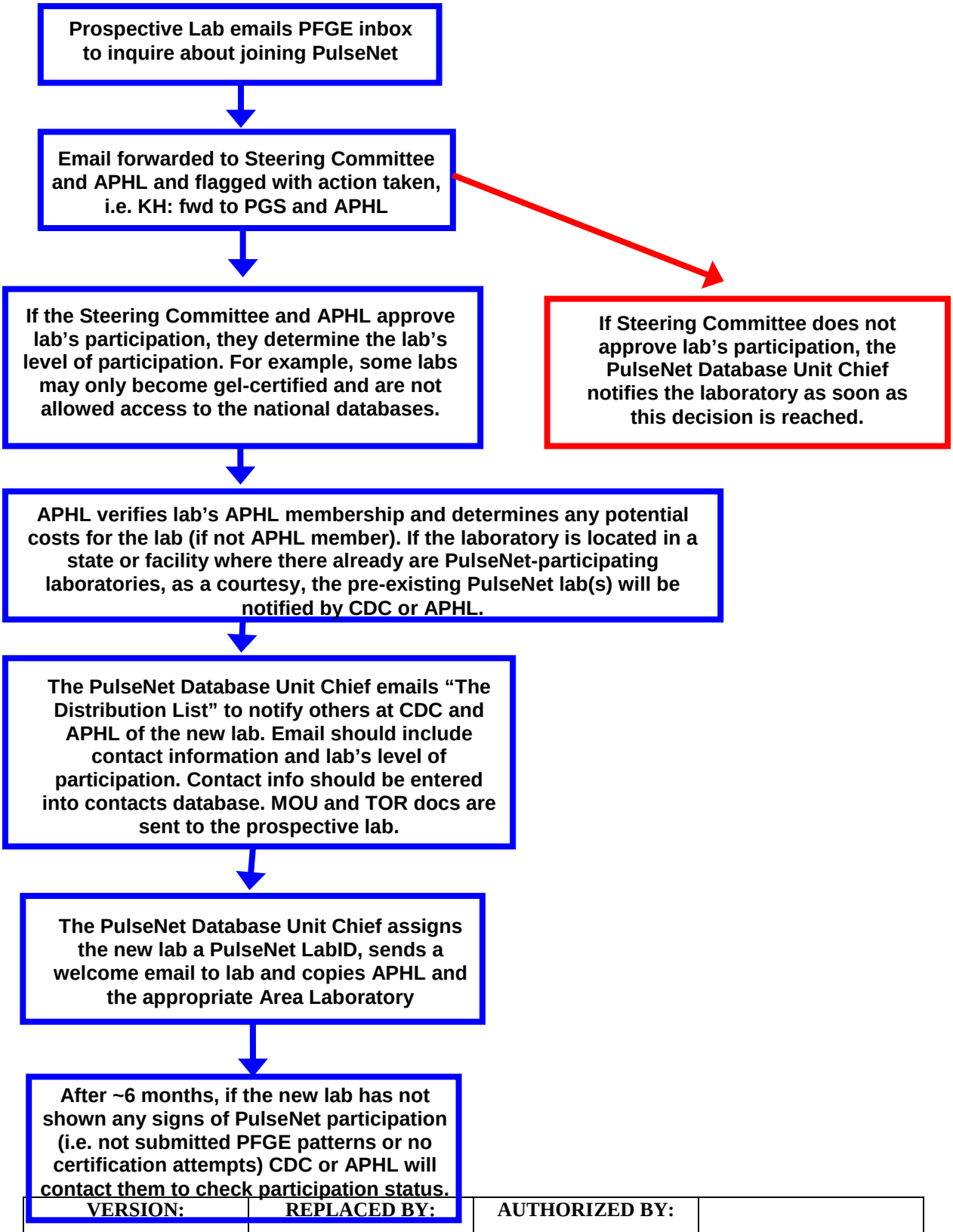
5.9.5. "Tips for Getting Started with PulseNet" (see appendix PNG08-6)

5.9.6. An excel file listing the PulseNet organisms, their differences, and database information. Document is updated for the annual EISO training and saved under \\cdc\project\CCID_NCZVED_DFBMD_PulseNet\Marketing\Presentations\EISO*yyyy*\Handouts\PulseNet Organisms_differences.xls.

5.10. After six months, if a new PulseNet laboratory does not seem to be participating by submitting certification sets and/or sending data to CDC, CDC and/or APHL will contact them to check on their status.

5.11. If a PulseNet laboratory can no longer perform PulseNet duties for any reason, the laboratory director must notify CDC in writing. Depending on the circumstances, arrangements will be made on a lab-to-lab basis.

6. FLOWCHART:


Prospective Lab emails PFGE inbox to inquire about joining PulseNet
Email forwarded to Steering Committee and APHL and flagged with action taken, i.e. KH: fwd to PGS and APHL
If the Steering Committee and APHL approve lab's participation, they determine the lab's level of participation. For example, some labs may only become gel-certified and are not allowed access to the national databases.
If Steering Committee does not approve lab's participation, the PulseNet Database Unit Chief notifies the laboratory as soon as this decision is reached.
APHL verifies lab's APHL membership and determines any potential costs for the lab (if not APHL member). If the laboratory is located in a state or facility where there already are PulseNet-participating laboratories, as a courtesy, the pre-existing PulseNet lab(s) will be notified by CDC or APHL.
The PulseNet Database Unit Chief emails "The Distribution List" to notify others at CDC and APHL of the new lab. Email should include contact information and lab's level of participation. Contact info should be entered into contacts database. MOU and TOR docs are sent to the prospective lab.
The PulseNet Database Unit Chief assigns the new lab a PulseNet LabID, sends a welcome email to lab and copies APHL and the appropriate Area Laboratory
After ~6 months, if the new lab has not shown any signs of PulseNet participation (i.e. not submitted PFGE patterns or no certification attempts) CDC or APHL will contact them to check participation status.

7. BIBLIOGRAPHY:

8. CONTACTS:

8.1. CDC PulseNet
Phone (404) 639-4558
Fax (404) 639-3333
PFGE@cdc.gov

9. AMENDMENTS:

9.1. January 2011: Updates made to the entire document to reflect CDC's reorganization, changes in position titles, and terms and definitions.

9.2. December 2012: Updates made to change CDC Team to SharePoint, Appendix PNG08-1 updated to reflect current charges and payment information for laboratories that are not APHL members.

9.3. October 2014: Updates made to enter new PulseNet MOU and ToR documents

APPENDIX PNG08-1
Payment information for federal participants and non-APHL members

PulseNet PFGE and MLVA Certifications and Proficiency Testing

Tier 1 laboratories will be billed $2,000 per year (October 1-September 30)
Includes:

- All PulseNet proficiency tests for organisms in which the laboratory is currently certified
- Up to 3 certifications submitted to PulseNet within the year as defined (these are use or lose, they do not accrue or roll over to the next year). Additional certifications may be purchased for $500 per organism per laboratorian.

Tier 2 laboratories will be billed $1,000 per year (October 1-September 30)
Includes:

- All PulseNet proficiency tests for organisms in which the laboratory is currently certified.
- Up to 1 certification submitted to PulseNet within the year as defined (these are use or lose, they do not accrue or roll over to the next year). Additional certifications may be purchased for $500 per organism per laboratorian.

Laboratories are billed for certification and proficiency testing according to a designated Tier classification. Tiers are determined by CDC and APHL based on several criteria including but not limited to the number of current employees performing PulseNet-related duties, the number of organisms in which the laboratory is certified (which also affects the number of proficiency tests), and the past charges incurred by the laboratory for PulseNet QA/QC related services. The past three years (fiscal years October 1-September 30) worth of certifications and proficiency tests performed by the laboratory are averaged to determine the tier classification a laboratory falls under. If a lab has averaged 3 or more proficiency tests and 1 or more certifications, then that lab is classified as a Tier 1. Those averaging below those numbers are designated as Tier 2. An annual review of Tier classifications will take place before the billing cycle begins. The laboratory will be notified by CDC and APHL in writing of the current tier status prior to billing. Laboratories will be billed by APHL within the first weeks of the yearly cycle. If a new laboratory joins PulseNet mid billing cycle, charges will be determined by CDC and APHL.

PulseNet Customized MasterScripts

As stated in the PulseNet SOP PNG05, Duties and Responsibilities of a PulseNet Laboratory, participating laboratories are expected to be using the latest version of the PulseNet MasterScripts. PulseNet MasterScripts are $1,000 per laboratory upon joining the network and an additional $1,000 for any future major upgrade. Laboratories will be notified by CDC and APHL when major upgrades have been made. APHL will bill laboratories once the MasterScripts have been distributed.

PulseNet Training

PulseNet Training is $250 per laboratorian per course. Training opportunities are announced on the PulseNet SharePoint site as information becomes available. All training course applicants are considered on an as-needed basis, charges will not be incurred if an applicant is not accepted into the course.

APPENDIX PNG08-2

MEMORANDUM OF UNDERSTANDING
Between:

National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), Centers for Disease Control and Prevention (CDC)

and

Participating PulseNet USA Public Health and State Agricultural Laboratories

WHEREAS The Enteric Diseases Laboratory Branch (EDLB), Division of Foodborne, Waterborne and Environmental Diseases, NCEZID, Centers for Disease Control and Prevention (CDC) relies on the participation of state, county, and local public health and state agricultural laboratories in PulseNet USA, a national network dedicated to the laboratory surveillance of enteric pathogens using standardized molecular subtyping methods; and

WHEREAS state, county, and local public health and state agricultural laboratories in the United States rely on the leadership of EDLB for participation in a network dedicated to the laboratory surveillance of enteric pathogens;

NOW THEREFORE NCEZID and participating state, county, and local PulseNet laboratories agree that it is in each of their interests to develop a joint cooperative program, namely the PulseNet USA network, for surveillance of enteric pathogens.

1. INTRODUCTION

1.1 This Memorandum of Understanding (MOU) between selected state, county, and local public health and state agricultural laboratories and NCEZID, hereinafter collectively referred to as the "Participating Laboratories," describes an arrangement to be willingly entered into by the Participating Laboratories. "Participants" refers to the individuals at the participating laboratories who perform PulseNet associated functions.

1.2 This MOU is not legally binding and places no legal obligation on the Participating Laboratories.

2. OBJECTIVES AND SCOPE

2.1 The objective of this MOU is to establish an understanding among the Participating Laboratories concerning their respective roles and responsibilities and to provide general guidelines under which cooperative activities may be implemented.

2.2 The purpose of any proposed joint program is to support collaborative activities related to the PulseNet USA enteric pathogens surveillance network.

3. ROLES AND RESPONSIBILITIES

3.1 Joint programs and projects will be consistent with the needs, merits, and goals of established programs within PulseNet USA (CDC, NCEZID and/or the state, county, and local public health laboratories). In general, the cooperative efforts described herein, and other joint cooperative programs and projects among the Participating Laboratories, may be initiated at any time that a specific agreement can be reached involving content, responsibility, liability, funding, intellectual property and other appropriate provisions, provided that the process is consistent with the administrative regulations and procedures of the Participating Laboratories.

3.2 The Participating Laboratories agree to comply, to the best of their ability, with the attached Terms of Reference (TOR), which details the collaborative activities of the Participating Laboratories and Participants.

3.3 The following cooperative activity areas have been identified:

3.3.1 Exchange information that is not publicly available, to the extent allowed by U.S. law and other Participating Laboratories'/Participants' governing law;

3.3.2 Coordinate and collaborate on public health activities related to foodborne disease investigation;

3.3.3 Share protocols, standards, strains and human resources;

3.3.4 Participate in, coordinate, and implement quality assurance and quality control programs.

4. FINANCIAL ARRANGEMENTS

4.1 No funds are authorized or guaranteed under this MOU. Qualified public health laboratories have received and may continue to receive support through the Epidemiology and Laboratory Capacity cooperative agreement mechanism and/or the Emerging Infections Program. It is recognized that all Participating Laboratories will, subject to the availability of appropriations, commit resources to keep PulseNet USA operational.

5. SETTLEMENT OF DISPUTES

5.1 Any disputes regarding the interpretation or implementation of this MOU will be resolved only by consultation between the Participating Laboratories and will not be referred to any third party for settlement.

6. AMENDMENT

6.1 This MOU may be amended by consent of all Participating Laboratories who have signed the MOU upon receipt of written notice.

7. DURATION AND TERMINATION

7.1 This MOU will remain in effect until further notice. It shall be reviewed and renewed at least every three (3) years by NCEZID and the Participating Laboratories.

7.2 This MOU may be terminated by any Participating Laboratories at any time provided that a 90 day written notice is given to the other Participating Laboratories and appropriate steps are taken to ensure an orderly termination of joint activities.

8. POINTS OF CONTACT

The National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention

Administrative

Name: Dr. Peter Gerner-Smidt

Title: Chief PulseNet USA

Phone: 404 639 3322

Fax: 404 639 3333

e-mail: PGernerSmidt@cdc.gov

Technical (laboratory methods)

Name: Dr. Efrain Ribot

Title: Chief PulseNet Methods Development and Validation Laboratory

Phone: 404 639 3521

Fax: 404 639 3333

e-mail: ERibot@CDC.GOV

Technical (database)

Name: Kelley Hise

Title: Chief Database Team

Phone: 404 639 0704

Fax: 404 639 3333

e-mail: KHise@CDC.GOV

Technical (software and database security)

Name: Brenda L. Brown

Title: PulseNet Technical and Security Steward

Phone: 404 639 3942

Fax: 404 639 3333

e-mail: BLBrown@CDC.GOV

Participating state, county or local public health or state agricultural laboratory

Administrative

Name:

Title:

Phone:

Fax:

e-mail:

Technical

Name:

Title:

Phone:

Fax:

e-mail:

9. EFFECTIVE DATE AND SIGNATURE

9.1 The effectiveness of this MOU does not depend upon the signature of every PulseNet USA Participating Laboratory. This MOU becomes effective when two of the Participating Laboratories have signed the MOU, upon the date of the later signature. The MOU will be effective only among the Participating Laboratories that have signed the MOU.

The National Center for Emerging and Zoonotic Infectious Diseases,

Centers for Disease Control and Prevention

_____

Dr. Beth Bell MD MPH, Director, NCEZID

_____

Date

Participating state, county or local public health laboratory

_____

Name of laboratory

_____

( )

_____

Date

Reviewed by

_____

Name of State Epidemiologist

_____

( )

_____

Date

APPENDIX PNG08-3
Terms of Reference for PulseNet USA

1. SCOPE: The National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), Centers for Disease Control and Prevention (CDC) and the participating PulseNet USA state, county, and local laboratories agree that it is in their mutual interest to develop a joint cooperative program to perform standardized DNA fingerprinting of foodborne disease-causing bacteria so that the participating organizations will be able to rapidly compare the DNA fingerprints of infectious agents from any laboratory participating in the PulseNet network. This will facilitate rapid and early identification of disease clusters and disease outbreaks at the national level and, when coordinated with PulseNet International networks, will serve as an effective global early alert system for foodborne disease outbreaks. The PulseNet network may be extended to cover surveillance for other pathogens.
2. PURPOSE: This Terms of Reference document (TOR) will define the rights and responsibilities of each participant in the PulseNet USA network.
3. DEFINITIONS: The following terms/abbreviations will be used throughout the document:
   - 3.1 DNA fingerprint – A subtype result obtained by a standardized DNA molecular method that has been adopted by the PulseNet network.
   - 3.2 TOR: Terms of Reference
   - 3.3 Participating Laboratory: A federal, state, county, or local public health, or state agricultural laboratory that engages in PulseNet activities.
   - 3.4 Participant: An individual at a Participating Laboratory who engages in PulseNet activities.
   - 3.5 PulseNet USA Steering Committee – A committee made up of selected members of the PulseNet USA network, from both CDC and state, county, or local laboratories and APHL.
4. BACKGROUND: Foodborne infections are a national and global problem. Trade of raw and processed food across borders within and between different regions of the world and international travel make it possible that the source of a foodborne infection may be located in a different country, state, or region than where the illnesses are observed. International trade and travel has grown rapidly during the past decades; consequently, foodborne infections increasingly show up in parts of the world differing from their origin.

A critical component in the investigation of foodborne outbreaks is the DNA fingerprinting of the causative organisms and comparison of the DNA fingerprints of strains isolated from ill persons who are considered to be part of a common source outbreak and the possible sources throughout the food chain. At present, Pulsed-Field Gel Electrophoresis (PFGE) is the gold standard routine method for comparison of most bacterial foodborne pathogens. CDC began setting up the PulseNet USA network in 1996 to facilitate rapid, standardized DNA fingerprinting of foodborne pathogens by state and local public health laboratories in the United States and their submission to a central electronic database at CDC for purposes of making the database of fingerprints available to PulseNet participants. Currently, PulseNet standardized protocols are available for Shiga toxin (Verocytotoxin) producing *E. coli* (STEC) O157:H7, non-O157 STEC, *Salmonella*, *Listeria monocytogenes*, *Shigella* species, *Shigella flexneri*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Clostridium perfringens*, *Clostridium botulinum*, *Yersinia pestis* and *Campylobacter* species. Next generation subtyping tools, e.g., Multi Locus VNTR Analysis (MLVA) has been implemented or is under development and validation for STEC O157, *Salmonella* serovars Enteritidis and Typhimurium and *Listeria monocytogenes.* More recently, PulseNet has begun exploring the use of whole genome sequencing (WGS) as a next-generation subtyping tool. The plan to use this technology has initiated a review of PulseNet data disclosure policy: optimal sharing of WGS data and a subset of associated metadata involves public disclosure while protecting patient confidentiality and avoiding inappropriate use of preliminary or potentially sensitive public health data. . The primary drivers and benefits to the public release of a subset of data include:

1) To conform with the President's Open Government Directive and the CDC/ATSDR Policy on Releasing and Sharing Data
2) To provide basic information on the frequency and genotypes of foodborne pathogens infecting people to the food industry, so they can potentially use this information in combination with their information to assess the possible risk posed by their products.
3) To provide researchers in microbial phylogenetics, molecular epidemiology and food microbiology access to data useful in their research endeavors.

In addition, the amount of sequence data potentially produced in PulseNet will require collaboration with partners with the capacity to store and manage extremely large datasets. This is accomplished in collaboration with the National Center for Biotechnology Information (NCBI). Data exchange may occur in the Sequence Read Archieve

(SRA) in the Genbank database. The metadata to be shared with the WGS data is presented in more detail in Section 6.B.4.

The PulseNet network has enabled CDC and its public health partners to detect and investigate outbreaks of foodborne infections in humans in the United States as well as international outbreaks in cooperation with public health colleagues in PulseNet International.

The objective of PulseNet USA is to use DNA fingerprinting to characterize foodborne pathogens in order to combat national and global outbreaks and to perform surveillance of the different foodborne pathogens throughout the food chain and of the infections they cause in humans. An ultimate goal is to help decision makers in establishing policies for safer food on the national and global level.

5. GOVERNANCE:

5.1 Activities of the PulseNet USA network are coordinated by the PulseNet USA Steering Committee. The PulseNet USA Steering Committee is chaired by the chief of the Enteric Diseases Laboratory Branch (EDLB) or his substitute at CDC and is comprised of participants from state and local PulseNet Laboratories, APHL and CDC.

5.2 Membership on the PulseNet USA Steering Committee is by invitation; terms on the Committee are open-ended. The Committee meets via conference call as necessary and generally once per quarter. The structure and membership of the committee may be changed upon agreement of the current members.

5.3 Decisions regarding topics brought before the committee are reached by consensus.

6. ROLES AND RESPONSIBILITIES OF PARTICIPANTS:

A. Coordination and collaboration relative to public health activities

It is mutually agreed that:

6.A.1 Each participating laboratory will utilize the expertise, resources, and relationships of the network in order to increase capability and readiness to respond to outbreaks. In addition, each participating laboratory will designate central contact points where communications dealing with matters covered by this agreement should be referred.

6.A.2 One or more participants from each participating laboratory will attend periodic joint meetings to

promote better communication and understanding of regulations, policies, and responsibilities and to address questions and issues that may arise through routine or critical operation of the network.

6.A.3 One or more participants at each participating laboratory will maintain knowledge of improvements to the communication methods, data utilization tools, and methodological developments within the network.

6.A.4 Each participating laboratory will notify the other participating laboratories as soon as possible when issues of mutual concern become evident. 6.A.5 Each participating laboratory will collaborate with the other participating laboratories in all investigations of mutual concern. Such collaboration may include providing alerts to the other participating laboratories regarding disease outbreaks encountered as part of its activities, providing technical advice in areas of recognized expertise, providing results of analysis, and exchanging information, e.g. identification at the state or local level of indistinguishable DNA fingerprints, developments of a subtyping method that potentially may be implemented in the network or some legal changes at the local level which may have consequences for the network as a whole.

6.A.6 Each participating laboratory will keep all information received from other participants confidential unless the information is already publicly available or written consent for distribution has been received from the originating laboratory.

6.A.7 This agreement does not preclude the participating laboratories and/or participants from entering into other agreements which may set forth procedures for special programs which can be handled more efficiently and expertly by other agreements.

B. Principles and Procedures for the Exchange of Information that is not Publicly Available

It is mutually agreed that:

6.B.1 Although there is no legal requirement for exchange of information, the PulseNet participating laboratories/participants agree in principle that there should be a presumption in favor of full and free sharing of information between them. The participants recognize and acknowledge, however, that it is essential that any confidential information that is shared between them must be protected from unauthorized public disclosure. Safeguards are important to protect the interests of, among others, owners and submitters of trade secrets and confidential commercial information, patient identities and other personal privacy information, privileged and/or pre-decisional agency records, and information protected for national security reasons.

Such safeguards also help guarantee the participant's compliance with applicable laws and regulations. A participant should only decide not to share information if credible information exists that the requesting participant may not be able to comply with applicable laws or regulations governing the protection of non-public information or with the principles or procedures set forth in this TOR.

6.B.2 All participants must implement appropriate data and information security systems. To facilitate the sharing of information, the participants must implement procedures to ensure, at a minimum, that such sharing of information is indeed appropriate and that the recipient guards the confidentiality of all information received. Document control procedures should also be implemented for the storage of any hard copy print outs of PulseNet data.

6.B.3 All PulseNet USA participating laboratories/participants shall limit the dissemination of shared information to those participants, internal agency offices, and/or individuals that reasonably require the information and will be responsible for ensuring that there are no other recipients of the information.

6.B.4 Sharing of whole genome sequence (WGS) data and a subset of associated metadata optimally involves public disclosure while protecting patient confidentiality and avoiding inappropriate use of preliminary or potentially sensitive public health data. In order to protect patient confidentiality the patient specific data to be released publically will be limited to clinical specimen type yielding the pathogen (i.e., blood, stool) the year of illness onset and region of country for patient residence (i.e., HHS region, see Appendix 1). To avoid inappropriate use of preliminary or potentially sensitive information, release of patient data will occur six months after the release of isolate data. Specifically, an isolate identifier, a taxonomic description of the isolate (genus and species), the source (i.e., clinical, food, or environmental), the country of origin along with the sequence information will be released to Genbank as soon as WGS data is available. After six months and if approved by the participant, information on isolation site of the organism, the serotype of the organism (if applicable),the year of isolation, the geographic region of patient residence (HHS region), and the age group of the patient will be released.

Each PulseNet participating laboratory must indicate by checking off the appropriate box and signing the form (Appendix 2) attached to this TOR by an appropriate official if the delayed metadata may be released.

6.B.5 All PulseNet USA participants shall keep any shared information confidential, to the extent permitted

by law, until otherwise agreed upon.

C. Protocols, Standards, Reference Strains and Quality Assurance

6.C.1 Each participant agrees to follow the standardized PulseNet protocol for subtyping of foodborne isolates, as distributed and/or published by CDC.

6.C.2 Each participant agrees to utilize the customized PulseNet scripts for analysis of PFGE patterns and other PulseNet subtyping data, assisted by the recommended version of the analysis software and according to the analysis guidelines distributed by CDC.

6.C.3 *Xba*I digested DNA of the strain *Salmonella* Braenderup H9812, provided to PulseNet networks participants by CDC and available through the American Type Culture Collection (ATCC number BAA-664), is the universal molecular size standard against which all PFGE profiles generated in the networks are normalized.

6.C.4 Each participating laboratory should establish a culture collection containing strains representing each unique pattern in their databases. The participants agree to share these strains with each other for network purposes at no cost or at the cost of shipping and handling.

6.C.5 Each participating laboratory agrees to participate in certification and proficiency testing programs designed to ensure comparability of profiles between the participants.

7. PULSENET NAME AND LOGO:

7.1 The PulseNet name and logo are trademarks that are owned by CDC. Signatories to this TOR may use the PulseNet name as needed for use in PulseNet USA activities. However, because the PulseNet logo contains the CDC logo signatories must obtain specific CDC approval before using the PulseNet logo.

7.2 When a participating laboratory exercises its option to terminate the PulseNet USA MOU, that participating laboratory immediately loses the right to use the PulseNet name and logo.

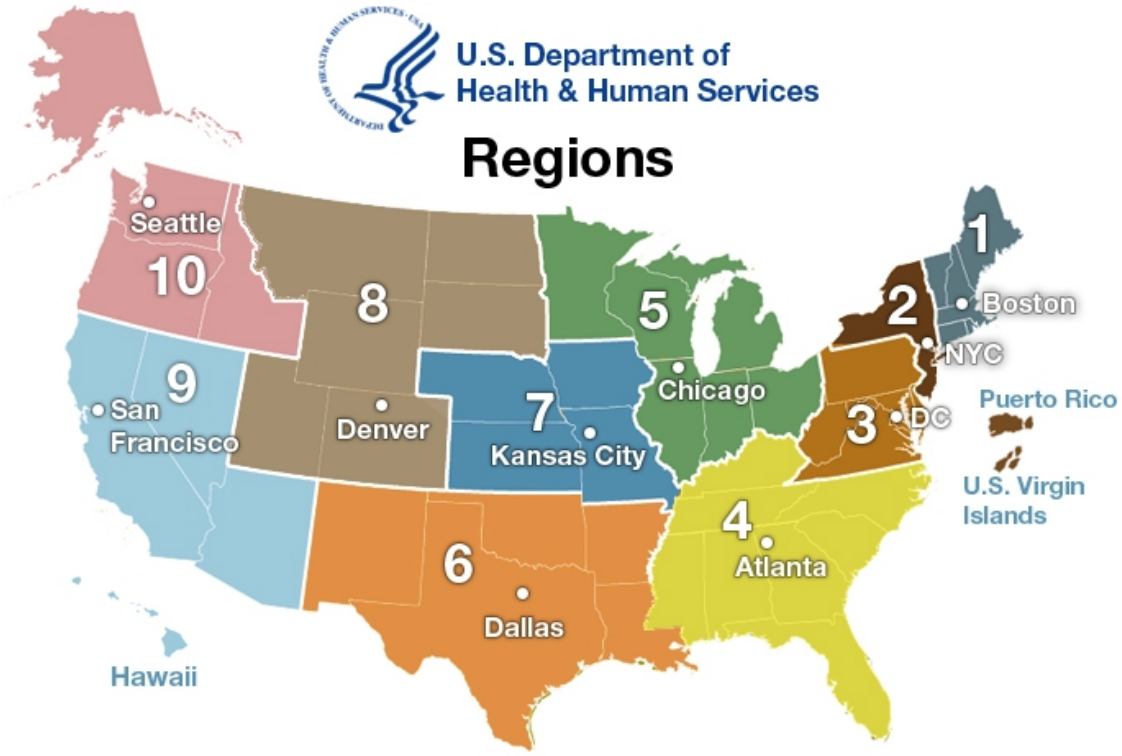
8. SYSTEMS AND DATA SECURITY MEASURES

8.1 All participants will install and configure system equipment capable of running the latest recommended version of the PulseNet Customized software. Data security will be maintained following the direction of the PulseNet Technical and Security Steward, including such mechanisms as storage in a locked room or locked file cabinet, shredding of discarded documents, and memory erasure upon replacement of hard

drives. Participants will not share logon identities, passwords, or SecurID devices that have been assigned to them by CDC.

8.2 All participants who have access to the PulseNet listserv (SharePoint or the most current communication mechanism) will sign a non-disclosure statement assuring that all information will be treated as confidential and only shared with appropriate public health personnel or others as may be required by law.

Appendix 1. HHS regions (from http://www.hhs.gov/about/regionmap.html)



- Region 1 - Boston
  Connecticut, Maine, Massachusetts, New Hampshire, Rhode Island, and Vermont
- Region 2 - New York
  New Jersey, New York, Puerto Rico, and the Virgin Islands
- Region 3 - Philadelphia
  Delaware, District of Columbia, Maryland, Pennsylvania, Virginia, and West Virginia
- Region 4 - Atlanta
  Alabama, Florida, Georgia, Kentucky, Mississippi, North Carolina, South Carolina, and Tennessee
- Region 5 - Chicago
  Illinois, Indiana, Michigan, Minnesota, Ohio, and Wisconsin
- Region 6 - Dallas
  Arkansas, Louisiana, New Mexico, Oklahoma, and Texas
- Region 7 - Kansas City
  Iowa, Kansas, Missouri, and Nebraska
- Region 8 - Denver
  Colorado, Montana, North Dakota, South Dakota, Utah, and Wyoming
- Region 9 - San Francisco
  Arizona, California, Hawaii, Nevada, American Samoa, Commonwealth of the Northern Mariana Islands, Federated States of Micronesia, Guam, Marshall Islands, and Republic of Palau
- Region 10 - Seattle
  Alaska, Idaho, Oregon, and Washington

Appendix 2

Metadata to be released immediately to NCBI's Genbank with a whole genome sequence (WGS) and with a 6 months delay following the upload of the sequence.

Metadata released immediately with the WGS:

- Unique sample ID (WGS_ID)
  - Created specifically for this purpose in order NOT to include information about isolation date or state of origin
- Organism genus and species (e.g., *Listeria monocytogenes*)
- Organism source (e.g., clinical, food, environment)
- Country of origin (USA)
- ID for laboratory submitting DNA sequence (e.g., CDC)

Metadata for release delayed 6 months* after upload of WGS:

- Site of isolation (e.g., blood, cerebral spinal fluid, stool)
- Organism serotype (if applicable and available)
- Collection year
- Geographic location (HHS Region of patient residence)
- Age category (in years: 0-4, 5-9, 10-19, 20- 29, 30- 39, 40- 49, 50- 59, 60- 69, 70- 79, 80+)

***** Six- seven months will be the usual period of delay; the update of metadata in GenBank should occur on a monthly basis.

___ Metadata may be submitted as described above for immediate and delayed release

___ Only metadata for immediate release as described above may be released

(Check one)

Signature Date

_____

Name, Affiliation, PulseNet laboratory

APPENDIX PNG08-4

Welcome E-mail

Greetings [***Lab Director***],

Welcome to PulseNet! We are excited about your laboratory's interest in PulseNet and pleased to include the [***XXXX***] lab in our constantly expanding network of participants. Please feel free to contact us with any questions.

We are looking forward to working with your laboratory!

Sincerely,

Kelley Hise, MPH
Chief, PulseNet Database Unit
Centers for Disease Control and Prevention
1600 Clifton Rd. NE
MS C-03
Atlanta, GA 30329
KHise@cdc.gov
Phone: (404) 639-4558
Fax: (404) 639-3333

Kristy Kubota, MPH
Senior Specialist, PulseNet Program
Association of Public Health Laboratories
8515 Georgia Avenue
Suite 700
Silver Spring, MD 20910
kristy.kubota@aphl.org
Phone: (240) 485-2720
Fax: (240) 485-2700

APPENDIX PNG08-5

Welcome Letter for New PulseNet Laboratories

[***Date***]

[***Lab Director***]
[***Lab Address/Information***]

Welcome to PulseNet! We are excited about your laboratory's interest in PulseNet and pleased to include the [***XXXX***] lab in our constantly expanding network of participants. As you read through the documents provided in this packet, along with those posted in the Library of PulseNet Documents on PulseNet's SharePoint site, please do not hesitate to contact CDC, APHL or your Area Laboratory with any questions.

Your PulseNet LabID is [***XXXX***]. You will need this LabID to install the PulseNet MasterScripts and also use it to name all TIFF and bundle files created in your lab.

Your PulseNet Area Laboratory is [***XXXX, contact info***]

Information provided in this packet for your reference:

- Standard Operating Procedures (SOPs) included in this packet
  - PNG05 Responsibilities of a PulseNet Laboratory
  - PND03 PulseNet SharePoint, Access and Use
  - PNQ02 Certification
- "Tips for Getting Started with PulseNet" is a document that was created to help answer some commonly asked questions
- PulseNet organisms and their differences

We look forward to working with you.

Kelley Hise, MPH
Chief, PulseNet Database Unit
Centers for Disease Control and Prevention
1600 Clifton Rd. NE
MS C-03
Atlanta, GA 30333
KHise@cdc.gov
Phone: (404) 639-4558
Fax: (404) 639-3333
Website: www.cdc.gov/pulsenet

Kristy Kubota, MPH
Senior Specialist, PulseNet Program
Association of Public Health Laboratories
8515 Georgia Avenue
Suite 700
Silver Spring, MD 20910
kristy.kubota@aphl.org
Phone: (240) 485-2720
Fax: (240) 485-2700
Website: www.aphl.org

APPENDIX PNG08-6

Tips for Getting Started with PulseNet

- **General**
  - Contact Information
    - PulseNet Database Team at CDC (404) 639-4558 or PFGE@cdc.gov
      - ✓ Please always use PFGE@cdc.gov when contacting CDC via email. Your emails will automatically be directed to the appropriate person. Using this email address will ensure a more prompt response in case you do not know who to contact for a specific question, someone is out of the office or no longer works at CDC.
    - Kristy Kubota, Senior Specialist, PulseNet Program at APHL (240) 485-2720 or kristy.kubota@aphl.org
  - There are currently eight PulseNet Area Laboratories. These are labs, designated by CDC and APHL that have agreed to assume responsibility for additional PulseNet duties for laboratories within their support region. The current Area Labs include MA, MN, WA, TX, VA, UT, MI and CDC. Refer to SOP PNG06 for more specific information on Area Lab responsibilities and how they may assist you.
  - Websites
    - PulseNet www.cdc.gov/pulsenet
    - APHL www.aphl.org
      - ✓ Visit APHL's website and click on "Conferences" at the top of the page to find information on the InFORM Conference
  - The PulseNet SharePoint site houses important PulseNet information including cluster and outbreak information, procedures, training documents, tips and reminders, and many more useful references. To request access to SharePoint, send an email to PFGE@cdc.gov with "SharePoint" in the subject line. Include your name, email, and position within the laboratory. Refer to SOP PND03 for more information, including how your epidemiologists may receive access.
  - Watch SharePoint postings for lab and software training opportunities at CDC. If no training courses are being advertised at the time, you may contact your Area Laboratory and/or CDC to discuss other possible opportunities.
- **Laboratory**
  - PFGE protocols are available on SharePoint within the "Library of PulseNet Documents/QA/QC Manual" section and on the PulseNet website www.cdc.gov/pulsenet
  - Troubleshooting advice is available within the troubleshooting discussions on SharePoint. Laboratories are also encouraged to contact their Area Laboratory and/or CDC for troubleshooting assistance.
- **Database**
  - A separate database must be created for each PulseNet organism run in your laboratory. Each organism is PFGE'd under different running conditions, therefore the reference standard (*Salmonella Braenderup* H9812) patterns look different. To take the different reference standard patterns into account, the PulseNet MasterScripts vary by organism and must only be run on the appropriate databases. If you ever need assistance with database setup, please contact CDC.
  - Laboratories are assigned a LabID upon joining PulseNet. Your LabID must be entered to run PulseNet MasterScripts in BioNumerics databases and used to name TIFF and bundle files submitted to PulseNet.
  - Name all files according to the standardized PulseNet naming system. The first two to four characters should be the LabID (i.e. CDC), the second two digits should be the year (i.e. 14), and

the last three digits should be the unique number of the TIFF or bundle file submitted that year from your laboratory (i.e. 0001).

- **QA/QC**
  - The PulseNet Standard Operating Procedures (SOPs) are provided on SharePoint within the "Library of PulseNet Documents/QA/QC Manual" and may be downloaded and/or printed for use in your laboratory. The SOPs are broken into four categories: General, Laboratory, Database, and QA/QC, to help locate procedures easily. For example, all of the PFGE laboratory protocols can be found within the Laboratory section.
  - Certification
    - Once a person is routinely producing good quality PFGE images, they may complete and submit for PFGE gel certification.
    - Once a person is comfortable with PFGE analysis and the creation of PulseNet bundle files in BioNumerics, they may complete and submit analysis certification.
    - Certification sets are available for *Salmonella*, *E. coli* O157, Non O157 STEC, *Shigella*, *Shigella flexneri*, *Listeria monocytogenes*, *Campylobacter jejuni*, *Vibrio cholerae*, *Vibrio parahaemolyticus* and *C. botulinum*. These sets may be requested by emailing PFGE@cdc.gov with "Certification" in the subject line. Participants may become gel-certified, analysis-certified, or both for each organism. Participants must be analysis-certified in an organism to receive access to that specific national database. Laboratories must have at least one person gel-certified in a specific organism before anyone in the lab may be analysis-certified for that organism.
  - Laboratories must participate and successfully complete annual proficiency testing (PT) to retain certification status for each organism.
    - Currently PT for *Salmonella*, *E. coli*, and *Shigella* is held in the Fall and *Campylobacter*, *Listeria* and *Vibrio* are held in the Spring.

READING CONTROL SHEET FOR:
GENERAL STANDARD OPERATING PROCEDURES (PNG)

NAME	DATE	COMMENTS	SIGNATURE

By signing above, you are indicating that you have read and understood all SOPs included in the PNG section of this manual.

READING CONTROL SHEET FOR:
STANDARD OPERATING PROCEDURES FOR THE PULSENET LABORATORY (PNL)

NAME	DATE	COMMENTS	SIGNATURE

By signing above, you are indicating that you have read and understood all SOPs included in the PNL section of this manual.

1. **PURPOSE:** To describe the laboratory equipment and supplies needed for molecular subtyping of foodborne bacterial pathogens by Pulsed-field Gel Electrophoresis (PFGE).

2. **SCOPE:** This procedure applies to all PulseNet participating laboratories performing PFGE.

3. **DEFINITIONS/TERMS:**

   3.1. PFGE: Pulsed-field Gel Electrophoresis
   3.2. TIFF: Tagged Image File Format. A file of a gel image that can be analyzed in BioNumerics
   3.3. BioNumerics: Gel analysis software used by PulseNet, developed by Applied Maths, Belgium

4. **RESPONSIBILITIES/PROCEDURE:**

Electrophoresis and Documentation Equipment:

Bio-Rad CHEF Mapper XA System, CHEF-DR III Variable Angle System, or GenePath Strain Typing System with PC customized software program. Include pump and chiller module.

Bio-Rad Gel Doc 2000 or ChemiDoc Documentation System

or

An equivalent documentation system that is equipped with a CCD camera that can provide IBM-compatible uncompressed TIFF images and resolution of ≥768 x 640 pixels, and which will allow comparison of images in the PulseNet database with BioNumerics software (Applied Maths, Inc.).

Other Equipment:

37EC Incubator (or appropriate temperature) - incubate cultures

Dade Microscan Turbidity Meter[1], Spectrophotometer, or bioMérieux Vitek Colorimeter
- adjust concentration of cell suspensions

Microwave - melt agarose

Water Bath with Shaker or Shaking Incubator
- lyse cells in agarose plugs (54°C)
- wash plugs with water and TE (50°C)

56°C Water Bath[2] - equilibrate and hold melted agarose
25°C, 30°C, 37°C Water Bath(s) - restriction digestion reactions
50°C Water Bath - heat water and TE that is used to wash plugs; restriction digestion reactions

Microcentrifuge - briefly centrifuge small vials of reagents such as restriction digestion buffer, Proteinase K, and other enzymes

[1] Dade Behring, Inc., 1717 Deerfield Rd. Deerfield, IL 60015
[2] At a minimum, two water baths are needed – one equilibrated to 56°C and one to 37°C. Temperature can be increased or decreased as needed. *Apa*I restriction of *Listeria* PFGE plugs slices requires a 30°C water bath.

Supplies and Selected Reagents:

SeaKem Gold Agarose (Cambrex Bio Sciences Rockland, Inc. 50152, 50150
- casting plugs in reusable or disposable plug molds
- PFGE gel

Sterile Ultrapure H_2O (Reagent Grade, Type 1)

Proteinase K Powder
Amresco - 0706
Invitrogen [Life Technologies] - 25530-031
Roche Molecular Biochemicals - 1 000 144, 1 092 766
or
Proteinase K Liquid
Amresco - E195
Invitrogen [Life Technologies] - 25530-049
Roche Molecular Biochemicals - 1 964 372, 1 964 399

5X TBE - dilute 1:10 to make 0.5X TBE
Amresco - J885
Sigma/Aldrich - T6400
or
10X TBE - dilute 1:20 to make 0.5X TBE
Amresco - 0658
Bio-Rad - 161-0733, 161-770
Invitrogen [Life Technologies] - 15581-044
Roche Molecular Biochemicals - 100 759, 1666 703
Sigma - T4415

Agar plates and slants
Trypticase Soy Agar with 5% defibrinated sheep blood (TSA-SB), Heart Infusion Agar (HIA), or TSA - ***E. coli***, ***Salmonella***, and ***Shigella***
TSA-SB, Brain Heart Infusion Agar (BHIA) - ***Listeria monocytogenes***
HIA with Rabbit Blood - ***Campylobacter jejuni, C. coli***

Restriction Enzymes and Appropriate Buffers (Roche Molecular Biochemicals, New England Biolabs, Promega, or other supplier) for each organism tested.

E. coli* O157:H7**, ***Salmonella, ***Shigella sonnei***
*Xba*I, *Avr*II (isoschizomer *Bln*I), *Spe*I
(*Not*I ***for S. sonnei***; do not use for *E. coli* O157:H7)
L. monocytogenes
*Asc*I, *Apa*I
Campylobacter jejuni, ***C. coli***
*Sma*I, *Kpn*I

PulseNet Standard Strain
Salmonella ser. Braenderup H9812 and PFGE plugs of H9812[3]

DNA Size Standard, Lambda Ladder
Bio-Rad - 170-3635
Cambrex Bio Sciences Rockland, Inc. - 50461
Roche Molecular Biochemicals - 1378 961, or other supplier
Ethidium bromide (Amresco - X328; Bio-Rad - 161-0433; Sigma - E-1510)

Miscellaneous Supplies:

Sterile clear polystyrene 12-mm x 75-mm (Falcon 2054) or 17-mm x 100-mm tubes (Falcon 2057) tubes with cap (or equivalent) - for cell suspensions

Sterile polyester-fiber or cotton swabs - remove growth from agar plates

Sterile transfer pipets or sterile Pasteur pipets and rubber bulbs - use when adjusting cell suspensions or to remove reagents from plug slices

Sterile 1.5 ml microcentrifuge tubes - mix cell suspensions with agarose; restriction digestions

Sterile 50 ml polypropylene screw-cap tubes (Falcon 2098, Corning 25330-50) or
50 ml Oak Ridge tubes (Nalgene 3118-50) - for plugs made in reusable or disposable molds

Green Screened Caps, Bio-Rad 1703711[4] - use when washing PFGE plugs

PFGE plug molds - 10-well reusable (2-cm x 1-cm x 1.5-mm; Bio-Rad 170-3622)
or
- 50-well disposable (1.5-mm x 10-mm x 5-mm, Bio-Rad 170-3713)

Single-edge razor blades, scalpels, glass cover slips, or equivalent - use to cut plug slices

Sterile disposable petri dishes or large glass slides - can use when cutting plugs

Flat spatulas with one wide and one tapered end

Standard Casting Stand (14 x 13 cm frame and platform) - Bio-Rad 170-3689

Wide/Long Combination Casting Stand (21 x 14 cm frame and platform) - Bio-Rad 170 -3704

Combination Comb Holder - Bio-Rad 170-3699

10-well Comb, 14 cm long, 1.5 mm wide - Bio-Rad 170-4326

[3] *S*. ser. Braenderup H9812 restricted with *Xba*I will be used as the standard strain for all organisms tested by PulseNet after March 15, 2003.

[4] These caps will not fit the 50 ml Oak Ridge tubes.

15-well Comb, 21 cm long, 1.5 mm wide - Bio-Rad 170-3627

Gel-leveling table

Plastic containers for staining gels with ethidium bromide

70% isopropanol, bleach (5% - 10%), or other appropriate disinfectant

Sterile screw cap flasks or bottles of various sizes (50 ml - 2000 ml)

Sterile graduated cylinders of various sizes (100 ml - 2000 ml)

Single-channel micropipetters - fixed and/or variable volume of various sizes

Sterile pipets (2 ml - 50 ml) and pipet tips (10 Φl - 1000 Φl)

Protective gloves (powder-free latex, vinyl, or nitrile)

Heat-resistant gloves

Ice Bucket

Use of trade names and commercial sources is for identification only and does not imply endorsement by the CDC or the U.S. Department of Health and Human Services.

5. FLOW CHART:

6. BIBLIOGRAPHY:

7. CONTACTS:

8. AMENDMENTS:

1. **PURPOSE:** To describe the formulas of stock reagents used for standardized PulseNet Pulsed-field Gel Electrophoresis (PFGE) protocols of foodborne bacterial pathogens.

2. **SCOPE:** To describe the procedures for preparation of reagents used in the PulseNet standardized PFGE protocols to be used by the PulseNet participants in order to assure inter-laboratory comparability of results generated.

3. **DEFINITIONS/TERMS:**
   3.1 PFGE: Pulsed-field Gel Electrophoresis
   3.2 DNA: Deoxyribonucleic acid

4. **RESPONSIBILITIES/PROCEDURE:**

Sterile Ultrapure or Reagent Grade Type 1 (NCCLS) Water* [1]

Autoclave or filter-sterilize in 100 ml, 500 ml, and/or 1 liter volumes in screw-cap bottles or flasks.

1 M Tris≅HCl, pH 8.0*

121.1 g Tris base
Dissolve in 650-700 ml Ultrapure H_2O
Add 80 ml 6 N HCl*
Let solution come to room temperature
Make final adjustments to pH
Dilute to 1000 ml with Ultrapure H_2O
Sterilize by autoclaving

or

157.6 g Tris-HCl
Dissolve in 800 ml Ultrapure H_2O
Let solution come to room temperature
Make final adjustments to pH
Dilute to 1000 ml with Ultrapure H_2O
Sterilize by autoclaving

10 N NaOH*

400 g NaOH
Carefully dissolve in 800 ml sterile Ultrapure H_2O
Cool solution to room temperature
Dilute to 1000 ml with sterile Ultrapure H_2O

0.5 M EDTA, pH 8.0*

186.1 g $Na_2EDTA \cong 2H_2O$
Add 800 ml Ultrapure H_2O

[1]Reagents or chemicals marked with * are available from commercial companies such as Amresco, Fisher, Invitrogen (Life Technologies), Roche Molecular Biochemicals, Sigma, Mediatech, Inc. (CellGro), and others.

Mix and adjust pH to 8.0 with 50 ml 10 N NaOH*****

Note: Add 10 N NaOH slowly to solution; check with pH meter.

Dilute to 1000 ml with Ultrapure H_2O
Dispense into aliquots. Sterilize by autoclaving

Phosphate Buffered Saline (PBS), 0.01M, pH 7.2 or pH 7.4* - use to make *Campylobacter* cell suspensions.
The following formula for PBS is from Molecular Cloning - A Laboratory Manual by J. Sambrook and D. Russell, 3rd edition.

137 mM NaCl (8 g)
2.7 mM KCl (0.2 g)
10 mM Na_2HPO_4 (1.44 g)
2 mM KH_2PO_4 (0.24 g)

Dissolve in 800 ml Ultrapure H_2O
Mix and adjust pH to 7.2 or 7.4 with HCl
Adjust final volume to 1000 ml with H_2O
Sterilize by autoclaving for 20 minutes at 15 lb/sq. in. on liquid cycle.
Store at room temperature

20mg/ml Lysozyme Stock Solution - use in *Listeria monocytogenes* standardized PFGE Protocol.

100 mg Lysozyme (Available from Sigma, L7651 or L6875)
5 ml TE Buffer
Mix and dispense in 200-250 µl volumes in 1.5 ml microcentrifuge tubes; store at -20°C

10mg/ml Lysozyme Stock Solution - use in *Listeria monocytogenes* standardized PFGE Protocol.

50 mg Lysozyme (Available from Sigma, L1667)
5 ml TE Buffer
Mix and dispense in 200-250 µl volumes in smaller microcentrifuge tubes; store at -20°C

20% Sodium Dodecyl Sulfate (SDS)***** - use in *Listeria* standardized PFGE protocol.

20 g SDS
80 ml sterile Ultrapure H_2O
Carefully add SDS to H_2O in sterile container; dissolve by mixing gently and warming to 35°- 45°C.

10% Sodium Dodecyl Sulfate (SDS)***** - use in *Listeria* standardized PFGE protocol.

10 g SDS
80 ml sterile Ultrapure H_2O
Carefully add SDS to H_2O in sterile container; dissolve by mixing gently and warming to 35°- 45°C.

20 mg/ml Proteinase K Stock Solution*****

100 mg Proteinase K powder
5 ml sterile Ultrapure H_2O
Mix and dispense in 500-600 µl volumes in 1.5 ml microcentrifuge tubes; store at -20°C.

10% N-Lauroylsarcosine, Sodium salt (Sarcosyl)[2]

10 g Sarcosyl (Available from Sigma, L-9150)
90 ml sterile Ultrapure H_2O
Carefully add Sarcosyl to H_2O in sterile container; dissolve by mixing gently and warming to 50° - 60°C

Safety note: Wear a mask when weighing Lysozyme, SDS, Proteinase K, and Sarcosyl; avoid creating aerosols; and wipe down balance and surrounding area after weighing.

10X Tris-Borate EDTA Buffer (TBE), pH .8.3*****

0.9 M Tris base (108 g)
0.9 M Boric Acid (55 g)
0.02 M EDTA, pH 8.0 (40 ml 0.5 M)
Dilute to 1000 ml with sterile Ultrapure H_2O
Sterilize by autoclaving

Note: Discard if buffer develops precipitate!

Note: The previous formula for TBE is from Molecular Cloning - A Laboratory Manual by J. Sambrook and D. Russell, 3rd Edition. It is similar to the formulas used by several commercial suppliers (Amresco10X - 0658, 5X - J885; Fisher 10X - BP1333-1, BP13334; Roche Molecular Biochemicals 10X - 100 661, 100 759, 1 666 703; Sigma-Aldrich 10X - T4415); it differs from 10X TBE from Invitrogen (Life Technologies) as documented in the formula below. These differences in formulation may affect the mobility of the DNA during electrophoresis.

Invitrogen 10X TBE Buffer, 15581-044 or 15581-028*****

1.0 M Tris base
0.9 M Boric Acid
0.01 M EDTA
pH of 10X solution = 8.4 ∀ 0.1

Ethidium Bromide*****

10 mg/ml stock solution
Dilute 1:10,000 with Ultrapure H_2O (10 µl in 100 ml H_2O).

Diluted Ethidium Bromide solution can be used for staining –up to 20 gels before discarding according to safety guidelines of your institution. See Section 10 of the PulseNet PFGE manual for further information.

[2]This chemical can be added directly to the other ingredients in the cell lysis buffer. See page 5.

WORKING REAGENTS

Use **sterile** glassware, plasticware, and Ultrapure (Reagent Grade Type 1) Water to make working reagents.

Tris:EDTA Buffer (TE), pH 8.0*

10 mM Tris-HCL:1 mM EDTA , pH 8.0[3]

10 ml 1 M Tris-HCL, pH 8.0
2 ml 0.5 M EDTA, pH 8.0
Dilute to 1000 ml with sterile Ultrapure H_2O

E. coli* O157:H7, *Salmonella* and *Shigella sonnei*, and *Campylobacter jejuni standardized PulseNet PFGE protocols - use this TE Buffer to:

a. Dissolve 1% SeaKem Gold
b. Dissolve 1% SeaKem Gold:0.5% SDS
c. Wash PFGE plugs after cell lysis.

L. monocytogenes standardized PulseNet PFGE protocol - use this TE Buffer to:

a. Suspend cells from the agar plates.
b. Wash PFGE plugs after cell lysis.

Cell Suspension Buffer (CSB) - use in ***E. coli* STEC, *Salmonella*, *Shigella* spp and *Vibrio* spp** standardized PulseNet PFGE protocols

100 mM Tris-HCl:100 mM EDTA, pH 8.0

10 ml 1 M Tris-HCl, pH 8.0
20 ml 0.5 M EDTA, pH 8.0
Dilute to 100 ml with sterile Ultrapure H_2O

Cell Lysis Buffer - use for lysing ***E. coli* STECs, *Salmonella*, *Shigella* spp, *Vibrio* spp, *Listeria monocytogenes* and *Campylobacter jejuni*** cells in PFGE plugs made in reusable (or disposable) plug molds; plugs are lysed in 50 ml screw-cap tubes.

50 mM Tris-HCl:50 mM EDTA, pH 8.0 + 1% N-Lauroyl-Sarcosine, Sodium salt (Sarcosyl)
0.1 mg/ml Proteinase K (add just before use).

25 ml 1 M Tris-HCl, pH 8.0
50 ml 0.5 M EDTA, pH 8.0

[3]This formula for TE is from Molecular Cloning - A Laboratory Manual by J. Sambrook and E. Russell, 3rd edition.

50 ml 10% Sarcosyl [3]
Dilute to 500 ml with sterile Ultrapure H_2O

Add **25 µl** Proteinase K stock solution (20 mg/ml) per 5 ml of cell lysis buffer **just before use**. The final concentration of Proteinase K is 0.1 mg/ml.

[3] **Note:** These reagents can also be made by adding 5g of Sarcosyl powder directly to the other three ingredients (Tris-HCl, EDTA, 400 ml H_2O). Warm the solution to 50°- 60°C for 30-60 minutes, or leave at room temperature for 1-2 hours to completely dissolve the Sarcosyl; adjust to the final volume with sterile Ultrapure H_2O.

0.5X TBE Buffer

200 ml **5X** TBE Buffer*
Dilute to 2000 ml with Ultrapure H_2O
or
100 ml **10X** TBE Buffer*
Dilute to 2000 ml with Ultrapure H_2O

Note: The water used to dilute concentrated 5X or 10X TBE buffer to 0.5X TBE does not have to be sterile.

Use of trade names and commercial sources is for identification only and does not imply endorsement by CDC or the U.S. Department of Health and Human Services.

5. FLOW CHART:

6. BIBLIOGRAPHY:

7. CONTACTS:

8. AMENDMENTS:

2010-05-07 Updated the Lysozyme Stock Solution and SDS for *Listeria monocytogenes* to reflect the changes made to the PFGE protocol PNL04.
2013-08-14 Updated Lysozyme Stock Solution and SDS for *Listeria monocytogenes* to reflect the changes made to the PFGE protocol PNL04.
2013-08-14 Clarified language regarding formula for TE.

1. **PURPOSE:** To describe the One-Day (24-26 h) Standardized Laboratory Protocol for Molecular Subtyping of *Campylobacter jejuni* by Pulsed-field Gel Electrophoresis (PFGE).

2. **SCOPE:** To provide the PulseNet participants with a standardized procedure for performing PFGE of *Campylobacter jejuni*, thus ensuring inter-laboratory comparability of the generated results.

3. **DEFNITIONS / TERMS:**
   3.1. PFGE: Pulsed-field Gel Electrophoresis
   3.2. DNA: Deoxyribonucleic acid
   3.3. CDC: Centers for Disease Control and Prevention
   3.4. CLRW: Clinical Laboratory Reagent Water

4. **RESPONSIBILITIES / PROCEDURE:**

BIOSAFETY WARNING: Please read all instructions carefully before starting protocol. Treat all plasticware, glassware, pipets, spatulas, etc. that come in contact with the cell suspensions or plugs as contaminated materials and dispose of, or disinfect according to the guidelines of your institution. Disinfect plug molds before they are washed. Contaminated items should be disinfected with 1% Lysol/Amphyll or 90% ethanol for at least 30 minutes if they will be washed and reused.

Day 0

Streak an isolated colony from test cultures onto Trypticase Soy Agar with 5% defibrinated sheep blood (TSA-SB) plates (or comparable non-selective media) for confluent growth. It is recommended that a storage vial of each culture be created. To do this, stab small screw cap tubes of TSA, HIA, or similar medium with the same inoculating loop used to streak the plate. This will ensure that the same colony can be retested if necessary. Incubate cultures at microaerobically 37ºC for 14-18 h.

Day 1

1. **Turn on shaker water bath or incubator (54-55ºC), stationary water baths (55-60ºC) and spectrophotometer** (or equivalent instrument such as the Dade Microscan Turbidity meter or bioMérieux Vitek colorimeter).

2. Prepare **TE Buffer** (**10 mM Tris:1 mM EDTA, pH 8.0**)[1] as follows:
   10 ml of 1 M Tris, pH 8.0
   2 ml of 0.5 M EDTA, pH 8.0
   Dilute to 1000 ml with sterile, Ultrapure Clinical Laboratory Reagent Water (CLRW)

Note: The TE Buffer used to make the plug agarose is also used to wash lysed PFGE plugs.

3. Prepare 1% SeaKem Gold agarose in **TE Buffer** (**10 mM Tris:1 mM EDTA, pH 8.0**) as follows:
   a. Weigh 0.50 g (or 0.25 g) SeaKem Gold (SKG) into 250 ml screw-cap flask.
   b. Add 50.0 ml (or 25.0 ml) TE Buffer; swirl gently to disperse agarose.
   c. Loosen cap or cover loosely with clear film and microwave for 30 sec; mix gently and repeat for 10 sec intervals until agarose is completely dissolved.
   d. Recap flask and return to a 55-60ºC water bath and equilibrate the agarose for 15 minutes or until ready to use.

SAFETY WARNING: Use heat-resistant gloves when handling hot flasks after microwaving.

[1]Additional information is found on page 11 of this document.
[2]The N-Lauroylsarcosine, Sodium salt can be added directly to the other ingredients and allowed to dissolve. See page 11 of this document.

Note: SeaKem Gold agarose works well for making PFGE plugs because it provides added strength to the plugs minimizing breakage of plugs during the lysis and washing steps. The time and temperature needed to completely dissolve the agarose is dependent on the specifications of the microwave used, and will have to be determined empirically in each laboratory.

4. Label small transparent tubes (12 mm x 75 mm Falcon 2054 tubes or equivalent) with culture numbers.

5. Transfer ~2 ml of **phosphate-buffered saline** (0.01 M PBS, pH 7.4) **or** 0.85% NaCl to small labeled tubes (Falcon 2054 tubes). Use sterile polyester-fiber or cotton swab that has been moistened with sterile PBS to remove some of the growth from agar plate; suspend cells in PBS by spinning swab gently so cells will be evenly dispersed and formation of aerosols is minimized.

Note: The minimum volume of the cell suspension needed will depend on size of the cuvettes or tubes used to measure the cell concentration and are dependent on the manufacturer's specifications for the spectrophotometer, turbidity meter, or colorimeter. Keep suspensions on ice if you have more than 6 cultures to process or refrigerate cell suspensions if you cannot adjust their concentration immediately.

6. Adjust concentration of cell suspensions to one of the values given below by diluting with sterile PBS or by adding additional cells:
   a. Spectrophotometer: 610 nm wavelength, absorbance (Optical Density) of **0.680** (**0.570 – 0.820).**
   b. Dade Microscan Turbidity Meter: **0.35 – 0.45** (measured in Falcon 2054 tubes).
      0.52 – 0.64 (measured in Falcon 2057 tubes).
   c. bioMérieux Vitek colorimeter: ≈ **20%** transmittance (measured in Falcon 2054 tubes)

Note: The values in Steps 6a, 6b and 6c give satisfactory results at CDC; each laboratory may need to establish the optimal concentration needed for satisfactory results.

CASTING PLUGS

Label wells of PFGE plug molds with culture number. When reusable plug molds are used, put strip of tape on lower part of reusable plug mold before labeling wells.

Note: Unused plug agarose can be kept at room temperature and reused 1-2 times. Microwave on low-medium power for 10-15 sec and mix; repeat for 5-10 sec intervals until agarose is completely melted. This agarose melts rapidly!

Note: Proteinase K solutions (20 mg/ml) are available commercially. Alternatively, a stock solution of Proteinase K can be prepared from the powder in sterile Ultrapure water (CLRW). For best results, aliquot 300-500µl into small tubes and store in a freezer (-20 ºC) until ready to use. Just before use, thaw appropriate number of vials needed for the samples; keep Proteinase K solutions on ice. If the Proteinase K stock solution was prepared from powder, discard any thawed solution at the end of the work day. Store commercially prepared Proteinase K solutions according to directions provided by the supplier.

1. Transfer 400µl (0.4 ml) of adjusted cell suspensions to labeled 1.5ml microcentrifuge tubes.

2. Add 20 µl of Proteinase K (20 mg/ml stock) to each tube and mix gently with pipet tip (200 µl is needed for 10 cell suspensions).

3. Add 400 µl (0.4 ml) melted 1% SeaKem Gold agarose to 400 µl cell suspension and mix gently by pipeting up and down two or three times. **Over-pipeting can cause DNA shearing**. Maintain temperature of melted agarose by keeping flask in beaker of warm water (55-60ºC).

4. Immediately, dispense part of mixture into appropriate well(s) of disposable plug mold. Do not allow bubbles

to form. Two plugs of each sample can be made from these amounts of cell suspension and agarose and are useful if repeat testing is required. Allow plugs to solidify at room temperature for 10-15 min. They can also be placed in the refrigerator (4ºC) for 5 minutes.

Note: If disposable plug molds are used for making plugs with 1% SeaKem Gold agarose, use 200 µl cell suspension, 10 µl of Proteinase K (20 mg/ml stock) and 200 µl of agarose; up to 4 plugs can be made from these amounts of cell suspension and agarose.

Note: The generation of cell suspension and the subsequent casting of the plugs should be performed as rapidly as possible in order to minimize premature cell lysis. If large numbers of samples are being prepared, it is recommended that they be processed in batches of ~10 samples at a time. Once the first batch of isolates are in the cell lysis incubation, then start preparing the cells suspensions the next group samples, and so on. All batches can be lysed and washed together, since additional lysis time will not affect the initial batches.

LYSIS OF CELLS IN AGAROSE PLUGS

Note: Two plugs (reusable plug molds) or up to four plugs (disposable plug molds) of the same strain can be lysed in the same 50 ml tube.

1. Label 50 ml polypropylene screw-cap or 50 ml Oak Ridge tubes with culture numbers.

2. Prepare **Cell Lysis Buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl)** as follows:
   25 ml of 1 M Tris, pH 8.0
   50 ml of 0.5 M EDTA, pH 8.0
   50 ml of 10 % Sarcosyl (N-Lauroylsarcosine, Sodium salt)[2]
   Dilute to 500 ml with sterile Ultrapure water (CLRW)

3. Calculate the total volume of **Cell Lysis**/**Proteinase K Buffer** needed as follows:
   a. 5 ml **Cell Lysis Buffer** (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl) is needed per tube (e. g., 5 ml x 10 tubes = 50 ml).
   b. 25 µl **Proteinase K** stock solution (20 mg/ml) is needed per tube of the cell lysis buffer (e. g., 25 µl x 10 tubes = 250 µl).
   c. Prepare the master mix by measuring the correct volume of Cell Lysis Buffer and Proteinase K into appropriate size test tube or flask and mix well.

Note: The final concentration of Proteinase K in lysis buffer is **0.1 mg/ml** and is different from the concentration that was added to the cell suspension (0.5 mg/ml).

4. Add 5 ml of Proteinase K/Cell Lysis Buffer to each labeled 50 ml tube.

5. Trim excess agarose from top of plugs with scalpel, razor blade or similar instrument. Open reusable plug mold and transfer plugs from mold with a 6 mm wide spatula to appropriately labeled tube. If disposable plug molds are used, remove white tape from bottom of mold and push out plug(s) into appropriately labeled tube. **Be sure plugs are under buffer and not on side of tube.**

Note: The excess agarose, scalpel, spatula, tape, etc. are contaminated. Dispose of or disinfect them appropriately.

6. **Remove tape from reusable mold.** Place both sections of plug mold, spatulas, and scalpel in 90% ethanol, 1% Lysol/Amphyll or other suitable disinfectant. **Soak them for 15 minutes before washing them.** Discard

[2]The N-Lauroylsarcosine, Sodium salt can be added directly to the other ingredients and allowed to dissolve. See page 11 of this document.

disposable plug molds.

7. Place tubes in rack and incubate in a 54-55ºC shaker water bath for 15-30 min with **constant and vigorous agitation** (175-200 rpm). If lysing in water bath, be sure water level in water bath is **above** level of lysis buffer in tubes.

8. Pre-heat enough sterile Ultrapure water (CLRW) to 54-55ºC so that plugs can be washed two times with 10-15 ml water (200-250 ml for 10 tubes).

WASHING OF AGAROSE PLUGS AFTER CELL LYSIS

Note: Most laboratories will find that their plugs are sufficiently stable to perform the following washing steps at 54-55ºC. However, if you notice that your plugs are nicked along the edges or breaking it will be necessary for your laboratory to lower the water bath or incubator to 50ºC for the following washing steps.

1. Remove tubes from water bath and carefully pour off lysis buffer. Plugs can be held in tubes with a screened cap (Bio-Rad) or spatula.

Note: Be sure to remove all of the liquid during this and subsequent wash steps by touching lip of tube onto an absorbent paper towel.

2. Add 10-15 ml of sterile Ultrapure water (CLRW) that has been pre-heated to 54-55°C to each tube and shake the tubes vigorously in a 54-55°C water bath or incubator for 10-15 min.

3. Pour off water from the plug and repeat wash step with pre-heated water (Step 2) one more time.
   a. Pre-heat enough sterile **TE Buffer** (10 mM Tris:1 mM EDTA, pH 8.0) in a 54-55°C water bath so that plugs can be washed 4 times with 10-15 ml TE (400-600 ml for 10 tubes) after beginning last water wash.

4. Pour off water, add 10-15 ml pre-heated (54-55°C) sterile TE Buffer, and shake the tubes vigorously in 54-55°C incubator or water bath for 10-15 min.

5. Pour off TE and repeat wash step with pre-heated TE three more times.

6. Decant last wash and add 5-10 ml sterile TE. Continue with step 1 in "Restriction Digestion" section or store plugs in TE Buffer at 4ºC until needed. Plugs can be transferred to smaller tubes for storage.

Note: If restriction digestion is to be done the same day, complete Steps 1-3 of next section (Restriction Digestion) during last TE wash step for optimal use of time.

RESTRICTION DIGESTION OF DNA IN AGAROSE PLUGS

Note: A small slice of the plug (not the entire plug) should be digested with the primary restriction enzyme *Sma*I because less enzyme is required and other slices of the plug can be subjected to restriction analysis with other enzymes. *Kpn*I is recommended as the secondary enzyme for analysis of *Campylobacter jejuni* isolates. The use of a secondary enzyme is useful in situations where the PFGE patterns obtained with the primary enzyme from two or more isolates are indistinguishable

1. Label 1.5 ml microcentrifuge tubes with culture numbers; label 3 (10-well gel) or 4 (15-well gel) tubes for *Salmonella* ser. Braenderup H9812[3] standards.

[3] Directions for making and testing PFGE plugs of *Salmonella* ser. Braenderup H9812 are in PNL05.

a. **Pre-Restriction Incubation Step (highly-recommended)**: Prepare a master mix by diluting the appropriate 10X restriction buffer (Roche Applied Science or equivalent) 1:10 with sterile Ultrapure water (CLRW) according to the following table:

Note: The appropriate restriction buffer will vary between vendors and may differ between enzymes from the same vendor. **Always** use the restriction buffer recommended by the vendor for the particular restriction enzyme.

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices
Sterile Clinical Laboratory Reagent Water (CLRW)	180 µl	1800 µl	2700 µl
10X Restriction Buffer	20 µl	200 µl	300 µl
Total Volume	200 µl	2000 µl	3000 µl

b. Add 200 µl diluted restriction buffer (1X) to labeled 1.5 ml microcentrifuge tubes.
c. Carefully remove plug from TE with spatula and place in a sterile disposable Petri dish or on large glass slide.
d. Cut a 2.0 to 2.5 mm wide slice from each test sample and the appropriate number of *S.* ser. Braenderup H9812 standards with a scalpel (or single edge razor blade, cover slip, etc.) and transfer to tube containing diluted restriction buffer. **Be sure plug slice is under buffer.** Replace rest of plug in original tube that contains 5 ml TE buffer and store at 4°C.

Note: PulseNet recommends that the combs with larger teeth (10 mm wide teeth) be used to cast the gels because computer analysis of the gel lanes is more accurate and less tedious than analysis of gel lanes cast with combs with the smaller teeth (5.5 mm). Using combs with smaller teeth is not advised. The number of slices that can be cut from the plugs will depend on the skill and experience of the operator, integrity of the plug, and whether the slices are cut vertically or horizontally (plugs made in disposable molds).

e. Incubate sample and control plug slices in water bath or incubator for 5-10 min or at room temperature for 10-15 min.
   i. Incubate samples to be restricted with *Sma*I at 25°C
   ii. Incubate samples to be restricted with *Kpn*I and *Xba*I at 37°C.
f. After incubation, remove buffer from plug slice using a pipet fitted with 200-250 µl tip all the way to bottom of tube and aspirate buffer. Be careful not to cut plug slice with pipet tip and that plug slice is not discarded with pipet tip.

2. Prepare the restriction enzyme master mix according to the following table. May mix in the same tube that was used for the diluted restriction buffer:

Note: Enzymes may be purchased in several different stock concentrations. The calculations below are based on using an enzyme at a concentration of 40 U/µl. If a different concentration of enzyme is used, make necessary adjustments to the volume of enzyme and water to achieve a final concentration of 40 U/ sample.

Note: Keep vial of restriction enzyme on ice or in insulated storage box (-20°C) at all times.

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices
Sterile Clinical Laboratory Reagent Water (CLRW)	177 µl	1770 µl	2655 µl
10X Restriction Buffer	20 µl	200 µl	300 µl
BSA (10mg/ml)	2 µl	20 µl	30 µl
***Sma*I (40 U/µl)**	1 µl	10 µl	15 µl

Total Volume	200 µl	2000 µl	3000 µl

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices
Sterile Clinical Laboratory Reagent Water (CLRW)	177 µl	1770 µl	2655 µl
10X Restriction Buffer	20 µl	200 µl	300 µl
BSA (10mg/ml)	2 µl	20 µl	30 µl
***Kpn*I (40 U/µl)**	1 µl	10 µl	15 µl
Total Volume	200 µl	2000 µl	3000 µl

Note: Addition of Bovine Serum Albumin (BSA; highly recommended): Several restriction enzyme vendors specifically recommend the addition of 1X BSA to enzyme restriction mixtures while others do not. PulseNet Central recommends adding BSA to all enzyme restriction mixtures to minimize the incidence of incomplete restriction.

3. Add 200 µl restriction enzyme master mix to each tube. Close tube and mix by tapping gently; **be sure plug slices are under enzyme mixture.**

4. Incubate sample and standard (control) plug slices for 2 hours (unless indicated otherwise) in a water bath or incubator at the appropriate temperature for the enzyme.
   a. Incubate samples restricted with *Sma*I at 25°C.
   b. Incubate samples restricted with *Kpn*I (4 – 6 hours) and *Xba*I at 37°C.

5. If plug slices will be loaded into the wells (Option B, page 7), continue with Steps 1-4 of the next section (**CASTING AGAROSE GEL**) approximately 1 h before restriction digest reaction is finished so the gel can solidify for at least 30 minutes before loading the restricted PFGE plugs.

CASTING AGAROSE GEL

A. Loading Restricted Plug Slices on the Comb:

1. Confirm that water bath is equilibrated to 55-60°C.

2. Make volume of 0.5X Tris-Borate EDTA Buffer (TBE) that is needed for both the gel and electrophoresis running buffer according to one of the following tables.

5X TBE:

Reagent	Volume in milliliters (ml)	
5X TBE	200	220
Clinical Laboratory Reagent Water (CLRW)	1800	1980
Total Volume of 0.5X TBE	2000	2200

10X TBE:

Reagent	Volume in milliliters (ml)

10X TBE	100	110
Clinical Laboratory Reagent Water (CLRW)	1900	2090
Total Volume of 0.5X TBE	2000	2200

3. Make 1% SeaKem Gold (SKG) Agarose in 0.5X TBE as follows:
   a. Weigh appropriate amount of SKG into 500 ml screw-cap flask.
   b. Add appropriate amount of 0.5X TBE; swirl gently to disperse agarose.
      i. Mix 1.0 g agarose with 100 ml 0.5X TBE for 14 cm wide gel form (10wells)
      ii. Mix 1.5 g agarose with 150 ml 0.5X TBE for 21 cm wide gel form (15 wells)
   c. Loosen cap or cover loosely with clear film and microwave for 60 sec; mix gently and repeat for 15 sec intervals until agarose is completely dissolved.
   d. Recap flask and return to 55-60ºC water bath and equilibrate the agarose in the water bath for 15 minutes or until ready to use.

SAFETY WARNING: Use heat-resistant gloves when handling hot flasks after microwaving.

Note: Agarose LF™ (Amresco, X174) is the only acceptable alternative to SeaKem Gold, at this time. The time and temperature needed to completely dissolve the agarose is dependent on the specifications of the microwave used and will have to be determined empirically in each laboratory. Similarly, the optimal running time for each agarose will have to be determined empirically in each laboratory.

4. A small volume (2-5 ml) of melted and cooled (50-60ºC) 1% SKG agarose may be added to fill wells after plugs are loaded. Prepare as described above. Unused SKG agarose can be kept at room temperature, melted, and reused several times.

Note: Place the gel form on a leveling table and adjust until perfectly leveled. Place the comb holder so the front part (side with small metal screws) and teeth face the bottom of gel frame and the comb teeth touch the gel platform**.**

5. Remove restricted plug slices from water bath. Remove enzyme/buffer mixture and add 200 µl 0.5X TBE. Incubate at room temperature for 5 min.

6. Remove plug slices from tubes; put comb on bench top and load plug slices on the bottom edge of the teeth in the following order:
   a. Load *Salmonella* serotype Branderup H9812 standards in lanes (teeth) 1, 5, 10 (10 well gel) or in lanes 1, 5, 10, 15 (15 well gel).
   b. Load samples on remaining teeth of the comb and note locations.

7. Remove excess buffer with tissue or kimwipe. Allow plug slices to air dry on the comb for 5-10 minutes or seal them to the comb with 1% SKG agarose (55-60°C).

8. Position comb in leveled gel form and confirm that the plugs slices are correctly aligned on the bottom of the comb teeth and that the lower edge of the plug slice is flush against the black platform.

9. Carefully pour the agarose (cooled to 55-60ºC) into the gel form and remove any bubbles or debris.

10. Put black gel frame in electrophoresis chamber. Add 2-2.2 L freshly prepared 0.5X TBE. Close cover of unit. The amount of buffer needed depends on whether residual buffer was left in tubing or if unit was flushed with water after the last gel was run.

11. Turn on power supply, pump calibrated to a flow rate of 1 liter/minute (setting of ≈70) and cooling module (14ºC).

12. Remove comb after gel solidifies, about 30-45 minutes.

13. Fill in wells of gel with melted and cooled (55-60ºC) 1% SKG Agarose (optional). Unscrew and remove end gates from gel form; remove excess agarose from sides and bottom of casting platform with a tissue or kimwipe. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber. Close cover of chamber.

B. Loading Restricted Plug Slices into the Wells:

1. Follow steps 1-4 in "Option A" above (**Loading Restricted Plug Slices on the Comb**).

Note: Place the gel form on a leveling table and adjust until perfectly leveled before pouring gel. Position the comb holder so that front part (side with small metal screws) and teeth face the bottom of the gel and the **bottom of the comb is 2mm above the surface of the gel platform**.

2. Cool melted SKG agarose in 55-60°C water bath for 15-20 min; carefully pour agarose into gel form (casting stand) fitted with comb. Be sure there are no bubbles.

3. Put black gel frame in electrophoresis chamber. Add 2-2.2 L freshly prepared 0.5X TBE. Close cover of unit. The amount of buffer depends on whether residual buffer was left in tubing, or if unit was flushed with water after the last gel was run.

4. Turn on power supply, pump calibrated to a flow rate of 1 liter/minute and cooling module (14°C) approximately 30 minutes before gel is to be run.

5. Remove restricted plug slices from water bath. Remove enzyme/buffer mixture and add 200 µl 0.5X TBE. Incubate at room temperature for 5 minutes.

6. Remove comb after gel solidifies, about30 – 45 minutes.

7. Remove restricted plug slices from tubes with tapered end of spatula and load into appropriate wells. Gently push plugs to bottom and front of wells with wide end of spatula. Manipulate position with spatula and be sure that are no bubbles.
   a. Load *Salmonella* serotype Branderup H9812 standards in lanes 1, 5, 10 (10-well gel) or in lanes 1, 5, 10, 15 (15-well gel).
   b. Load samples in remaining wells.

Note: Loading the plug slices can be tedious; each person has to develop his/her own technique for consistently placing the plug slices in the wells so the lanes will be straight and the bands sharp.

8. Fill in wells of gel with melted 1% SKG Agarose (equilibrated to 55-60°C). Allow to harden for 3-5 min. Unscrew and remove end gates from gel form; remove excess agarose from sides and bottom of casting platform with a tissue or kimwipe. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber. Close cover of chamber.

ELECTROPHORESIS CONDITIONS

1. Select following conditions for *Campylobacter jejuni* restricted with ***Sma*I**.

   a. Select following conditions on **Chef Mapper**
      Auto Algorithm
      50 kb - low MW
      400 kb - high MW
      Select default values except where noted by pressing "enter"
      Change run time to 18 – 19 hours (see note below)

(Default values: Initial switch time = 6.76 s; Final switch time = 35.38 s)

b. Select following conditions on **CHEF-DR III**
Initial switch time: 6.8 s
Final switch time: 35.4 s
Voltage: 6 V
Included Angle: 120°
Run time: 18 – 19 h (see note below)

c. Select following conditions on **CHEF-DR II**
Initial A time: 6.8 s
Final A time: 35.4 s
Start ratio: 1.0 (if applicable)
Voltage: 200 V
Run time: 19 – 20 h (see note below)

2. Select following conditions on Chef Mapper for *Campylobacter jejuni* restricted with ***Kpn*I.**

a. Select following conditions on **Chef Mapper**
Auto Algorithm
50 kb - low MW
475 kb - high MW
Select default values except where noted by pressing "enter"
Change run time to 18 – 19 hours (see note below)
Change initial switch time = 5.2 s. Accept default Final switch time = 42.34 s.

b. Select following conditions on **CHEF-DR III**
Initial switch time = 5.2 s
Final Switch time = 42.3 s
Voltage: 6 V
Included Angle: 120°
Run time: 18 – 19 h (see note below)

c. Select following conditions on **CHEF-DR II**
Initial A time: 5.2 s
Final A time: 42.3 s
Start ratio: 1.0 (if applicable)
Voltage: 200 V
Run time: 19 – 20 h (see note below)

Note: The electrophoresis running times recommended above are based on the equipment and reagents used at the CDC. **Run times may be different in your laboratory and will have to be optimized for your gels so that the lowest band in the *S.* ser. Braenderup H9812 standard migrates 1.0 - 1.5 cm from the bottom of the gel.**

Note: Make note of the initial milliamp (mAmp) reading on the instrument. The initial mAmps should be between 110-150 mAmps. A reading outside of this range may indicate that the 0.5X TBE buffer was prepared improperly and the buffer should be remade.

Day 2

STAINING AND DOCUMENTATION OF PFGE AGAROSE GEL

Note: The following staining procedure describes the use of ethidium bromide to stain PFGE gels. Alternate DNA stains may be used. Please see the "Alternate DNA Stains-Results and Recommendations" posting within the PulseNet Documents forum on the SharePoint site for additional information.

1. When electrophoresis run is over, turn off equipment; remove and stain gel with ethidium bromide by diluting 40 µl of ethidium bromide stock solution (10 mg/ml) with 400 ml of reagent grade water. This volume is for a staining box that is approximately 14 cm x 24 cm; a larger container may require a larger amount of staining solution. Stain gel for 20-30 min in covered container.

Note: Ethidium bromide is toxic and a mutagen. Stock solutions of 10 mg/ml Ethidium Bromide (EtBr) in water are available from several commercial companies (Amresco X328; Bio-Rad, 161-0433; Sigma, E-1510). The diluted solution can be kept in dark bottle and reused 6-8 times before discarding according to your institution's guidelines for hazardous waste. CDC does not recommend disposing of EtBr down the drain. Aqueous solutions containing EtBr can be filtered through charcoal or degraded using activated carbon destaining or "tea" bags from Amresco (E732-25 Destaining Bags) or other companies, which effectively and safely remove EtBr from solutions and gels. Once the EtBr is removed, the treated aqueous solutions can be discarded down the drain. If you have further questions about EtBr please refer to the Material Safety Data Sheets (MSDS) provided by the vendor or manufacturer.

Note: Currently, the only acceptable alternative stain options are GelRed™ (Biotium, 31010), SYBR® Safe (Invitrogen, S-33102) and SYBR® Gold (Invitrogen, S-11494). Labs are strongly encouraged to follow manufacturer's instructions and test stains in their labs before adopting them for routine use. If one of the alternative stains is used, the destaining steps should be omitted.

2. Destain gel in approximately 500 ml CLRW for 60-90 min, changing water every 20 minutes. Capture image on Gel Doc 1000, 2000, EQ, XR, or equivalent documentation system. If background interferes with resolution, destain for an additional 30-60 min.

3. Follow directions given with the imaging equipment to save gel image as an ***.1sc** file; convert this file to ***.tif** file for analysis with BioNumerics software program. The gel image should fill the entire window of the imaging equipment (computer) screen (without cutting off wells or lower bands). Ensure that the image is in focus and that there is little to no saturation (over-exposure) in the bands (signified by red pixilation in the QuantityOne or ImageLab software). Additional instructions are provided in PNL07 of the PulseNet QA/QC manual.

4. Drain buffer from electrophoresis chamber and discard. Rinse chamber with 2 L CLRW or, if unit is not going to be used for several days, flush lines with water by letting pump run for 5-10 min **before** draining water from chamber and tubing.

5. If the lowest band in the H9812 standard does not migrate within 1-1.5 cm of the bottom of the gel, the proper run time will need to be determined empirically for the conditions in each laboratory.

Note: The following options are available if PFGE results do not have to be available within 24 hours:
- Plugs can be lysed for longer periods of time (up to 2 hours).
- The washing steps with TE to remove the lysis buffer from the PFGE plugs can be done for longer periods of time (15-30 min) and at lower temperatures (37ºC or room temperature). They can be started on Day 1 and finished the morning of Day 2 after overnight refrigeration of the plugs in TE.

Use of trade names and commercial sources is for identification purposes only and does not imply endorsement by CDC or the U.S. Department of Health and Human Services.

NOTE: CLIA LABORATORY PROCEDURE MANUAL REQUIREMENTS

Efforts have been made to assure that the procedures described in this protocol have been written in accordance with the 1988 Clinical Laboratory Improvement Amendments (CLIA) requirements for a procedure manual (42 CFR 493.1211). However, due to the format required for training, the procedures will require some modifications and additions to customize them for your particular laboratory operation.

Any questions regarding the CLIA requirements for a procedure manual, quality control, quality assurance, etc., should be directed to the agency or accreditation organization responsible for performing your laboratory's CLIA inspection. In addition, some states and accreditation organizations may have more stringent requirements that will need to be addressed.

Formulas of Selected Reagents used in PulseNet Standardized Laboratory Protocol for PFGE

Tris:EDTA Buffer, pH 8.0 (TE, 10 mM Tris:1 mM EDTA, pH 8.0)

10 ml of 1 M Tris, pH 8.0
2 ml of 0.5 M EDTA, pH 8.0
Dilute to 1000 ml with sterile Ultrapure water (CLRW)

Cell Lysis Buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosine + 0.1 mg/ml Proteinase K)

25 ml (50 ml) of 1 M Tris, pH 8.0
50 ml (100 ml) of 0.5 M EDTA, pH 8.0
50 ml (100 ml) 10% N-Lauroylsarcosine, Sodium salt (Sarcosyl)

OR

5 g (10 g) of N-Lauroylsarcosine, Sodium salt (Sarcosyl)[4]
Dilute to 500 ml (1000 ml) with Sterile Ultrapure water (CLRW)

Add **25 µl** Proteinase K stock solution (20 mg/ml) per 5 ml of cell lysis buffer just before use for a final concentration in the lysis buffer of 0.1 mg/ml Proteinase K.

5. FLOW CHART:

6. BIBLIOGRAPHY:

7. CONTACTS:

8. AMENDMENTS:

8.1 The phrase "Type I Water" has been changed to "Ultrapure Clinical Laboratory Reagent Water (CLRW)." The water composition is the same, but this reflects a change in the terminology used by the Clinical Laboratory Standards Institute (CLSI).

8.2 2011-08 changes:

- The wording for programming electrophoresis conditions was updated to standardize this section and make it the same as other PFGE laboratory SOPs.
- The wording for washing the agarose plugs after cell lysis was also updated to standardize the section.

8.3 2013-03 changes:

- Corrected formula for TE buffer. TE used at CDC is 10 mM for Tris and 1 mM for EDTA.
- Recommended disinfectant changed from 10% bleach to 1% Lysol/Amphyll or 90% ethanol.
- Volume of TE needed for washing plugs was correted from 300 – 350 ml to 400 – 600 ml.
- A statement was added to clarify that using combs with small teeth (5.5 mm) was not advised.
- Use of pre-restriction step and BSA was changed from optional to highly recommended. Calculation for including BSA in restriction enzyme master mix was added.

[4]If Sarcosyl powder is added directly to the other components of this reagent, warm the solution to 50- 60ºC for 30-60 minutes, or leave at room temperature for ≈2 hours to completely dissolve the Sarcosyl; adjust to the final volume with sterile Ultrapure water (CLRW).

- Statement allowing of Megbase agarose (BioRad) was deleted. Additional testing revealed run time and normalization were negatively impacted by this agarose.
- A statement was included to allow the use of alternative DNA stains that are equivalent to EtBr. Labs are strongly urged to follow manufacturer's instructions as well as test stains in their own labs to gain experience using alternative agarose stains. Additional stain alternatives may be tested and deemed acceptable at a later date.
- The option to allow incubation times for restriction digestion to be increased longer than recommended was deleted.

1. **PURPOSE:** To describe the One-Day (24-26 h) standardized laboratory protocol for molecular subtyping of *Listeria monocytogenes* by Pulsed-field Gel Electrophoresis (PFGE).

2. **SCOPE:** To provide the PulseNet participants with a standardized procedure for performing PFGE of *Listeria monocytogenes* thus ensuring inter-laboratory comparability of the generated results.

3. **DEFINITIONS/TERMS:**
   3.1 PFGE: Pulsed-field Gel Electrophoresis
   3.2 DNA: Deoxyribonucleic acid
   3.3 CDC: Centers for Disease Control and Prevention
   3.4 CLRW: Clinical Laboratory Reagent Water

4. **RESPONSIBILITIES/PROCEDURE:**

PREPARATION OF PFGE PLUGS FROM AGAR CULTURES

BIOSAFETY WARNING: The infectious dose for listeriosis has not been determined and it may depend, in part, on the susceptibility of the host. Groups at highest risk of acquiring infection are pregnant women, neonates, immunocompromised patients, and the elderly. Therefore, laboratorians working with *Listeria monocytogenes*, particularly those who may be at increased risk of acquiring listeriosis should be made aware of this potential and advised to be particularly cautious when working with this organism.

Please read all instructions carefully before starting protocol. Treat all plasticware, glassware, pipets, spatulas, etc. that come in contact with the cell suspensions or plugs as contaminated materials and dispose of, or disinfect according to the guidelines of your institution. Disinfect reusable plug molds before they are washed; the disposable plug molds, including the tape and the tab that is used to push the plugs out of the wells, are also contaminated and should be disinfected with 1% Lysol/Amphyll or 90% ethanol for at least 30 minutes if they will be washed and reused.

Day 0

Streak an isolated colony from test cultures onto Brain Heart Infusion Agar (BHIA) plates (or comparable non-selective media) for confluent growth. It is recommended that a storage vial of each culture be created. To do this, stab small screw cap tubes of TSA, HIA, or similar medium with the same inoculating loop used to streak the plate. This will ensure that the same colony can be retested if necessary. Incubate cultures at 37ºC for 14-18 h.

Day 1

1. **Turn on shaker water bath or incubator (54-55ºC), stationary water baths (55-60ºC) and spectrophotometer** (or equivalent instrument such as the Dade Microscan Turbidity meter or bioMérieux Vitek colorimeter).

2. Prepare **TE Buffer** (**10 mM Tris:1 mM EDTA, pH 8.0**)[1] as follows:
   10 ml of 1 M Tris, pH 8.0
   2 ml of 0.5 M EDTA, pH 8.0
   Dilute to 1000 ml with sterile Ultrapure water (Clinical Laboratory Reagent Water (CLRW))

Note: The TE Buffer is used to make the plug agarose, suspend the cells, and to wash lysed PFGE plugs.

3. Prepare Lysozyme stock solution as follows:

[1]Additional information is found on page 12 of this document.

a. For 20mg/ml stock, weigh out 100 mg Lysozyme (Sigma L7651 or L6875). Keep the container of Lysozyme on ice.
b. Add 5 mL TE buffer, swirl to mix,
c. Aliquot 250 µL amounts into sterile eppendorf tubes and freeze for future use.
d. Or for 10mg/ml stock, weight out 50 mg Lysozyme (Sigma L1667). Keep the container of Lysozyme on ice.
e. Add 5 mL TE buffer, swirl to mix.
f. Aliquot 250 µL amounts into sterile eppendorf tubes and freeze for future use.

4. Prepare 1% SeaKem Gold agarose + 0.5% Sodium Dodecyl Sulfate (SDS) in **TE Buffer** (**10 mM Tris:1 mM EDTA, pH 8.0**) for PFGE plugs as follows:
   a. Place 10% SDS stock solution (GibcoBRL #15553-035) into 55- 60ºC water bath to warm.
   b. Weigh 0.50 g (or 0.25 g) SeaKem Gold (SKG) agarose into 250 ml screw-cap flask.
   c. Add 47.5 ml (or 23.75 ml) TE Buffer; swirl gently to disperse agarose.
   d. Loosen or remove cap and cover loosely with clear film, and microwave for 30 sec; mix gently and repeat for 10 sec intervals until agarose is completely dissolved.
   e. Add 2.5 mL (or 1.25 mL) of warm 10% SDS stock solution, swirl to mix.
   f. Recap flask and return to 55- 60ºC water bath and equilibrate the agarose in the water bath for 15 minutes or until ready to use.

SAFETY WARNING: Use heat-resistant gloves when handling hot flasks after microwaving.

Note: SeaKem Gold agarose works well for making PFGE plugs because it provides added strength to the plugs minimizing breakage of plugs during the lysis and washing steps. The time and temperature needed to completely dissolve the agarose is dependent on the specifications of the microwave used, and will have to be determined empirically in each laboratory.

5. Label small transparent tubes (12 mm x 75 mm Falcon 2054 tubes or equivalent) with culture numbers.

6. Transfer ≈2 ml of **TE Buffer** to small labeled tubes. Use a sterile polyester-fiber or cotton swab that has been moistened with sterile TE to remove some of the growth from the agar plate; suspend cells in TE by spinning swab gently so cells will be evenly dispersed and formation of aerosols is minimized.

Note: The minimum volume of the cell suspension needed will depend on size of the cuvettes or tubes used to measure the cell concentration and are dependent on the manufacturer's specifications for the spectrophotometer, turbidity meter, or colorimeter. Keep suspensions on ice if you have more than 6 cultures to process at one time.

7. Adjust concentration of cell suspensions to one of values given below by diluting with sterile TE or by adding additional cells.
   a. Spectrophotometer: 610 nm wavelength, absorbance (Optical Density) of **1.00**
   b. Dade Microscan Turbidity Meter: **0.40 - 0.45** (measured in Falcon 2054 tubes)
      0.58 - 0.63 (measured in Falcon 2057 tubes)
   c. bioMérieux Vitek colorimeter: **≈17-18%** transmittance (measured in Falcon 2054 tubes)

Note: Cell suspensions need to be at room temperature when concentration is checked. The values in Steps 7a, 7b and 7c give satisfactory results at CDC; if different instruments or tubes are used, each laboratory may need to establish the concentration needed for satisfactory results.

CASTING PLUGS

Label wells of PFGE plug molds with culture number. When reusable plug molds are used, put strip of tape on lower part of reusable plug mold before labeling wells.

Note: Unused plug agarose can be kept at room temperature and reused 1-2 times. Microwave on low-medium power for 10 -15 sec and mix; repeat for 5-10 sec intervals until agarose is completely melted. This agarose melts rapidly!

Note: Proteinase K solutions (20 mg/ml) are available commercially. Alternatively, a stock solution of Proteinase K can be prepared from the powder in sterile Ultrapure water (CLRW). For best results, aliquot 300-500 µl into small tubes and store in a freezer (-20ºC) until ready to use. Just before use, thaw appropriate number of vials needed for the samples; keep Proteinase K solutions on ice. If the Proteinase K stock solution was prepared from powder, discard any thawed solution at the end of the work day. Store commercially prepared Proteinase K solutions according to directions provided by the supplier.

1. Transfer 400 µl (0.4 ml) adjusted cell suspensions to labeled 1.5 ml microcentrifuge tubes.

2. Add 20 µL thawed Lysozyme stock solution (20 mg/mL or 10 mg/mL[2]) to each tube and mix gently. Place tubes into a 55-60ºC water bath for 10-20 minutes. Discard unused thawed Lysozyme solution.

3. Add 20 µl of Proteinase K (20 mg/ml stock) to each tube and mix gently with pipet tip. (200 µl is needed for 10 cell suspensions.)

4. Add 400 µl (0.4 ml) melted 1% SeaKem Gold agarose to 400 µl cell suspension and mix by gently pipetting mixture up and down a few times. **Over-pipeting can cause DNA shearing**. Maintain temperature of melted agarose by keeping flask in beaker of warm water (55-60ºC).

5. Immediately, dispense part of mixture into appropriate well(s) of reusable plug mold. Do not allow bubbles to form. Two plugs of each sample can be made from these amounts of cell suspension and agarose. Allow plugs to solidify at room temperature for 10-15 min. They can also be placed in the refrigerator (4ºC) for 5 minutes.

Note: If disposable plug molds are used for making plugs with 1% SeaKem Gold agarose, use 200 µl cell suspension, 10 µl of Lysozyme (20 mg/ml),10 µl of Proteinase K (20 mg/ml stock), and 200 µl of agarose; up to 4 plugs can be made from these amounts of cell suspension and agarose.

Note: The generation of cell suspension and the subsequent casting of the plugs should be performed as rapidly as possible in order to minimize premature cell lysis. If large numbers of samples are being prepared, it is recommended that they be processed in batches of ~10 samples at a time. Once the first batch of isolates are in the cell lysis incubation, then start preparing the cells suspensions the next group samples, and so on. All batches can be lysed and washed together, since additional lysis time will not affect the initial batches.

LYSIS OF CELLS IN AGAROSE PLUGS

Note: Two plugs (reusable plug molds) or up to four plugs (disposable plug molds) of the same strain can be lysed in the same 50 ml tube.

1. Label 50 ml polypropylene screw-cap or 50 ml Oak Ridge tubes with culture numbers.

[2] The same volume is added of either stock solution because L1667 has approximately twice the activity of L6875 or L7651, according to Sigma.

2. Prepare **Cell Lysis Buffer** (**50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl**) as follows:
   25 ml of 1 M Tris, pH 8.0
   50 ml of 0.5 M EDTA, pH 8.0
   50 ml of 10 % Sarcosyl (N-Lauroylsarcosine, Sodium salt)[3]
   Dilute to 500 ml with sterile Ultrapure water (CLRW)

3. Calculate the total volume of **Cell Lysis**/**Proteinase K Buffer** needed as follows:
   a. 5 ml **Cell Lysis Buffer** (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl) is needed per tube (e. g., 5 ml x 10 tubes = 50 ml).
   b. 25 µl **Proteinase K** stock solution (20 mg/ml) is needed per tube of the cell lysis buffer (e. g., 25 µl x 10 tubes = 250 µl).
   c. Prepare the master mix by measuring the correct volume of Cell Lysis Buffer and Proteinase K into appropriate size test tube or flask and mix well.

Note: The final concentration of Proteinase K in the lysis buffer is **0.1 mg/ml** and is different from the concentration that was added to the cell suspension (0.5 mg/ml).

4. Add 5 ml of Proteinase K/Cell Lysis Buffer to each labeled 50 ml tube.

5. Trim excess agarose from top of plugs with scalpel, razor blade or similar instrument. Open reusable plug mold and transfer plugs from mold with a 6 mm wide spatula to appropriately labeled tube. If disposable plug molds are used, remove white tape from the bottom of mold and push out plug(s) into appropriately labeled tube**. Be sure plugs are under buffer and not on side of tube.**

Note: The excess agarose, plug mold, spatula, etc. are contaminated. Discard or disinfect appropriately.

6. **Remove tape from reusable mold.** Place both sections of the plug mold, spatulas, and scalpel in 90% ethanol, 1% Lysol/Amphyll or other suitable disinfectant. **Soak them for 15 minutes before washing them.** Discard disposable plug molds.

7. Place tubes in rack and incubate in a 54-55ºC shaker water bath or incubator for 2 h with **constant and vigorous agitation** (150-175 rpm). If lysing in water bath, be sure water level is **above** level of lysis buffer in tubes.

8. Pre-heat enough sterile Ultrapure water (CLRW) to 54-55ºC so that plugs can be washed two times with 10-15 ml water (200-250 ml for 10 tubes).

WASHING OF AGAROSE PLUGS AFTER CELL LYSIS

Note: Most laboratories will find that their plugs are sufficiently stable to perform the following washing steps at 54-55ºC. However, if you notice that your plugs are nicked along the edges or breaking it will be necessary for your laboratory to lower the water bath or incubator to 50ºC for the following washing steps.

1. Remove tubes from water bath or incubator, and carefully pour off lysis buffer into an appropriate discard container; plugs can be held in tubes with a screened cap or spatula.

[3]The N-Lauroylsarcosine, Sodium salt can be added directly to the other ingredients and allowed to dissolve. See page 12 of this document.

Note: It is important to remove all of the liquid during this and subsequent wash steps by touching edge of tube or screened cap on an absorbent paper towel.

2. Add at 10-15 ml sterile Ultrapure water (CLRW) that has been pre-heated to 54-55ºC to each tube and shake the tubes in a 54-55ºC water bath or incubator for 10-15 min.

3. Pour off water from the plugs and repeat wash step with pre-heated water (Step 2) one more time.
   a. Pre-heat enough sterile **TE Buffer** (10 mM Tris:1 mM EDTA, pH 8.0) in a 54-55ºC water bath so that plugs can be washed four times with 10-15 ml TE (400-600 ml for 10 tubes) after beginning last water wash.

4. Pour off water, add 10-15 ml pre-heated (54-55ºC) sterile TE Buffer, and shake the tubes in 54-55ºC water bath or incubator for 10-15 min.

5. Pour off TE and repeat wash step with pre-heated TE three more times.

6. Decant last wash and add 5-10 ml sterile TE. Continue with step 1 in "Restriction Digestion" section or store plugs in TE Buffer at 4ºC until needed. Plugs can be transferred to smaller tubes for storage.

Note: If restriction digestion is to be done the same day, complete Steps 1-3 of next section (**RESTRICTION DIGESTION OF DNA IN AGAROSE PLUGS WITH *Asc*I or *Apa*I**) during last TE wash step for optimal use of time.

RESTRICTION DIGESTION OF DNA IN AGAROSE PLUGS

Note: A small slice of the plug (not the entire plug) should be digested with the primary restriction enzyme *Asc*I because less enzyme is required and other slices of the plug can be subjected to restriction analysis with other enzymes. *Apa*I is recommended as the secondary enzyme for analysis of *Listeria monocytogenes* isolates. The use of a secondary enzyme is useful in situations where the PFGE patterns obtained with the primary enzyme are also indistinguishable.

1. Label 1.5 ml microcentrifuge tubes with culture numbers; label 3 (10-well gel) or 4 (15-well gel) tubes for *Salmonella* ser. Braenderup H9812[4] standards.
   a. **Pre-Restriction Incubation Step (highly recommended)**: Prepare a master mix by diluting the appropriate 10X restriction buffer (Roche Applied Science or equivalent) 1:10 with sterile Ultrapure water (CLRW) according to the following table:

Note: Use the appropriate buffer supplied by the enzyme manufacturer. For NEB enzymes, the corresponding buffer for *Asc*I and *Apa*I is NEBuffer 4. For Roche the corresponding buffer for *Apa*I is buffer A. Roche does not sell *Asc*I.

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices
Sterile Clinical Laboratory Reagent Water (CLRW)	180 µl	1800 µl	2700 µl
10X Restriction Buffer	20 µl	200 µl	300 µl

[4]Directions for making and testing PFGE plugs of *Salmonella* ser. Braenderup H9812 are in PNL05.

Total Volume	200 µl	2000 µl	3000 µl

b. Add 200 µl diluted restriction buffer (1X) to labeled 1.5 ml microcentrifuge tubes.
c. Carefully remove plug from TE with spatula and place in a sterile disposable Petri dish or on large glass slide.
d. Cut a 2.0 to 2.5 mm-wide slice from each test sample and the appropriate number of *S.* ser. Braenderup H9812 standards with a single edge razor blade (or scalpel, cover slip, etc.) and transfer to tube containing diluted restriction buffer. **Be sure plug slice is under buffer.** Replace rest of plug in the original tube that contains 5 ml TE buffer and store at 4ºC.

Note: PulseNet recommends that the combs with larger teeth (10 mm-wide teeth) be used to cast the gels because computer analysis of the gel lanes is more accurate and less tedious than analysis of gel lanes cast with combs with the smaller teeth (5.5 mm). Using combs with smaller teeth is not advised. The number of slices that can be cut from the plugs will depend on the skill and experience of the operator, integrity of the plug, and whether the slices are cut vertically or horizontally (plugs made in disposable molds).

e. Incubate sample and control plug slices in water bath or incubator for 5-10 min or at room temperature for 10-15 min.
   i. Incubate samples to be restricted with *Asc*I and *Xba*I at 37°C.
   ii. Incubate samples to be restricted with *Apa*I at 30°C (Roche) or 25°C (New England Biolabs).
f. After incubation, remove buffer from plug slice using a pipet fitted with 200-250 µl tip all the way to bottom of tube and aspirate buffer. Be careful not to damage the plug slice with pipet tip and that plug slice is not discarded with pipet tip.

2. Prepare the restriction enzyme master mix according to the following table. May mix in the same tube that was used for the diluted restriction buffer:

Note: Enzymes may be purchased in several different stock concentrations. The calculations outlined here are based on using an enzyme at a concentration of 10 or 50 U/µl. If the enzyme used is of a different concentration, make necessary adjustments to the volume of enzyme and water to achieve a final concentration of **25 U/ sample**.

Note: Keep vial of restriction enzyme on ice or in insulated storage box (-20˚C) at all times.

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices
Sterile Clinical Laboratory Reagent Water (CLRW)	175.5 µl	1755 µl	2632.5 µl
10X Corresponding Restriction Buffer	20 µl	200 µl	300 µl
BSA (10mg/ml)	2 µl	20 µl	30 µl
***Asc*I (10 U/µl)**	2.5 µl	25 µl	37.5 µl
Total Volume	200 µl	2000 µl	3000 µl

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices

Sterile Clinical Laboratory Reagent Water (CLRW)	177.5 µl	1775 µl	2662.5 µl
10X Corresponding Restriction Buffer	20 µl	200 µl	300 µl
BSA (10mg/ml)	2 µl	20 µl	30 µl
***Apa*I (50 U/µl)**	0.5 µl	5 µl	7.5 µl
Total Volume	200 µl	2000 µl	3000 µl

Note: Addition of Bovine Serum Albumin (BSA; highly recommended): Several restriction enzyme vendors specifically recommend the addition of 1X BSA to enzyme restriction mixtures while others do not. PulseNet Central recommends adding BSA to all enzyme restriction mixtures to minimize the incidence of incomplete restriction.

3. Add 200 µl restriction enzyme master mix to each tube. Close tube and mix by tapping gently; **be sure plug slices are under enzyme mixture.**

4. Incubate sample and standard (control) plug slices for 2 hours in a water bath at the appropriate temperature for the enzyme.
   a. Incubate samples restricted with *Apa*I at 25°C.
   b. Incubate samples restricted with *Asc*I and *Xba*I at 37°C.

5. If plug slices will be loaded into the wells (Option B, page 9), continue with Steps 1-4 of the next section (**CASTING AGAROSE GEL**) approximately 1 h before restriction digest reaction is finished so the gel can solidify for at least 30 minutes before loading the restricted PFGE plugs.

CASTING AGAROSE GEL

A. Loading Restricted Plug Slices on the Comb:

1. Confirm that water bath is equilibrated to 55-60ºC.

2. Make volume of 0.5X Tris-Borate EDTA Buffer (TBE) that is needed for both the gel and electrophoresis running buffer according to one of the following tables.

5X TBE:

Reagent	Volume in milliliters (ml)	
5X TBE	200	220
Clinical Laboratory Reagent Water (CLRW)	1800	1980
Total Volume of 0.5X TBE	2000	2200

10X TBE:

Reagent	Volume in milliliters (ml)	
10X TBE	100	110
Clinical Laboratory Reagent Water (CLRW)	1900	2090
Total Volume of 0.5X TBE	2000	2200

3. Make 1% SeaKem Gold (SKG) agarose in 0.5X TBE as follows:
   a. Weigh appropriate amount of SKG into 500 ml screw-cap flask.
   b. Add appropriate amount of 0.5X TBE; swirl gently to disperse agarose.
      i. Mix 1.0 g agarose with 100 ml 0.5X TBE for 14 cm wide gel form (10 wells)
      ii. Mix 1.5 g agarose with 150 ml 0.5X TBE for 21 cm wide gel form (15 wells)
   c. Loosen cap and microwave for 60 sec; mix gently and repeat for 15 sec intervals until agarose is completely dissolved.
   d. Recap flask and return to 55-60ºC water bath and equilibrate the agarose in the water bath for 15 minutes or until ready to use.

SAFETY WARNING: Use heat-resistant gloves when handling hot flasks after microwaving.

Note: Agarose LF™ (Amresco, X174) is the only acceptable alternative to SeaKem Gold, at this time. The time and temperature needed to completely dissolve the agarose is dependent on the specifications of the microwave used and will have to be determined empirically in each laboratory. Similarly, the optimal running time for each agarose will have to be determined empirically in each laboratory.

4. A small volume (2-5 ml) of melted and cooled (50-60ºC) 1% SKG agarose may be wanted to seal wells after plugs are loaded. Prepare as described above. Unused SKG agarose can be kept at room temperature, melted, and reused several times.

Note: Place the gel form on a leveling table and adjust until perfectly leveled. Place the comb holder so the front part (side with small metal screws) and teeth face the bottom of gel frame and the comb teeth touch the gel platform.

5. Remove restricted plug slices from the water bath. Remove enzyme/buffer mixture and add 200 µl 0.5X TBE. Incubate at room temperature for 5 min.

6. Remove plug slices from tubes; put comb on bench top and load plug slices on the bottom of the comb teeth as follows:
   a. Load *S.* ser. Braenderup H9812 standards on teeth (lanes) 1, 5, 10 (10-well gel) or on teeth 1, 5, 10, 15 (15-well gel).
   b. Load samples on remaining teeth and note locations.

7. Remove excess buffer with tissue or kimwipe. Allow plug slices to air dry on the comb for 5-10 minutesor seal them to the comb with 1% SKG agarose (55-60ºC).

8. Position comb in leveled gel form and confirm that the plugs slices are correctly aligned on the bottom of the comb teeth, and that the lower edge of the plug slice is flush against the black platform..

9. Carefully pour the agarose (cooled to 55-60ºC) into the gel form and remove any bubbles or debris.

10. Put black gel frame in electrophoresis chamber. Add 2 -2.2 L freshly prepared 0.5X TBE. Close cover of unit. The amount of buffer needed depends on whether residual buffer was left in tubing or if unit was flushed with water after the last gel was run.

11. Turn on power supply, pump calibrated to a flow rate of 1 liter/minute (setting of ~70) and cooling module (14ºC) approximately 30 minutes before gel is to be run.

12. Remove comb after gel solidifies, about 30-45 minutes.

13. Fill in wells of gel with melted and cooled (55-60ºC) 1% SKG Agarose (optional). Unscrew and remove end gates from gel form; remove excess agarose from sides and bottom of casting platform with a tissue or kimwipe. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber. Close cover of chamber.

B. Loading Restricted Plug Slices into the Wells:

1. Follow steps 1-4 in Option A on pages 7 and 8 (**Loading Restricted Plug Slices on the Comb**).

Note: Place the gel form on a leveling table and adjust until perfectly leveled before pouring gel. Position the comb holder so that front part (side with small metal screws) and teeth face the bottom of gel and the **bottom edge of the comb is 2 mm above the surface of the gel platform.**

2. Cool melted SKG agarose in 55-60ºC water bath for 15-20 min; carefully pour agarose into gel form (casting stand) fitted with comb. Be sure there are no bubbles.

3. Put black gel frame in electrophoresis chamber. Add 2-2.2 L freshly prepared 0.5X TBE. Close cover of unit. (The amount of buffer depends on whether residual buffer was left in tubing, or if unit was flushed with water after the last gel was run.)

4. Turn on power supply, pump calibrated to a flow rate of 1 liter/minute (set at ~70) and cooling module (14ºC) approximately 30 min before gel is to be run.

5. Remove restricted plug slices from 37ºC water bath. Remove enzyme/buffer mixture and add 200 µl 0.5X TBE. Incubate at room temperature for 5 minutes.

6. Remove comb after gel solidifies for at least 30 minutes.

7. Remove restricted plug slices from tubes with tapered end of spatula and load into appropriate wells. Gently push plugs to bottom and front of wells with wide end of spatula. Manipulate position with spatula and be sure that are no bubbles.
   a. Load *S.* ser. Braenderup H9812 standards in wells (lanes) 1, 5, 10 (10 well gel) or in wells 1, 5, 10, 15 (15 well gel).
   b. Load samples in remaining wells.

Note: Loading the plug slices can be tedious; each person has to develop his/her own technique for consistently placing the plug slices in the wells so the lanes will be straight and the bands sharp.

8. Fill in wells of gel with melted 1% SKG agarose (equilibrated to 55-60ºC). Allow to harden for 3-5 min. Unscrew and remove end gates from gel form; remove excess agarose from sides and bottom of casting platform with a tissue or kimwipe. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber. Close cover of chamber.

ELECTROPHORESIS CONDITIONS

1. Select following conditions for *Listeria monocytogenes* strains restricted with *Asc*I or *Apa*I:
    a. Select following conditions on **CHEF Mapper**
        Auto Algorithm
        49 kb - low MW
        450 kb - high MW
        Select default values except where noted by pressing "enter."
        Initial switch time = 4.0 s
        Final switch time = 40.0 s
        Change run time to 18 - 19 h (See note below)

    b. Select following conditions on **CHEF-DR III**
        Initial switch time: 4.0 s
        Final switch time: 40.0 s
        Voltage: 6 V
        Included Angle: 120°
        Run time: 18-19 h (See note below)

    c. Select following conditions on **CHEF-DR II**
        Initial A time: 4.0 s
        Final A time: 40.0 s
        Start ratio: 1.0 (if applicable)
        Voltage: 200 V
        Run time: 19-20 h (See note below)

Note: The electrophoresis running times recommended above are based on the equipment and reagents used at the CDC. **Run times may be different in your laboratory and will have to be optimized for your gels so that the lowest band in the *S.* ser. Braenderup H9812 standard migrates 1.0 - 1.5 cm from the bottom of the gel.**

Note: Make note of the initial milliamp (mAmp) reading on the instrument. The initial mAmps should be between 110-150 mAmps. A reading outside of this range may indicate that the 0.5X TBE buffer was prepared improperly and the buffer should be remade.

Day 2

STAINING AND DOCUMENTATION OF PFGE AGAROSE GEL

Note: The following staining procedure describes the use of ethidium bromide to stain PFGE gels. Alternate DNA stains may be used. Please see the "Alternate DNA Stains-Results and Recommendations" posting within the PulseNet Documents forum on the SharePoint site for additional information.

1. When electrophoresis run is over, turn off equipment; remove and stain gel with ethidium bromide by diluting 40 µl of ethidium bromide stock solution (10 mg/ml) with 400 ml CLRW. This volume is for a staining box that is approximately 14 cm x 24 cm; a larger container may require a larger amount of staining solution). Stain gel for 20 - 30 min in covered container.

Note: Ethidium bromide is toxic and a mutagen. Stock solutions of 10 mg/ml Ethidium Bromide (EtBr) in water are available from several commercial companies (Amresco X328; Bio-Rad, 161-0433; Sigma, E-1510). The diluted solution can be kept in dark bottle and reused 6-8 times before discarding according to your institution's guidelines for hazardous waste. CDC does not recommend disposing of EtBr down the drain. Aqueous solutions containing EtBr can be filtered through charcoal or degraded using activated carbon destaining or "tea" bags from Amresco (E732-25 Destaining Bags) or other companies, which effectively and safely remove EtBr from solutions and gels. Once the EtBr is removed, the treated aqueous solutions can be discarded down the drain. If you have further questions about EtBr please refer to the **Material Safety Data Sheets** (**MSDS**) provided by the vendor or manufacturer.

Note: Currently, the only acceptable alternative stain options are GelRed™ (Biotium, 31010), SYBR® Safe (Invitrogen, S-33102) and SYBR® Gold (Invitrogen, S-11494). Labs are strongly encouraged to follow manufacturer's instructions and test stains in their labs before adopting them for routine use. If one of the alternative stains is used, the destaining steps should be omitted.

2. Destain gel in approximately 500 ml CLRW for 60 - 90 min, changing water every 20 minutes. Capture image using a Gel Doc 1000, 2000, EQ, XR, or equivalent documentation system. If too much background is observed destain for an additional 30-60 min.

3. Follow directions given with the imaging equipment to save gel image as a ***.1sc** file; convert this file to ***.tif** file for analysis with the BioNumerics software program. The gel image should fill the entire window of the imaging equipment (computer) screen (without cutting off wells or lower bands). Ensure that the image is in focus and that there is little to no saturation (over-exposure) in the bands (signified by red pixilation in the QuantityOne or ImageLab software). Additional instructions are provided in PNL07 of the PulseNet QA/QC manual.

4. Drain buffer from electrophoresis chamber and discard. Rinse chamber with 2 L CLRW or, if unit is not going to be used for several days, flush lines with water by letting pump run for 5-10 min **before** draining water from chamber and hoses.

5. If the lowest band in the H9812 standard does not migrate within 1-1.5 cm of the bottom of the gel, the proper run time will need to be determined empirically for the conditions in each laboratory.

Note: The following options are available if PFGE results do not have to be available within 24-28 hours:
- Plugs can be lysed for longer periods of time (3-16 hours).
- The washing steps with TE to remove the lysis buffer from the PFGE plugs can be done for longer periods of time (30-45 min) and at lower temperatures (37°C or room temperature). They can be started on Day 1 and finished on Day 2 after overnight refrigeration of the plugs in TE.

Use of trade names and commercial sources is for identification purposes only and does not imply endorsement by CDC or the U.S. Department of Health and Human Services.

NOTE: CLIA LABORATORY PROCEDURE MANUAL REQUIREMENTS

Efforts have been made to assure that the procedures described in this protocol have been written in accordance with the 1988 Clinical Laboratory Improvement Amendments (CLIA) requirements for a procedure manual (42 CFR 493.1211). However, due to the format required for training, the procedures will require some modifications and additions to customize them for your particular laboratory operation.

Any questions regarding the CLIA requirements for a procedure manual, quality control, quality assurance, etc., should be directed to the agency or accreditation organization responsible for performing your laboratory's CLIA inspection. In addition, some states and accreditation organizations may have more stringent requirements that will need to be addressed.

Formulas of Selected Reagents used in PulseNet Standardized Laboratory Protocol for PFGE

Tris:EDTA Buffer, pH 8.0 (TE, 10 mM Tris:1 mM EDTA, pH 8.0)

10 ml of 1 M Tris, pH 8.0
2 ml of 0.5 M EDTA, pH 8.0
Dilute to 1000 ml with sterile Ultrapure Water (CLRW)

Cell Lysis Buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosine + 0.1 mg/ml Proteinase K)

25 ml (50 ml) of 1 M Tris, pH 8.0
50 ml (100 ml) of 0.5 M EDTA, pH 8.0
50 ml (100 ml) 10% N-Lauroylsarcosine, Sodium salt (Sarcosyl)
or
5 g (10 g) of N-Lauroylsarcosine, Sodium salt (Sarcosyl)[5]
Dilute to 500 ml (1000 ml) with Sterile Ultrapure Water (CLRW)

Add **25 µl** Proteinase K stock solution (20 mg/ml) per 5 ml of cell lysis buffer **just before use** for a final concentration in the lysis buffer of 0.1 mg/ml Proteinase K.

5. FLOW CHART:

6. BIBLIOGRAPHY:

7. CONTACTS:

8. AMENDMENTS:

8.1 The above protocol was revised and distributed on October 30, 2008, modifications as follows:
- Reduction of cell suspension concentration
- Solubilization of the Lysozyme in TE rather than water
- Volumes used for plug preparation
- Incubation of the cell suspensions with the Lysozmye for 10-20 minutes in a 56°C water bath rather than 37°C
- Restriction enzyme units used for *Apa*I (50 U/sample) and *Asc*I (40 U/ sample) for two to three hours

8.2 2011-07 A note was added to clarify which buffers to use during the restriction digestion "step 2" under the enzyme mixture charts.

[5]If Sarcosyl powder is added directly to the other components of this reagent, warm the solution to 50-60ºC for 30-60 minutes, or leave at room temperature for ≈2 hours to completely dissolve the Sarcosyl; adjust to the final volume with sterile Sterile Clinical Laboratory Reagent Water.

8.3 2013-03 changes:

- Corrected formula for TE buffer. TE used at CDC is 10mM for Tris and 1mM for EDTA.
- Recommended disinfectant changed from 10% bleach to 1% Lysol/Amphyll or 90% ethanol.
- Corrected calculations for plug agarose.
- New options for Lysozyme were added because Sigma L7651 was discontinued.
- A note was added to provide guidance when working with large numbers of isolates (>10).
- Volume of TE needed for washing plugs was corrected from 300 – 350 ml to 400 – 600 ml.
- Use of pre-restriction step was changed from optional to highly recommended.
- A statement was added to clarify that using combs with smaller teeth (5.5 mm) was not advised.
- Units of enzyme for *Asc*I and *Apa*I was reduced from 40 units and 50 units, respectively, to 25 units for each. Incubation time was changed to 2 hours, instead of a range of 2 – 3 hours.
- A statement was included to allow the use of an alternative agarose for casting the running gel, along with recommendations strongly urging each lab to optimize the run time. Internal and external validation showed that run times could be affected by agarose type, but no trends were noted so a blanket recommendation on run times cannot be made. Additional agarose alternatives may be tested and deemed acceptable at a later date.
- A statement was included to allow the use of alternative DNA stains that are equivalent to EtBr. Labs are strongly urged to follow manufacturer's instructions as well as test stains in their own labs to gain experience using alternative agarose stains. Additional stain alternatives may be tested and deemed acceptable at a later date.

1. **PURPOSE:** To describe the One-Day (24-26 h) Standardized Laboratory Protocol for Molecular Subtyping of *E. coli* O157:H7, *E. coli* Non-O157 (STEC), *Salmonella*, *Shigella sonnei* and *Shigella flexneri* by Pulsed-field Gel Electrophoresis (PFGE).

2. **SCOPE:** To provide the PulseNet participants with a standardized procedure for performing PFGE of *E. coli* O157:H7, *E. coli* Non-O157 (STEC), *Salmonella*, *Shigella sonnei* and *Shigella flexneri*, thus ensuring inter-laboratory comparability of the generated results.

3. **DEFINITIONS/TERMS:**
   3.1 PFGE: Pulsed-field Gel Electrophoresis
   3.2 DNA: Deoxyribonucleic acid
   3.3 CDC: Centers for Disease Control and Prevention
   3.4 CLRW: Clinical Laboratory Reagent Water

4. **RESPONSIBILITIES/PROCEDURE:**

BIOSAFETY WARNING: *Escherichia coli* O157:H7, *Salmonella* serotypes, *Shigella sonnei*, and *Shigella flexneri* are human pathogens and can cause serious disease. It has been reported that less than 100 cells of *E. coli* O157:H7 may cause infection. *Shigella* species also have a low infectious dose and are demonstrated hazards to laboratory personnel. Always use Biosafety Level 2 practices (at a minimum) and extreme caution when transferring and handling strains of these genera. Work in a biological safety cabinet when handling large amounts of cells. Disinfect or dispose of all plasticware and glassware that come in contact with the cultures in a safe manner.

Please read all instructions carefully before starting protocol. Treat all plasticware, glassware, pipets, spatulas, etc. that come in contact with the cell suspensions or plugs as contaminated materials and dispose of, or disinfect according to the guidelines of your institution. Disinfect reusable plug molds before they are washed; the disposable plug molds, including the tape and the tab that is used to push the plugs out of the wells, are also contaminated and should be disinfected 1% Lysol/Amphyll or 90% ethanol for at least 30 minutes if they will be washed and reused.

Day 0

Streak an isolated colony from test cultures onto Trypticase Soy Agar with 5% defibrinated sheep blood (TSA-SB) plates (or comparable non-selective media) for confluent growth. It is recommended that a storage vial of each culture be created. To do this stab small screw cap tubes of TSA, HIA, or similar medium with the same inoculating loop used to streak the plate. This will ensure that the same colony can be retested if necessary. Incubate cultures at 37ºC for 14-18 h.

Day 1

1. **Turn on shaker water bath or incubator (54-55ºC), stationary water baths (55-60ºC) and spectrophotometer** (or equivalent instrument such as the Dade Microscan Turbidity meter or bioMérieux Vitek colorimeter).

2. Prepare **TE Buffer** (**10 mM Tris:1 mM EDTA, pH 8.0**)[1] as follows:
   10 ml of 1 M Tris, pH 8.0
   2 ml of 0.5 M EDTA, pH 8.0
   Dilute to 1000 ml with sterile Ultrapure Clinical Laboratory Reagent Water (CLRW)

Note: The TE Buffer is used to make the plug agarose and also to wash lysed PFGE plugs.

[1]Additional information is found on page 13 of this document.

3. Prepare 1% SeaKem Gold agarose in **TE Buffer** (**10 mM Tris:1 mM EDTA, pH 8.0**) for PFGE plugs as follows:
   a. Weigh 0.50 g (or 0.25 g) SeaKem Gold (SKG) agarose into 250 ml screw-cap flask.
   b. Add 50.0 ml (or 25.0 ml) TE Buffer; swirl gently to disperse agarose.
   c. Loosen or remove cap, cover loosely with clear film, and microwave for 30sec; mix gently and repeat for 10sec intervals until agarose is completely dissolved.
   d. Recap flask and return to 55-60ºC water bath and equilibrate the agarose in the water bath for 15 minutes or until ready to use.

SAFETY WARNING: Use heat-resistant gloves when handling hot flasks after microwaving.

Note: SeaKem Gold agarose works well for making PFGE plugs because it provides added strength to the plugs minimizing breakage of plugs during the lysis and washing steps. The time and temperature needed to completely dissolve the agarose is dependent on the specifications of the microwave used, and will have to be determined empirically in each laboratory.

4. Label small transparent tubes (12 mm x 75 mm Falcon 2054 tubes or equivalent) with culture numbers.

5. Prepare **Cell Suspension Buffer** (**100 mM Tris:100 mM EDTA, pH 8.0**) as follows:
   10 ml of 1 M Tris, pH 8.0
   20 ml of 0.5 M EDTA, pH 8.0
   Dilute to 100 ml with sterile Ultrapure water (CLRW)

6. Transfer ≈2 ml of **Cell Suspension Buffer** (CSB) to small labeled tubes. Use a sterile polyester-fiber or cotton swab that has been moistened with sterile CSB to remove some of the growth from agar plate; suspend cells in CSB by spinning swab gently so cells will be evenly dispersed and formation of aerosols is minimized.

Note: The minimum volume of the cell suspension needed will depend on size of the cuvettes or tubes used to measure the cell concentration and are dependent on the manufacturer's specifications for the spectrophotometer, turbidity meter, or colorimeter. Keep suspensions on ice if you have more than 6 cultures to process or refrigerate cell suspensions if you cannot adjust their concentration immediately.

7. Adjust concentration of cell suspensions to one of values given below by diluting with sterile CSB or by adding additional cells.
   a. Spectrophotometer: 610 nm wavelength, absorbance (Optical Density) of 1.00 (range of 0.8-1.0)
   b. Dade Microscan Turbidity Meter: **0.40 - 0.45** (measured in Falcon 2054 tubes)
      0.58 - 0.63 (measured in Falcon 2057 tubes)
   c. bioMérieux Vitek colorimeter: **≈17-18%** transmittance (measured in Falcon 2054 tubes)

Note: The values in Steps 7a, 7b and 7c give satisfactory results at CDC; each laboratory may need to establish the optimal concentration needed for satisfactory results.

CASTING PLUGS

Label wells of PFGE plug molds with culture number. When reusable plug molds are used, put strip of tape on lower part of reusable plug mold before labeling wells.

Note: Unused plug agarose can be kept at room temperature and reused 1-2 times. Microwave on low-medium power for 10 -15 sec and mix; repeat for 5-10 sec intervals until agarose is completely melted. This agarose melts rapidly!

Note: Proteinase K solutions (20 mg/ml) are available commercially. Alternatively, a stock solution of Proteinase K can be prepared from the powder in sterile Ultrapure water (CLRW). For best results, aliquot in 300-500 µl into small tubes and store in a freezer (-20ºC) until ready to use. Just before use, thaw appropriate number of vials needed for the samples; keep Proteinase K solutions on ice. If the Proteinase K stock solution was prepared from powder, discard any thawed solution at the end of the work day. Store commercially prepared Proteinase K solutions according to directions provided by the supplier.

1. Transfer 400 µl (0.4 ml) adjusted cell suspensions to labeled 1.5 ml microcentrifuge tubes.

2. Add 20 µl of Proteinase K (20 mg/ml stock) to each tube and mix gently with pipet tip. (200 µl are needed for 10 cell suspensions.)

3. Add 400 µl melted 1% SeaKem Gold agarose to 400 µl cell suspension; mix by gently pipetting mixture up and down a few times. **Over-pipeting can cause DNA shearing**. Maintain temperature of melted agarose by keeping flask in beaker of warm water (55-60ºC).

4. Immediately, dispense part of mixture into appropriate well(s) of reusable plug mold. Do not allow bubbles to form. Two plugs of each sample can be made from these amounts of cell suspension and agarose and are useful if repeat testing is required. Allow plugs to solidify at room temperature for 10-15 min. They can also be placed in the refrigerator (4ºC) for 5 minutes

Note: If disposable plug molds are used for making plugs with 1% SeaKem Gold agarose, use 200 µl cell suspension, 10 µl of Proteinase K (20 mg/ml stock) and 200 µl of agarose; up to 4 plugs can be made from these amounts of cell suspension and agarose.

Note: The generation of cell suspension and the subsequent casting of the plugs should be performed as rapidly as possible in order to minimize premature cell lysis. If large numbers of samples are being prepared, it is recommended that they be processed in batches of ~10 samples at a time. Once the first batch of isolates are in the cell lysis incubation, then start preparing the cells suspensions the next group samples, and so on. All batches can be lysed and washed together, since additional lysis time will not affect the initial batches.

LYSIS OF CELLS IN AGAROSE PLUGS

Note: Two plugs (reusable molds) or up to four plugs (disposable molds) of the same strain can be lysed in the same 50ml tube.

1. Label 50ml polypropylene screw-cap or 50ml Oak Ridge tubes with culture numbers.

2. Prepare **Cell Lysis Buffer** (**50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl**) as follows:
   25 ml of 1 M Tris, pH 8.0
   50 ml of 0.5 M EDTA, pH 8.0
   50 ml of 10 % Sarcosyl (N-Lauroylsarcosine, Sodium salt)[2]
   Dilute to 500 ml with sterile Ultrapure water (CLRW)

3. Calculate the total volume of **Cell Lysis**/**Proteinase K Buffer** needed as follows:

[2]The N-Lauroylsarcosine, Sodium salt can be added directly to the other ingredients and allowed to dissolve. See page 13 of this document.

a. 5 ml **Cell Lysis Buffer** (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl) is needed per tube (e. g., 5 ml x 10 tubes = 50 ml).
b. 25 µl **Proteinase K** stock solution (20 mg/ml) is needed per tube of the cell lysis buffer (e. g., 25 µl x 10 tubes = 250 µl).
c. Prepare the master mix by measuring the correct volume of Cell Lysis Buffer and Proteinase K into appropriate size test tube or flask and mix well.

Note: The final concentration of Proteinase K in the lysis buffer is **0.1 mg/ml** and is different from the concentration that was added to the cell suspension (0.5 mg/ml).

4. Add 5 ml of Proteinase K/Cell Lysis Buffer to each labeled 50 ml tube.

5. Trim excess agarose from top of plugs with scalpel, razor blade or similar instrument. Open reusable plug mold and transfer plugs from mold with a 6-mm wide spatula to appropriately labeled tube. If disposable plug molds are used, remove white tape from bottom of mold and push out plug(s) into appropriately labeled tube. **Be sure plugs are under buffer and not on side of tube**.

Note: The excess agarose, plug mold, spatula, etc. are contaminated. Discard or disinfect appropriately.

6. **Remove tape from reusable mold.** Place both sections of the plug mold, spatulas, and scalpel in 90% ethanol, 1% Lysol/Amphyll other suitable disinfectant. **Soak them for 15 minutes before washing them.** Discard disposable plug molds or disinfect them in or 90% ethanol for 30-60 minutes if they will be washed and reused.

7. Place tubes in rack and incubate in a 54-55ºC shaker water bath or incubator for 1.5 - 2 h with **constant and vigorous agitation** (150-175 rpm). If lysing in water bath, be sure water level is **above** level of lysis buffer in tubes.

8. Pre-heat enough sterile Ultrapure water (CLRW) to 54-55ºC so that plugs can be washed two times with 10-15 ml water (200-250 ml for 10 tubes).

WASHING OF AGAROSE PLUGS AFTER CELL LYSIS

Note: Most laboratories will find that their plugs are sufficiently stable to perform the following washing steps at 54-55ºC. However, if you notice that your plugs are nicked along the edges or breaking it will be necessary for your laboratory to lower the water bath or incubator to 50ºC for the following washing steps.

1. Remove tubes from water bath or incubator, and carefully pour off lysis buffer into an appropriate discard container; plugs can be held in tubes with a screened cap or spatula.

Note: It is important to remove all of the liquid during this and subsequent wash steps by touching edge of tube or screened cap on an absorbent paper towel.

2. Add at 10-15 ml sterile Ultrapure water (CLRW) that has been pre-heated to 54-55ºC to each tube and shake the tubes in a 54-55ºC water bath or incubator for 10-15 min.

3. Pour off water from the plugs and repeat wash step with pre-heated water (Step 2) one more time.
   a. Pre-heat enough sterile **TE Buffer** (10 mM Tris:1 mM EDTA, pH 8.0) in a 54-55ºC water bath so that plugs can be washed four times with 10-15 ml TE (400-600 ml for 10 tubes) after beginning last water wash.

4. Pour off water, add 10-15 ml pre-heated (54-55ºC) sterile TE Buffer, and shake the tubes in 54-55ºC water bath or incubator for 10-15 min.

5. Pour off TE and repeat wash step with pre-heated TE three more times.

6. Decant last wash and add 5-10 ml sterile TE. Continue with step 1 in "Restriction Digestion" section or store plugs in TE Buffer at 4ºC until needed. Plugs can be transferred to smaller tubes for long term storage.

Note: If restriction digestion is to be done the same day, complete Steps 1-3 of next section (Restriction Digestion) during last TE wash step for optimal use of time.

RESTRICTION DIGESTION OF DNA IN AGAROSE PLUGS

Note: A small slice of the plug (not the entire plug) should be digested with the primary restriction enzyme because less enzyme is required and other slices of the plug can be subjected to restriction analysis with secondary or tertiary enzymes, according to the table below. *E. coli* species, *Salmonella*, and *Shigella sonnei* utilize *Xba*I as the primary enzyme and *Bln*I as the secondary enzyme. *Shigella flexneri* are tested with *Not*I as the primary enzyme and *Xba*I as the secondary enzyme. The use of a secondary (or tertiary) enzyme is useful in situations where the PFGE patterns obtained with the primary enzyme from two or more isolates are indistinguishable.

Organism	Primary Enzyme (Concentration)	Secondary Enzyme (Concentration)	Tertiary Enzyme (Concentration
***E. coli* O157**	*Xba*I (50 U/sample)	*Bln*I/*Avr*II (30U/sample)	*Spe*I (30 U/sample)
***E. coli* non-O157**	*Xba*I (50 U/sample)	*Bln*I/*Avr*II (30U/sample)	*Spe*I (30 U/sample)
Salmonella	*Xba*I (50 U/sample)	*Bln*I/*Avr*II (30U/sample)	*Spe*I (30 U/sample)
Shigella sonnei	*Xba*I (50 U/sample)	*Bln*I/*Avr*II (30U/sample)	*Spe*I (30 U/sample)
Shigella flexneri	*Not*I (50 U/sample)	*Xba*I (50 U/ sample)	*Spe*I (30 U/sample)

1. Label 1.5 ml microcentrifuge tubes with culture numbers; label 3 (10-well gel) or 4 (15-well gel) tubes for *Salmonella* ser. Braenderup H9812 standards.
   a. **Pre-Restriction Incubation Step (highly recommended)**: Prepare a master mix by diluting the appropriate 10X restriction buffer (Roche Applied Science or equivalent) 1:10 with sterile Ultrapure water (CLRW) according to the following table:

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices
Sterile Clinical Laboratory Reagent Water (CLRW)	180 µl	1800 µl	2700 µl
10X Restriction Buffer	20 µl	200 µl	300 µl
Total Volume	200 µl	2000 µl	3000 µl

b. Add 200 µl diluted restriction buffer (1X) to labeled 1.5 ml microcentrifuge tubes.
c. Carefully remove plug from TE with spatula and place in a sterile disposable Petri dish or on large glass slide.
d. Cut a 2.0 to 2.5 mm wide slice from each test samples and the appropriate number of *S.* ser. Braenderup H9812 standards with a single edge razor blade (or scalpel, cover slip, etc.) and transfer to tube containing diluted restriction buffer. **Be sure plug slice is under buffer.** Replace rest of plug into the original tube that contains 5 ml TE buffer and store at 4ºC.

Note: PulseNet recommends that the combs with larger teeth (10 mm wide teeth) be used to cast the gels because computer analysis of the gel lanes is more accurate and less tedious than analysis of gel lanes cast with combs with the smaller teeth (5.5 mm). Using combs with smaller teeth is not advised. The number of slices that can be cut from the plugs will depend on the skill and experience of the operator, integrity of the plug, and whether the slices are cut vertically or horizontally (plugs made in disposable molds).

e. Incubate sample and control plug slices in a 37ºC water bath for 5-10 min or at room temp for 10-15 min.
f. After incubation, remove buffer from plug slice using a pipet fitted with 200-250 µl tip all the way to bottom of tube and aspirate buffer. Be careful not to damage the plug slice with pipet tip and that plug slice is not discarded with pipet tip.

2. Prepare the restriction enzyme master mix according to the following table[3]. May mix in the same tube that was used for the diluted restriction buffer.

Note: Enzymes may be purchased in several different stock concentrations. The calculations outlined here are based on using an enzyme at a concentration of 10 U/µl. If the enzyme used is of a different concentration, make necessary adjustments to the volume of enzyme and water to achieve a final concentration of 50 U/ sample.

Note: Keep vial of restriction enzyme on ice or in insulated storage box (-20˚C) at all times.

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices
Sterile Clinical Laboratory Reagent Water (CLRW)	173 µl	1730 µl	2595 µl
10X Restriction Buffer	20 µl	200 µl	300 µl
BSA (10mg/ml)	2 µl	20 µl	30 µl
Enzyme (10 U/µl)	5 µl	50 µl	75 µl
Total Volume	200 µl	2000 µl	3000 µl

Note: Addition of Bovine Serum Albumin (BSA; highly recommended): Several restriction enzyme vendors specifically recommend the addition of 1X BSA to enzyme restriction mixtures while others do not. PulseNet Central recommends adding BSA to all enzyme restriction mixtures to minimize the incidence of incomplete restriction.

3. Add 200 µl restriction enzyme master mix to each tube. Close tube and mix by tapping gently; **be sure plug slices are under enzyme mixture**.

4. Incubate sample and control plug slices in 37˚C water bath for 1.5-2 h.

5. If plug slices will be loaded into the wells (Option B, page 8), continue with Steps 1-4 of the next section (**CASTING AGAROSE GEL**) approximately 1 h before restriction digest reaction is finished so the gel can solidify for at least 30 minutes before loading the restricted PFGE plugs.

CASTING AGAROSE GEL

A. Loading Restricted Plug Slices on the Comb:

1. Confirm that water bath is equilibrated to 55-60ºC.

2. Make volume of 0.5X Tris-Borate EDTA Buffer (TBE) that is needed for both the gel and electrophoresis running buffer according to one of the following tables.

5X TBE:

Reagent	Volume in milliliters (ml)	
5X TBE	200	220
Clinical Laboratory Reagent Water (CLRW)	1800	1980
Total Volume of 0.5X TBE	2000	2200

10X TBE:

Reagent	Volume in milliliters (ml)	
10X TBE	100	110
Clinical Laboratory Reagent Water (CLRW)	1900	2090
Total Volume of 0.5X TBE	2000	2200

3. Make 1% SeaKem Gold (SKG) Agarose in 0.5X TBE as follows:
   a. Weigh appropriate amount of SKG into 500 ml screw-cap flask.
   b. Add appropriate amount of 0.5X TBE; swirl gently to disperse agarose.
      i. Mix 1.0 g agarose with 100 ml 0.5X TBE for 14 cm wide gel form (10 wells)
      ii. Mix 1.5 g agarose with 150 ml 0.5X TBE for 21 cm wide gel form (15 wells)
   c. Loosen or remove cap and cover loosely with clear film, and microwave for 60 sec; mix gently and repeat for 15 sec intervals until agarose is completely dissolved.
   d. Recap flask and return to 55-60ºC water bath and equilibrate the agarose in the water bath for 15 minutes or until ready to use.

SAFETY WARNING: Use heat-resistant gloves when handling hot flasks after microwaving.

Note: Agarose LF™ (Amresco, X174) is the only acceptable alternative to SeaKem Gold, at this time. The time and temperature needed to completely dissolve the agarose is dependent on the specifications of the microwave used and will have to be determined empirically in each laboratory. Similarly, the optimal running time for each agarose will have to be determined empirically in each laboratory.

4. A small volume (2-5 ml) of melted and cooled (55-60ºC) 1% SKG agarose may be wanted to seal wells after plugs are loaded. Prepare as described above. Unused SKG agarose can be kept at room temperature, melted, and reused several times.

Note: Place the gel form on a leveling table and adjust until perfectly leveled. Place the comb holder so the front part (side with small metal screws) and teeth face the bottom of gel frame and the comb teeth touch the gel platform.

5. Remove restricted plug slices from 37ºC water bath. Remove enzyme/buffer mixture and add 200 µl 0.5X TBE. Incubate at room temperature for 5 min.

6. Remove plug slices from tubes; put comb on bench top and load plug slices on the bottom of the comb teeth as follows:
   a. Load *S.* ser. Braenderup H9812 standards on teeth (lanes) 1, 5, 10 (10 well gel) or on teeth 1, 5, 10, 15 (15 well gel).
   b. Load samples on remaining teeth and note locations.

7. Remove excess buffer with tissue or kimwipe. Allow plug slices to air dry on the comb for 5-10 minutes or seal them to the comb with 1% SKG agarose (55-60ºC).

8. Position comb in leveled gel form and confirm that the plugs slices are correctly aligned on the bottom of the comb teeth, and that the lower edge of the plug slice is flush against the black platform.

9. Carefully pour the agarose (cooled to 55-60ºC) into the gel form and remove any bubbles or debris.

10. Put black gel frame in electrophoresis chamber. Add 2-2.2 L freshly prepared 0.5X TBE. Close cover of unit. The amount of buffer needed depends on whether residual buffer was left in tubing or if unit was flushed with water after the last gel was run.

11. Turn on power supply, pump calibrated to a flow rate of 1 liter/minute (setting of ≈70) and cooling module (14ºC) approximately 30 minutes before gel is to be run.

12. Remove comb after gel solidifies, about 30-45 minutes.

13. Fill in wells of gel with melted and cooled (55- 60ºC) 1% SKG Agarose (optional). Unscrew and remove end gates from gel form; remove excess agarose from sides and bottom of casting platform with a tissue or kimwipe. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber. Close cover of chamber.

B. Loading Restricted Plug Slices into the Wells:

1. Follow steps 1-4 in Section A on page 7-8 (**Loading Restricted Plug Slices on the Comb**).

Note: Place the gel form on a leveling table and adjust until perfectly leveled before pouring gel. Position the comb holder so that front part (side with small metal screws) and teeth face the bottom of gel and the **bottom edge of the comb is 2 mm above the surface of the gel platform**.

2. Cool melted SKG agarose in 55-60ºC water bath for 15-20 min; carefully pour agarose into gel form (casting stand) fitted with comb. Be sure there are no bubbles.

3. Put black gel frame in electrophoresis chamber. Add 2-2.2 L freshly prepared 0.5X TBE. Close cover of unit. (The amount of buffer depends on whether residual buffer was left in tubing, or if unit was flushed with water after the last gel was run).

4. Turn on power supply, pump calibrated to a flow rate of 1 liter/minute (setting at ~70), and cooling module (14ºC) approximately 30 minutes before gel is to be run.

5. Remove restricted plug slices from 37ºC water bath. Remove enzyme/buffer mixture and add 200 µl 0.5X TBE. Incubate at room temperature for 5 minutes.

6. Remove comb after gel solidifies, about30 – 45 minutes.

7. Remove restricted plug slices from tubes with tapered end of spatula and load into appropriate wells. Gently push plugs to bottom and front of wells with wide end of spatula. Manipulate position with spatula and be sure that are no bubbles.
   a. Load *S.* ser. Braenderup H9812 standards in wells (lanes) 1, 5, 10 (10 well gel) or in wells 1, 5, 10, 15 (15 well gel).
   b. Load samples in remaining wells.

Note: Loading the plug slices can be tedious; each person has to develop his/her own technique for consistently placing the plug slices in the wells so the lanes will be straight and the bands sharp.

8. Fill in wells of gel with melted 1% SKG Agarose (equilibrated to 55-60ºC). Allow to harden for 3-5 min. Unscrew and remove end gates from gel form; remove excess agarose from sides and bottom of casting platform with a tissue or kimwipe. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber. Close cover of chamber.

ELECTROPHORESIS CONDITIONS

1. Select following conditions for ***Escherichia coli* O157:H7 and *Shigella sonnei*** strains restricted with *Xba*I or *Avr*II (*Bln*I):
   a. Select following conditions on **CHEF Mapper**
      Auto Algorithm
      30 kb - low MW
      600 kb - high MW
      Select default values except where noted by pressing "enter."
      Change run time to 18 - 19 h (See note below)
      (Default values: Initial switch time = 2.16 s; Final switch time = 54.17 s)

   b. Select following conditions on **CHEF-DR III**
      Initial switch time: 2.2 s
      Final switch time: 54.2 s
      Voltage: 6 V
      Included Angle: 120°
      Run time: 18-19 h (See note below)

   c. Select following conditions on **CHEF-DR II**
      Initial A time: 2.2 s
      Final A time: 54.2 s

Start ratio: 1.0 (if applicable)
Voltage: 200 V
Run time: 19-20 h (See note below)

2. Select following conditions for *Salmonella* strains restricted with *Xba*I or *Avr*II (*Bln*I):
   a. Select following conditions on **CHEF Mapper**
      Auto Algorithm
      30 kb - low MW
      700 kb - high MW
      Select default values except where noted by pressing "Enter."
      Change run time to 18 - 19 h (See note below)
      (Default values: Initial switch time = 2.16 s; Final switch time = 63.8 s)

   b. Select following conditions on **CHEF DR-III**
      Initial switch time: 2.2 s
      Final switch time: 63.8 s
      Voltage: 6 V
      Included Angle: 120°
      Run time: 18-19 h (See note below)

   c. Select following conditions on **CHEF DR-II**
      Initial A time: 2.2s
      Final A time: 63.8 s
      Start Ratio: 1.0 (if applicable)
      Voltage: 200 V
      Run time: 19-20 h (See note below)

3. Select following conditions for Non-O157 Shiga Toxin-Producing ***Escherichia coli* (STEC)** strains restricted with *Xba*I or *Avr*II (*Bln*I):
   a. Select following conditions on **CHEF Mapper**
      Auto Algorithm
      50 kb - low MW
      400 kb - high MW
      Select default values except where noted by pressing "Enter."
      Change run time to 18 - 19 h (See note below)
      (Default values: Initial switch time = 6.76 s; Final switch time = 35.38 s)

   b. Select following conditions on **CHEF DR-III**
      Initial switch time: 6.76 s
      Final switch time: 35.38 s
      Voltage: 6 V
      Included Angle: 120°
      Run time: 18-19 h (See note below)

   c. Select following conditions on **CHEF DR-II**
      Initial A time: 6.76 s
      Final A time: 35.38 s
      Start Ratio: 1.0 (if applicable)
      Voltage: 200 V
      Run time: 19-20 h (See note below)

4. Select following conditions for *Shigella flexneri* strains restricted with *Not*I or *Xba*I :
    a. Select following conditions on **CHEF Mapper**
        Auto Algorithm
        50 kb - low MW
        400 kb - high MW
        Select default values except where noted by pressing "Enter."
        Change the switch times to the following values:
        Initial switch time: 5 seconds
        Final switch time: 35 seconds
        Change run time to 18 - 19 h (See note below)
        (Default values: Initial switch time = 6.76 s; Final switch time = 35.38 s)

    b. Select following conditions on **CHEF DR-III**
        Initial switch time: 5 s
        Final switch time: 35 s
        Voltage: 6 V
        Included Angle: 120°
        Run time: 18-19 h (See note below)

    c. Select following conditions on **CHEF DR-II**
        Initial A time: 5 s
        Final A time: 35 s
        Start Ratio: 1.0 (if applicable)
        Voltage: 200 V
        Run time: 19-20 h (See note below)

Note: The electrophoresis running times recommended above are based on the equipment and reagents used at the CDC. **Run times may be different in your laboratory and will have to be optimized for your gels so that the lowest band in the *S.* ser. Braenderup H9812 standard migrates 1.0 - 1.5 cm from the bottom of the gel.**

Note: Make note of the initial milliamp (mAmp) reading on the instrument. The initial mAmps should be between 110-150 mAmps. A reading outside of this range may indicate that the 0.5X TBE buffer was prepared improperly and the buffer should be remade.

Day 2

STAINING AND DOCUMENTATION OF PFGE AGAROSE GEL

Note: The following staining procedure describes the use of ethidium bromide to stain PFGE gels. Alternate DNA stains may be used. Please see the "Alternate DNA Stains-Results and Recommendations" posting within the PulseNet Documents forum on the SharePoint site for additional information.

1. When electrophoresis run is over, turn off equipment; remove and stain gel with ethidium bromide by diluting 40 µl of ethidium bromide stock solution (10 mg/ml) with 400 ml of Ultrapure water (CLRW). This volume is for a staining box that is approximately 14 cm x 24 cm; a larger container may require a larger amount of staining solution. Stain gel for 20-30 min in covered container.

Note: Ethidium bromide is toxic and a mutagen. Stock solutions of 10 mg/ml Ethidium Bromide (EtBr) in water are available from several commercial companies (Amresco X328; Bio-Rad, 161-0433; Sigma, E-1510). The diluted solution can be kept in dark bottle and reused 6-8 times before discarding according to your institution's guidelines for hazardous waste. CDC does not recommend disposing of EtBr down the drain. Aqueous solutions containing EtBr can be filtered through charcoal or degraded using activated carbon destaining or "tea" bags from Amresco (E732-25 Destaining Bags) or other companies, which effectively and safely remove EtBr from solutions and gels. Once the EtBr is removed, the treated aqueous solutions can be discarded down the drain. If you have further questions about EtBr please refer to the **Material Safety Data Sheets** (**MSDS**) provided by the vendor or manufacturer.

Note: Currently, the only acceptable alternative stain options are GelRed™ (Biotium, 31010), SYBR® Safe (Invitrogen, S-33102) and SYBR® Gold (Invitrogen, S-11494). Labs are strongly encouraged to follow manufacturer's instructions and test stains in their labs before adopting them for routine use. If one of the alternative stains is used, the destaining steps should be omitted.

2. Destain gel in approximately 500 ml CLRW for 60 - 90 min, changing water every 20 minutes. Capture image using a Gel Doc 1000, 2000, EQ, XR, or equivalent documentation system. If too much background is observed destain for an additional 30-60 min.

3. Follow directions given with the imaging equipment to save gel image as a ***.1sc** file; convert this file to ***.tif** file for analysis with the BioNumerics software program. The gel image should fill the entire window of the imaging equipment (computer) screen (without cutting off wells or lower bands). Ensure that the image is in focus and that there is little to no saturation (over-exposure) in the bands (signified by red pixilation in the QuantityOne or ImageLab software). Additional instructions are provided in PNL07 of the PulseNet QA/QC manual.

4. Drain buffer from electrophoresis chamber and discard. Rinse chamber with 2 L Ultrapure water (CLRW) or, if unit is not going to be used for several days, flush lines with water by letting pump run for 5-10 min **before** draining water from chamber and tubing.

5. If the lowest band in the H9812 standard does not migrate within 1-1.5 cm of the bottom of the gel, the proper run time will need to be determined empirically for the conditions in each laboratory.

Note: The following options are available if PFGE results do not have to be available within 24-28 hours:

- Plugs can be lysed for longer periods of time (3-16 hours).
- The washing steps with TE to remove the lysis buffer from the PFGE plugs can be done for longer periods of time (30-45 min) and at lower temperatures (37°C or room temperature). They can be started on Day 1 and finished on Day 2 after overnight refrigeration of the plugs in TE.

Use of trade names and commercial sources is for identification purposes only and does not imply endorsement by CDC or the U.S. Department of Health and Human Services.

NOTE: CLIA LABORATORY PROCEDURE MANUAL REQUIREMENTS

Efforts have been made to assure that the procedures described in this protocol have been written in accordance with the 1988 Clinical Laboratory Improvement Amendments (CLIA) requirements for a procedure manual (42 CFR 493.1211). However, due to the format required for training, the procedures will require some modifications and additions to customize them for your particular laboratory operation.

Any questions regarding the CLIA requirements for a procedure manual, quality control, quality assurance, etc., should be directed to the agency or accreditation organization responsible for performing your laboratory's CLIA

inspection. In addition, some states and accreditation organizations may have more stringent requirements that will need to be addressed.

Formulas of Selected Reagents used in PulseNet Standardized Laboratory Protocol for PFGE

Tris:EDTA Buffer, pH 8.0 (TE, 10 mM Tris:1 mM EDTA, pH 8.0)

10 ml of 1 M Tris, pH 8.0
2 ml of 0.5 M EDTA, pH 8.0
Dilute to 1000 ml with sterile Ultrapure water (CLRW)

Cell Lysis Buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosine + 0.1 mg/ml Proteinase K)

25 ml (50 ml) of 1 M Tris, pH 8.0
50 ml (100 ml) of 0.5 M EDTA, pH 8.0
50 ml (100 ml) 10% N-Lauroylsarcosine, Sodium salt (Sarcosyl)

OR

5 g (10 g) of N-Lauroylsarcosine, Sodium salt (Sarcosyl)[3]
Dilute to 500 ml (1000 ml) with Sterile Ultrapure water (CLRW)

Add **25 µl** Proteinase K stock solution (20 mg/ml) per 5 ml of cell lysis buffer **just before use** for a final concentration in the lysis buffer of 0.1 mg/ml Proteinase K.

Use the following calculations for *Avr*II (*Bln*I) or *Spe*I (30 Units/plug slice):

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices
Sterile Clinical Laboratory Reagent Water (CLRW)	175 µl	1750 µl	2625 µl
10X Restriction Buffer	20 µl	200 µl	300 µl
BSA (10mg/ml)	2 µl	20 µl	30 µl
***Avr*II or *Bln*I (10 U/µl)**	3 µl	30 µl	45 µl
Total Volume	200 µl	2000 µl	3000 µl

Note: Keep vial of restriction enzyme on ice or in insulated storage box (-20ºC) at all times.

5. FLOW CHART:

6. BIBLIOGRAPHY:

7. CONTACTS:

8. AMENDMENTS:

8.1 In May 2007, two revisions were made to the Standardized PFGE Protocol for *E. coli* O157:H7, *Salmonella*, and *Shigella sonnei*, including reduction in cell suspension concentrations and the removal of

[3]If Sarcosyl powder is added directly to the other components of this reagent, warm the solution to 50-60ºC for 30-60 minutes, or leave at room temperature for ≈2 hours to completely dissolve the Sarcosyl; adjust to the final volume with sterile Ultrapure water (CLRW).

SDS from the plug agarose. These revisions were adopted following an extensive evaluation and validation process which demonstrated that the revised protocol remained robust and reproducible in multiple laboratories.

8.2 The phrase "Type I Water" has been changed to "Ultrapure Clinical Laboratory Reagent Water (CLRW)." The water composition is the same, but this reflects a change in the terminology used by the Clinical Laboratory Standards Institute (CLSI).

8.3 August 2010 this protocol was revised to include *E. coli* Non-O157 (STEC) and *Shigella flexnei* instructions.

8.4 August 2010 changes:

- Plug washing steps can be performed at 54-55 ºC rather than lowering to 50 ºC.
- References to specific restriction buffers have been removed.
- Moved reference to BSA out of footnotes and into the main text of the protocol.
- The word "Sterile" has been deleted in reference to diluting 5X or 10X TBE to 0.5X TBE. Non-sterile CLRW is acceptable.
- Added a recommendation for laboratories to monitor the initial mAmps when electrophoresis is started.

8.5. August 2010: A statement was included to allow the use of alternative agaroses for casting the running gel, along with recommendations strongly urging each lab to optimize the run time. Internal and external validation showed that run times could be affected by agarose type, but no trends were noted so a blanket recommendation on run times cannot be made. Additional agarose alternatives may be tested and deemed acceptable at a later date.

8.6. August 2010: A statement was included to allow the use of alternative DNA stains that are equivalent to EtBr. Labs are strongly urged to follow manufacturer's instructions as well as test stains in their own labs to gain experience using alternative agarose stains. Additional stain alternatives may be tested and deemed acceptable at a later date.

8.7. March 2013 changes:

- Corrected formula for TE buffer. TE used at CDC is 10 mM for Tris and 1 mM for EDTA.
- Recommended disinfectant changed from 10% bleach to 1% Lysol/Amphyll or 90% ethanol.
- Volume of TE needed for washing plugs was corrected from 300 – 350 ml to 400 – 600 ml.
- A statement was added to clarify that using combs with small teeth (5.5 mm) was not advised.
- Use of pre-restriction step and BSA was changed from optional to highly recommended. Calculation for including BSA in restriction enzyme master mix was added.
- Statement allowing of Megbase agarose (BioRad) was deleted. Additional testing revealed run time and normalization were negatively impacted by this agarose.
- Upper limit for starting mAmps was reduced from 170 to 150mAmps.
- The option to allow incubation times for restriction digestion to be increased longer than recommended was deleted.

1. PURPOSE: To describe the One-Day (24-26 h) standardized laboratory protocol for molecular subtyping of *Vibrio cholerae* and *Vibrio parahaemolyticus* by Pulsed-field Gel Electrophoresis (PFGE).

2. SCOPE: To provide the PulseNet participants with a standardized procedure for performing PFGE of *Vibrio cholerae* and *Vibrio parahaemolyticus* thus ensuring inter-laboratory comparability of the generated results.

3. DEFINITIONS/TERMS:

3.1 PFGE: Pulsed-field Gel Electrophoresis
3.2 DNA: Deoxyribonucleic acid
3.3 CDC: Centers for Disease Control and Prevention
3.4 CLRW: Clinical Laboratory Reagent Water

4. RESPONSIBILITIES/PROCEDURE:

PREPARATION OF PFGE PLUGS FROM AGAR CULTURES

BIOSAFETY WARNING: *Vibrio cholerae* and *Vibrio parahaemolyticus* are human pathogens and can cause serious disease. Always use Biosafety Level 2 practices and extreme caution when transferring and handling strains of these genera. Work in a biological safety cabinet when handling large amounts of cells. Disinfect or dispose of all plasticware and glassware that come in contact with the cultures in a safe manner.

Please read all instructions carefully before starting protocol. Treat all plasticware, glassware, pipets, spatulas, etc. that come in contact with the cell suspensions or plugs as contaminated materials and dispose of, or disinfect according to the guidelines of your institution. Disinfect reusable plug molds before they are washed; the disposable plug molds, including the tape and the tab that is used to push the plugs out of the wells, are also contaminated and should be disinfected with 1% Lysol/Amphyll or 90% ethanol for at least 30 minutes if they will be washed and reused.

Day 0

Streak an isolated colony from test cultures to Trypticase Soy Agar with 5% defibrinated sheep blood (TSA-SB) plates (or comparable non-selective media) for confluent growth. It is recommended that a storage vial of each culture be created. To do this, stab small screw cap tubes of Marine motility agar or similar medium with the same inoculating loop used to streak the plate. This will ensure that the same colony can be retested if necessary Incubate cultures at 37ºC for 14-18 h.

Day 1

1. **Turn on shaker water bath (54-55ºC), stationary water baths (55-60ºC) and spectrophotometer** (or equivalent instrument such as the Dade Microscan Turbidity meter or bioMérieux Vitek colorimeter).

2. Prepare **TE Buffer** (**10 mM Tris:1 mM EDTA, pH 8.0**)[1] as follows:
   10 ml of 1 M Tris, pH 8.0
   2 ml of 0.5 M EDTA, pH 8.0
   Dilute to 1000 ml with sterile Ultrapure Clinical Laboratory Reagent Water (CLRW)

Note: The TE Buffer is used to make the plug agarose and also to wash lysed PFGE plugs.

[1]Additional information is found on page 13 of this document.

3. Prepare 1% SeaKem Gold agarose in **TE Buffer** (**10 mM Tris:1 mM EDTA, pH 8.0**) for PFGE plugs as follows:
   a. Weigh 0.50 g (or 0.25 g) SeaKem Gold (SKG) into 250 ml screw-cap flask.
   b. Add 50 ml (or 25 ml) TE Buffer; swirl gently to disperse agarose.
   c. Loosen or remove cap and cover loosely with clear film, and microwave for 30 sec; mix gently and repeat for 10 sec intervals until agarose is completely dissolved.
   d. Recap flask and return to 55- 60ºC water bath and equilibrate the agarose for 15 minutes or until ready to use.

SAFETY WARNING: Use heat-resistant gloves when handling hot flasks after microwaving.

Note: SeaKem Gold agarose works well for making PFGE plugs because it provides added strength to the plugs that are cast in reusable plug molds, minimizing breakage of plugs during the lysis and washing steps. The time and temperature needed to completely dissolve the agarose is dependent on the specifications of the microwave used, and will have to be determined empirically in each laboratory.

4. Label small transparent tubes (12 mm x 75 mm Falcon 2054 tubes or equivalent) with culture numbers.

5. Prepare **Cell Suspension Buffer** (**100 mM Tris:100 mM EDTA, pH 8.0**) as follows:
   10 ml of 1 M Tris, pH 8.0
   20 ml of 0.5 M EDTA, pH 8.0
   Dilute to 100 ml with sterile Ultrapure water (CLRW)

6. Transfer ≈2 ml of **Cell Suspension Buffer** (CSB) to small labeled tubes. Use a sterile polyester-fiber or cotton swab that has been moistened with sterile CSB to remove some of the growth from agar plate; suspend cells in CSB by spinning swab gently so cells will be evenly dispersed and formation of aerosols is minimized.

Note: The minimum volume of the cell suspension needed will depend on size of the cuvettes or tubes used to measure the cell concentration and are dependent on the manufacturer's specifications for the spectrophotometer, turbidity meter, or colorimeter.

7. Adjust concentration of cell suspensions to one of values given below by diluting with sterile CSB or by adding additional cells.
   a. Spectrophotometer: 610 nm wavelength, absorbance (Optical Density) of 0.9 (range of 0.8-1.0)
   b. Dade Microscan Turbidity Meter: **0.35 – 0.45** (measured in Falcon 2054 tubes)
      0.52 – 0.64 (measured in Falcon 2057 tubes; *V. cholerae*)
      0.55 – 0.65 (measured in Falcon 2057 tubes; *V. parahaemolyticus*)
   c. bioMérieux Vitek colorimeter: ≈ **20%** transmittance (measured in Falcon 2054 tubes)

Note: Cell suspensions need to be at room temperature when concentration is checked. The values in Steps 7a, 7b and 7c give satisfactory results at CDC; if different instruments or tubes are used, each laboratory may need to establish the concentration needed for satisfactory results.

CASTING PLUGS

Label wells of PFGE plug molds with culture number. When reusable plug molds are used, put strip of tape on lower part of reusable plug mold before labeling wells.

Note: Unused plug agarose can be kept at room temperature and reused 1-2 times. Microwave on low-medium power for 10 -15 sec and mix; repeat for 5-10 sec intervals until agarose is completely melted. This agarose melts rapidly!

Note: Proteinase K solutions (20 mg/ml) are available commercially. Alternatively, a stock solution of Proteinase K can be prepared from the powder in sterile Ultrapure water (CLRW). For best results, aliquot 300-500 µl into small tubes

and store in a freezer (-20ºC) until ready to use. Just before use, thaw appropriate number of vials needed for the samples; keep Proteinase K solutions on ice. If the Proteinase K stock solution was prepared from powder, discard any thawed solution at the end of the work day. Store commercially prepared Proteinase K solutions according to directions provided by the supplier.

1. Transfer 400 µl (0.4 ml) adjusted cell suspensions to labeled 1.5 ml microcentrifuge tubes.

2. Add 20 µl of Proteinase K (20 mg/ml stock) to each tube and mix gently with pipet tip. (200 µl is needed for 10 cell suspensions.)

3. Add 400 µl (0.4 ml) melted 1% SeaKem Gold agarose to 400 µl cell suspension; mix by gently pipetting mixture up and down a few times. **Over-pipeting can cause DNA shearing.** Maintain temperature of melted agarose by keeping flask in beaker of warm water (55-60ºC).

4. Immediately, dispense part of mixture into appropriate well(s) of reusable plug mold. Do not allow bubbles to form. Two plugs of each sample can be made from these amounts of cell suspension and agarose and are useful if repeat testing is required. Allow plugs to solidify at room temperature for 10-15 min. They can also be placed in the refrigerator (4ºC) for 5 minutes.

Note: If disposable plug molds are used for making plugs with 1% SeaKem Gold agarose, use 200 µl cell suspension, 10 µl of Proteinase K (20 mg/ml stock) and 200 µl of agarose; up to 4 plugs can be made from these amounts of cell suspension and agarose.

Note: The generation of cell suspension and the subsequent casting of the plugs should be performed as rapidly as possible in order to minimize premature cell lysis. If large numbers of samples are being prepared, it is recommended that they be processed in batches of ~10 samples at a time. Once the first batch of isolates are in the cell lysis incubation, then start preparing the cells suspensions the next group samples, and so on. All batches can be lysed and washed together, since additional lysis time will not affect the initial batches.

LYSIS OF CELLS IN AGAROSE PLUGS

Note: Two plugs (reusable plug molds) or up to four plugs (disposable plug molds) of the same strain can be lysed in the same 50 ml tube.

1. Label 50 ml polypropylene screw-cap or 50ml Oak Ridge tubes with culture numbers.

2. Prepare **Cell Lysis Buffer** (**50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl**) as follows:
   25 ml of 1 M Tris, pH 8.0
   50 ml of 0.5 M EDTA, pH 8.0
   50 ml of 10 % Sarcosyl (N-Lauroylsarcosine, Sodium salt)[2]
   Dilute to 500 ml with Ultrapure water (CLRW)

3. Calculate the total volume of **Cell Lysis**/**Proteinase K Buffer** needed as follows:
   a. 5 ml **Cell Lysis Buffer** (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl) is needed per tube (e. g., 5 ml x 10 tubes = 50 ml).

[2]The N-Lauroylsarcosine, Sodium salt can be added directly to the other ingredients and allowed to dissolve. See page 13 of this document.

b. 25 µl **Proteinase K** stock solution (20 mg/ml) is needed per tube of the cell lysis buffer (e. g., 25 µl x 10 tubes = 250 µl).
c. Prepare the master mix by measuring the correct volume of Cell Lysis Buffer and Proteinase K into appropriate size test tube or flask and mix well.

Note: The final concentration of Proteinase K in the lysis buffer is **0.1 mg/ml**, and is different from the concentration that was added to the cell suspension (0.5 mg/ml).

4. Add 5 ml of Proteinase K/Cell Lysis Buffer to each labeled 50 ml tube.

5. Trim excess agarose from top of plugs with scalpel or razor blade (optional). Open reusable plug mold and transfer plugs from mold with a 6-mm wide spatula to appropriately labeled tube. If disposable plug molds are used, remove white tape from bottom of mold and push out plug(s) into appropriately labeled tube. **Be sure plugs are under buffer and not on side of tube**.

Note: The excess agarose, plug mold, spatula, etc. are contaminated. Discard or disinfect appropriately.

6. **Remove tape from reusable mold.** Place both sections of the plug mold, spatulas, and scalpel in 90% ethanol, 1% Lysol/Amphyll or other suitable disinfectant. **Soak them for 15 minutes before washing them.** Discard disposable plug molds.

7. Place tubes in rack and incubate in a 54-55ºC shaker water bath or incubator for 1 hour with constant and vigorous agitation (150-175 rpm). If lysing in water bath, be sure water level in water bath is above the level of lysis buffer in tubes.

8. Pre-heat enough sterile Ultrapure water (CLRW) to 54-55ºC so that plugs can be washed two times with 10-15 ml water (200-250 ml for 10 tubes).

WASHING OF AGAROSE PLUGS AFTER CELL LYSIS

Note: Most laboratories will find that their plugs are sufficiently stable to perform the following washing steps at 54-55ºC. However, if you notice that your plugs are nicked along the edges or breaking it will be necessary for your laboratory to lower the water bath or incubator to 50ºC for the following washing steps.

1. Remove tubes from water bath, and carefully pour off lysis buffer into an appropriate discard container; plugs can be held in tubes with a screened cap or spatula.

Note: It is important to remove all of the liquid during this and subsequent wash steps by touching edge of tube or screened cap on an absorbent paper towel.

2. Add at 10-15 ml sterile Ultrapure water (CLRW) that has been pre-heated to 54-55ºC to each tube and shake the tubes vigorously in a 54-55ºC water bath or incubator for 10-15 min.

3. Pour off water from the plugs and repeat wash step with pre-heated water (Step 2) one more time.
   a. Pre-heat enough sterile **TE Buffer** (10 mM Tris:1 mM EDTA, pH 8.0) in a 54-55ºC water bath so that plugs can be washed four times with 10-15 ml TE (400-600 ml for 10 tubes) after beginning last water wash.

4. Pour off water, add 10-15 ml pre-heated (54-55ºC) sterile TE Buffer, and shake the tubes vigorously in 54-55ºC water bath or incubator for 10-15 min.

5. Pour off TE and repeat wash step with pre-heated TE three more times.

6. Decant last wash and add 5-10 ml sterile TE. Continue with step 1 in "Restriction Digestion" section or store plugs in TE Buffer at 4ºC until needed. Plugs can be transferred to smaller tubes for storage.

Note: If restriction digestion is to be done the same day, complete Steps 1-3 of next section (**RESTRICTION DIGESTION OF DNA IN AGAROSE PLUGS WITH *Sfi*I or *Not*I**) during last TE wash step for optimal use of time.

RESTRICTION DIGESTION OF DNA IN AGAROSE PLUGS

Note: A small slice of the plug (not the entire plug) should be digested with the primary restriction enzyme because less enzyme is required and other slices of the plug can be subjected to restriction analysis with other enzymes. *Sfi*I is recommended as the secondary enzyme for analysis of *Vibrio cholerae* and *Vibrio parahaemolyticus*. The use of a secondary enzyme is useful in situations where the PFGE patterns obtained with the primary enzyme from two or more isolates are indistinguishable.

1. Label 1.5 ml microcentrifuge tubes with culture numbers; label 3 (10-well gel) or 4 (15-well gel) tubes for *Salmonella* ser. Braenderup H9812[3] standards.

   a. **Pre-Restriction Incubation Step (highly recommended)**: Prepare a master mix by diluting the appropriate 10X restriction buffer (Roche Applied Science or equivalent) 1:10 with sterile Ultrapure water (CLRW) according to the following table:

Note: The appropriate restriction buffer will vary between vendors and may differ between enzymes from the same vendor. **Always** use the restriction buffer recommended by the vendor for the particular restriction enzyme.

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices
Sterile Clinical Laboratory Reagent Water (CLRW)	180 µl	1800 µl	2700 µl
10X Restriction Buffer	20 µl	200 µl	300 µl
Total Volume	200 µl	2000 µl	3000 µl

   b. Add 200 µl diluted restriction buffer (1X) to labeled 1.5 ml microcentrifuge tubes.
   c. Carefully remove plug from TE with spatula and place in a sterile disposable Petri dish or on large glass slide.
   d. Cut a 2.0 to 2.5 mm-wide slice from test samples with a single edge razor blade (or scalpel, cover slip, etc.) and transfer to tube containing diluted restriction buffer. **Be sure plug slice is under buffer.** Replace rest of plug in original tube that contains 5 ml TE buffer and store at 4ºC.

Note: PulseNet recommends that the combs with larger teeth (10 mm-wide teeth) be used to cast the gels because computer analysis of the gel lanes is more accurate and less tedious than analysis of gel lanes cast with combs with the smaller teeth (5.5 mm). Using combs with smaller teeth is not advised. The number of slices that can be cut from the plugs will depend on the skill and experience of the operator, integrity of the plug, and whether the slices are cut vertically or horizontally (plugs made in disposable molds).

[3] Directions for making and testing PFGE plugs of *Salmonella* ser. Braenderup H9812 are in PNL05.

e. Incubate sample and control plug slices in water bath or incubator for 5-10 min or at room temperature for 10-15 min.
   i. Incubate samples to be restricted with *Sfi*I at 50°C.
   ii. Incubate samples to be restricted with *Not*I and *Xba*I at 37ºC.
f. After incubation, remove buffer from plug slice using a pipet fitted with 200-250 µl tip all the way to bottom of tube and aspirate buffer. Be careful not to cut plug slice with pipet tip and that plug slice is not discarded with pipet tip.

2. Prepare the restriction enzyme master mix according to the following table. May mix in the same tube that was used for the diluted restriction buffer:

Note: Enzymes may be purchased in several different stock concentrations. The *Sfi*I stock enzyme should be ordered in concentrated form (40 U/µl) rather than unconcentrated form (10 U/µl). Either form is acceptable for *Not*I restriction. The calculations below are based on using an enzyme at a concentration of 40 U/µl. If a different concentration of enzyme is used, make necessary adjustments to the volume of enzyme and water to achieve a final concentration of 40 U/ sample.

Note: Keep vial of restriction enzyme on ice or in insulated storage box (-20˚C) at all times.

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices
Sterile Clinical Laboratory Reagent Water (CLRW)	177µl	1777 µl	2655 µl
10X Restriction Buffer	20 µl	200 µl	300 µl
BSA (10mg/ml)	2 µl	20 µl	30 µl
***Sfi*I (40 U/µl)**	1µl	10 µl	15 µl
Total Volume	200 µl	2000 µl	3000 µl

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices
Sterile Clinical Laboratory Reagent Water (CLRW)	177 µl	1777 µl	2655 µl
10X Restriction Buffer	20 µl	200 µl	300 µl
BSA (10mg/ml)	2 µl	20 µl	30 µl
***Not*I (40 U/µl)**	1 µl	10 µl	15 µl
Total Volume	200 µl	2000 µl	3000 µl

Note: Addition of Bovine Serum Albumin (BSA; highly recommended): Several restriction enzyme vendors specifically recommend the addition of 1X BSA to enzyme restriction mixtures while others do not. PulseNet Central recommends adding BSA to all enzyme restriction mixtures to minimize the incidence of incomplete restriction.

3. Add 200 µl restriction enzyme master mix to each tube. Close tube and mix by tapping gently; confirm that plug slices are under enzyme mixture.

4. Incubate sample and standard (control) plug slices for 4 hours in a water bath at the appropriate temperature for the enzyme.
   a. Incubate samples restricted with *Sfi*I at 50°C
   b. Incubate samples restricted with *Not*I and *Xba*I at 37°C.

5. If plug slices will be loaded into the wells (Option B, page 9), continue with Steps 1-4 of the next section (**CASTING AGAROSE GEL**) approximately 1 h before restriction digest reaction is finished so the gel can solidify for at least 30 minutes before loading the restricted PFGE plugs.

CASTING AGAROSE GEL

A. Loading Restricted Plug Slices on the Comb:

1. Confirm that water bath is equilibrated to 55 - 60ºC.

2. Make volume of 0.5X Tris-Borate EDTA Buffer (TBE) that is needed for both the gel and electrophoresis running buffer according to one of the following tables.

5X TBE:

Reagent	Volume in milliliters (ml)	
5X TBE	200	220
Clinical Laboratory Reagent Water (CLRW)	1800	1980
Total Volume of 0.5X TBE	2000	2200

10X TBE:

Reagent	Volume in milliliters (ml)	
10X TBE	100	110
Clinical Laboratory Reagent Water (CLRW)	1900	2090
Total Volume of 0.5X TBE	2000	2200

3. Make 1% SeaKem Gold (SKG) Agarose in 0.5X TBE as follows:
   a. Weigh appropriate amount of SKG into 500 ml screw-cap flask.
   b. Add appropriate amount of 0.5X TBE; swirl gently to disperse agarose.
      i. Mix 1.0 g agarose with 100 ml 0.5X TBE for 14 cm-wide gel form (10 wells)
      ii. Mix 1.5 g agarose with 150 ml 0.5X TBE for 21 cm-wide gel form (15 wells)
   c. Remove cap or cover loosely with clear film, and microwave for 60 sec; mix gently and repeat for 15 sec intervals until agarose is completely dissolved.
   d. Recap flask and return to 55- 60ºC water bath and equilibrate the agarose in the water bath for 15 minutes or until ready to use.

SAFETY WARNING: Use heat-resistant gloves when handling hot flasks after microwaving.

Note: Agarose LF™ (Amresco, X174) is the only acceptable alternative to SeaKem Gold at this time. The time and temperature needed to completely dissolve the agarose is dependent on the specifications of the microwave used and will have to be determined empirically in each laboratory. Similarly, the optimal running time for each agarose will have to be determined empirically in each laboratory.

4. A small volume (2-5 ml) of melted and cooled (50-60ºC) 1% SKG agarose may be wanted to seal wells after plugs are loaded. Prepare as described above. Unused SKG agarose can be kept at room temperature, melted, and reused several times.

Note: Place the gel form on a leveling table and adjust until perfectly leveled. Place the comb holder so the front part (side with small metal screws) and teeth face the bottom of gel frame and the comb teeth touch the gel platform.

5. Remove restricted plug slices from 50°C or 37ºC water bath. Remove enzyme/buffer mixture and add 200 µl 0.5X TBE. Incubate at room temperature for 5 min.

6. Remove plug slices from tubes; put comb on bench top and load plug slices on the bottom of the comb teeth as follows:
   a. Load *S.* ser. Braenderup H9812 standards on teeth (lanes) 1, 5, 10 (10-well gel) or on teeth 1, 5, 10, 15 (15-well gel).
   b. Load samples on remaining teeth and note locations.

7. Remove excess buffer with tissue or kimwipe. Allow plug slices to air dry on the comb for 5-10 minutes or seal them to the comb with 1% SKG agarose (55-60ºC).

8. Position comb in leveled gel form and confirm that the plugs slices are correctly aligned on the bottom of the comb teeth, and that the lower edge of the plug slice is flush against the black platform.

9. Carefully pour the agarose (cooled to 55-60ºC) into the gel form and remove any bubbles or debris.

10. Put black gel frame in electrophoresis chamber. Add 2 -2.2 L freshly prepared 0.5X TBE. Close cover of unit. (The amount of buffer needed depends on whether residual buffer was left in tubing or if unit was flushed with water after the last gel was run.)

11. Turn on power supply, pump calibrated to a flow rate of 1 liter/minute (setting of ~70) and cooling module (14ºC) approximately 30 minutes before gel is to be run.

12. Remove comb after gel solidifies, about 30-45 minutes.

13. Fill in wells of gel with melted and cooled (55- 60ºC) 1% SKG Agarose (optional). Unscrew and remove end gates from gel form; remove excess agarose from sides and bottom of casting platform with a tissue or kimwipe. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber. Close cover of chamber.

B. Loading Restricted Plug Slices into the Wells:

1. Follow steps 1-4 in Section A on pages 7 and 8 (**Loading Restricted Plug Slices on the Comb**).

Note: Place the gel form on a leveling table and adjust until perfectly leveled before pouring gel. Position the comb holder so that the front part (side with small metal screws) and teeth face the bottom and the bottom of the comb is 2 mm above the surface of the gel platform.

2. Cool melted SKG agarose in 55-60ºC water bath for 15-20 min; carefully pour agarose into gel form (casting stand) fitted with comb. Be sure there are no bubbles.

3. Put black gel frame in electrophoresis chamber. Add 2-2.2 L freshly prepared 0.5X TBE. Close cover of unit. (The amount of buffer depends on whether residual buffer was left in tubing, or if unit was flushed with water after the last gel was run).

4. Turn on power supply, pump calibrated to a flow rate of 1 liter/minute (setting of ~70) and cooling module (14ºC) approximately 30 minutes before gel is to be run.

5. Remove restricted plug slices from water bath. Remove enzyme/buffer mixture and add 200 µl 0.5X TBE. Incubate at room temperature for 5 minutes.

6. Remove comb after gel solidifies, about 30 – 45 minutes.

7. Remove restricted plug slices from tubes with tapered end of spatula and load into appropriate wells. Gently push plugs to bottom and front of wells with wide end of spatula. Manipulate position with spatula and be sure that are no bubbles.
   a. Load *S.* ser. Braenderup H9812 standards in wells (lanes) 1, 5, 10 (10-well gel) or in wells 1, 5, 10, 15 (15-well gel).
   b. Load samples in remaining wells.

Note: Loading the plug slices can be tedious; each person has to develop his/her own technique for consistently placing the plug slices in the wells so the lanes will be straight and the bands sharp.

8. Fill in wells of gel with melted 1% SKG Agarose (equilibrated to 55-60ºC). Allow to harden for 3-5 min. Unscrew and remove end gates from gel form; remove excess agarose from sides and bottom of casting platform with a tissue or kimwipe. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber. Close cover of chamber.

ELECTROPHORESIS CONDITIONS

1. Select following conditions for *V. cholerae* strains restricted with *Sfi*I and *Not*I :
   a. Select following conditions on the **CHEF Mapper with a two-block program**
      Block 1: 2 s - 10 s, 13 hours
      Block 2: 20 s - 25 s, 6 hours
      1. Press the Multi-State button on the Chef Mapper.
      2. Program with Interrupts?
         0 = No

      Note: Press 'Enter' after each value or command is entered.

      3. Block 1 Runtime?
         13 hours
      4. Block 1, State 1: (Fill in the blanks appropriately)
         a. 6.0 volts
         b. angle = 60.0
         c. Initial switch time = 2 s

d. Final switch time = 10 s
e. Ramping factor, a = 0 (linear)

5. Continue with another state (Vector)?
   1 = Yes
6. Block 1, State 2: (Fill in the blanks appropriately)
   a. 6.0 volts
   b. **angle = - 60.0**

Note: The angle for State 2 is Negative

   c. Initial switch time = 2 s
   d. Final switch time = 10 s
   e. Ramping factor, a = 0 (linear)
7. Continue with another state (Vector)?
   0 = No
8. Continue with another Block?
   1 = Yes
9. Block 2 Runtime?
   6 hours
10. Block 2, State 1: (Fill in the blanks appropriately)
    a. 6.0 volts
    b. angle = 60.0
    c. Initial switch time = 20 s
    d. Final switch time = 25 s
    e. Ramping factor, a = 0 (linear)
11. Continue with another state (Vector)? 1 = Yes
12. Block 2, State 2: fill in the blanks appropriately.
    a. 6.0 volts
    b. angle = - 60.0

Note: The angle for State 2 is Negative

    c. Initial switch time = 20 s
    d. Final switch time = 25 s
    e. Ramping factor, a = 0 (linear)
13. Continue with another state (Vector)? 0 = No
14. Continue with another Block? 0 = No
15. A program is in memory, please enter another command.
16. Press the **Start Run** Button

b. Select the following conditions on **CHEF DR-III**

Block I:
- Initial switch time: 2s
- Final Switch time: 10s
- Voltage: 6V
- Included Angle: 120°
- Run time: 13 h

Block II:
- Initial switch time: 20s
- Final switch time: 25s
- Voltage: 6V
- Included Angle: 120°
- Run time: 6 h

2. Select following conditions for ***V. parahaemolyticus*** **strains restricted with *Sfi*I and *Not*I** :
   a. Select following conditions on the **CHEF Mapper**

Auto Algorithm
78 kb - low MW
396 kb - high MW
Select default values except where noted by pressing "enter."
Change run time to 18 – 19 hr (See note below)
(Default values: Initial switch time = 10 s; Final switch time = 35.03 s)
Linear ramping factor

b. Select the following conditions on **CHEF DR-III**
Initial switch time: 10 s
Final switch time: 35 s
Voltage: 6 V
Included Angle: 120°
Run time: 18 - 19 hr

c.Select the following conditions on **CHEF DR-II**
Initial A time: 10 s
Final A time: 35 s
Start Ragio: 1.0 (if applicable)
Voltage: 200 V
Run time: 18 - 19 hr

Note: The electrophoresis running times recommended above are based on the equipment and reagents used at the CDC. **Run times may be different in your laboratory and will have to be optimized for your gels so that the lowest band in the *S.* ser. Braenderup H9812 standard migrates 1.0 - 1.5 cm from the bottom of the gel.**

Note: Make note of the initial milliamp (mAmp) reading on the instrument. The initial mAmps should be between 110-150 mAmps. A reading outside of this range may indicate that the 0.5X TBE buffer was prepared improperly and the buffer should be remade.

Day 2

STAINING AND DOCUMENTATION OF PFGE AGAROSE GEL

Note: The following staining procedure describes the use of ethidium bromide to stain PFGE gels. Alternate DNA stains may be used. Please see the "Alternate DNA Stains-Results and Recommendations" posting within the PulseNet Documents forum on the SharePoint site for additional information.

1. When electrophoresis run is over, turn off equipment; remove and stain gel with ethidium bromide by diluting40 µl of ethidium bromide stock solution (10 mg/ml) with 400 ml of Ultrapure water (CLRW). This volume is for a staining box that is approximately 14 cm x 24 cm; a larger container may require a larger amount of staining solution. Stain gel for 20-30 min in covered container.

Note: Ethidium bromide is toxic and a mutagen. Stock solutions of 10 mg/ml Ethidium Bromide (EtBr) in water are available from several commercial companies (Amresco X328; Bio-Rad, 161-0433; Sigma, E-1510). The diluted solution can be kept in dark bottle and reused 6-8 times before discarding according to your institution's guidelines for hazardous waste. CDC does not recommend disposing of EtBr down the drain. Aqueous solutions containing EtBr can be filtered through charcoal or degraded using activated carbon destaining or "tea" bags from Amresco (E732-25 Destaining Bags) or other companies, which effectively and safely remove EtBr from solutions and gels. Once the EtBr is removed, the treated aqueous solutions can be discarded down the drain. If you have further

questions about EtBr please refer to the Material Safety Data Sheets (MSDS) provided by the vendor or manufacturer.

Note: Currently, the only acceptable alternative stain options are GelRed™ (Biotium, 31010), SYBR® Safe (Invitrogen, S-33102) and SYBR® Gold (Invitrogen, S-11494). Labs are strongly encouraged to follow manufacturer's instructions and test stains in their labs before adopting them for routine use. If one of the alternative stains is used, the destaining steps should be omitted.

2. Destain gel in approximately 500 ml Ultrapure water (CLRW) for 60 - 90 min, changing water every 20 minutes. Capture image on a Gel Doc 1000, Gel Doc 2000, or equivalent documentation system. If background interferes with resolution, destain for an additional 30-60 min.

3. Follow directions given with the imaging equipment to save gel image as an ***.1sc** file; convert this file to ***.tif** file for analysis with the BioNumerics software program. The gel image should fill the entire window of the imaging equipment (computer) screen (without cutting off wells or lower bands). Ensure that the image is in focus and that there is little to no saturation (over-exposure) in the bands (signified by red pixilation in the QuantityOne or ImageLab software). Additional instructions are provided in PNL07 of the PulseNet QA/QC manual.

4. Drain buffer from electrophoresis chamber and discard. Rinse chamber with 2 L Ultrapure water (CLRW) or, if unit is not going to be used for several days, flush lines with water by letting pump run for 5-10 min **before** draining water from chamber and tubing.

5. If the lowest band in the H9812 standard does not migrate within 1-1.5 cm of the bottom of the gel, the proper run time will need to be determined empirically for the conditions in each laboratory.

Note: The following options are available if PFGE results do not have to be available within 24-28 hours:
- Plugs can be lysed for longer periods of time (5-16 hours).
- The washing steps with TE to remove the lysis buffer from the PFGE plugs can be done for longer periods of time (30-45 min) and at lower temperatures (37°C or room temperature). They can be started on Day 1 and finished on Day 2 after overnight refrigeration of the plugs in TE.

Use of trade names and commercial sources is for identification purposes only and does not imply endorsement by CDC or the U.S. Department of Health and Human Services.

NOTE: CLIA LABORATORY PROCEDURE MANUAL REQUIREMENTS

Efforts have been made to assure that the procedures described in this protocol have been written in accordance with the 1988 Clinical Laboratory Improvement Amendments (CLIA) requirements for a procedure manual (42 CFR 493.1211). However, due to the format required for training, the procedures will require some modifications and additions to customize them for your particular laboratory operation.

Any questions regarding the CLIA requirements for a procedure manual, quality control, quality assurance, etc., should be directed to the agency or accreditation organization responsible for performing your laboratory's CLIA inspection. In addition, some states and accreditation organizations may have more stringent requirements that will need to be addressed.

Formulas of Selected Reagents used in PulseNet Standardized Laboratory Protocol for PFGE

Tris:EDTA Buffer, pH 8.0 (TE, 10 mM Tris:1 mM EDTA, pH 8.0)[4]

10 ml of 1 M Tris, pH 8.0
2 ml of 0.5 M EDTA, pH 8.0
Dilute to 1000 ml with sterile Ultrapure water (CLRW)

Cell Lysis Buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosine + 0.1 mg/ml Proteinase K)

25 ml (50 ml) of 1 M Tris, pH 8.0
50 ml (100 ml) of 0.5 M EDTA, pH 8.0
50 ml (100 ml) 10% N-Lauroylsarcosine, Sodium salt (Sarcosyl)

OR

5 g (10 g) of N-Lauroylsarcosine, Sodium salt (Sarcosyl)[5]
Dilute to 500 ml (1000 ml) with sterile Ultrapure water (CLRW)

Add **25 µl** Proteinase K stock solution (20 mg/ml) per 5 ml of cell lysis buffer **just before use** for a final concentration in the lysis buffer of 0.1 mg/ml Proteinase K.

5. FLOW CHART:

6. BIBLIOGRAPHY:

7. CONTACTS:

8. AMENDMENTS:

8.1. The phrase "Type I Water" has been changed to "Ultrapure Clinical Laboratory Reagent Water (CLRW)." The water composition is the same, but this reflects a change in the terminology used by the Clinical Laboratory Standards Institute (CLSI).

8.2. March 2013 changes:

- Protocol was revised to combine *Vibrio cholerae and parahaemolyticus* instructions.
- Corrected formula for TE buffer. TE used at CDC is 10mM for Tris and 1 mM for EDTA.
- Recommended disinfectant changed from 10% bleach to 1% Lysol/Amphyll or 90% ethanol.
- Corrected 1% SKG / TE plug agarose recipe.
- A note was added to provide guidance when working with large numbers of isolates (>10).
- Plug washing steps can be performed at 54-55ºC rather than lowering to 50ºC.
- Volume of TE needed to wash 10 plugs was corrected from 300 – 350 ml to 400 – 600 ml.
- A statement was added to clarify that using combs with small teeth (5.5 mm) was not advised.
- References to specific restriction buffers have been removed.
- Moved reference to BSA out of footnotes and into the main text of the protocol. Use of pre-restriction step and BSA was changed from optional to highly recommended. Calculation for including BSA in restriction enzyme master mix was added.

[4] TE Buffer used at CDC is 10 mM for Tris and 1mM for EDTA

[5] If Sarcosyl powder is added directly to the other components of this reagent, warm the solution to 50- 60ºC for 30-60 minutes, or leave at room temperature for ≈2 hours to completely dissolve the Sarcosyl; adjust to the final volume with sterile Ultrapure Water.

- The word "Sterile" has been deleted in reference to diluting 5X or 10X TBE to 0.5X TBE. Non-sterile CLRW is acceptable.
- A statement was included to allow the use of an alternative agarose for casting the running gel, along with recommendations strongly urging each lab to optimize the run time. Internal and external validation showed that run times could be affected by agarose type, but no trends were noted so a blanket recommendation on run times cannot be made. Additional agarose alternatives may be tested and deemed acceptable at a later date.
- Added parameters for programing a CHEF DR-II for *Vibrio parahaemolyticus.*
- Added a recommendation for laboratories to monitor the initial mAmps when electrophoresis is started.
- A statement was included to allow the use of alternative DNA stains that are equivalent to EtBr. Labs are strongly urged to follow manufacturer's instructions as well as test stains in their own labs to gain experience using alternative agarose stains. Additional stain alternatives may be tested and deemed acceptable at a later date.
- The option to allow incubation times for restriction digestion to be increased longer than recommended was deleted.

1. **PURPOSE**: To establish guidelines for the standardization of gel image acquisition using the GelDoc 1000, 2000, EQ, or XR Systems; and production of TIFF files for data analysis.

2. **SCOPE:** This procedure applies to all gels which will be analyzed using the BioNumerics software.

3. **DEFINITIONS/TERMS:**

   3.1. SOP: Standard Operating Procedure
   3.2. DI: distilled water

4. **RESPONSIBILITIES:**

5. **PROCEDURE:**

   5.1. GelDoc 1000

   5.1.1. After adequate staining and destaining of the agarose gel, a TIFF image of the gel is required for analysis using the BioNumerics software. Handle the gel with untreated gloves (no powder or aloe) at all times; remove or change gloves when using the computer keyboard or mouse.

   5.1.2. Turn on the computer and printer connected with the GelDoc 1000 system. Open the Windows directory. Using the mouse, double click on the ***Molecular Analyst*** icon. Click on ***File → New***. The default for the View window is ***Live***.

   5.1.3. Open the door of the GelDoc 1000 and carefully remove the gel from the appropriate container with gloved hands or gel scoop; drain excess liquid from gel and place on the transilluminator platform inside the GelDoc 1000. Use the black gel frame (with the pegs removed) to help prevent the gel from sliding on the transilluminator platform. Turn on the white light using the switch on the upper right hand corner of the GelDoc 1000. Using the computer monitor to visualize the gel, center the gel on screen with the wells parallel to the top of the screen so that the wells are still visible. The camera aperture (F stop) should be barely open (setting of 2-3) to allow for proper exposure of the gel (adjustment is done with the **top ring on the camera**).

   5.1.4. Make sure that the image completely fills the window and includes the wells on the top of the screen. Slowly turn the **middle ring of the camera** to zoom in (or out) as close as possible to eliminate blank space around the top, bottom and sides of the gel, but do not cut off blank wells or the bottom of the gel. Using a flat ruler or grid, focus the image until it is sharp by turning the **bottom ring of the camera**. If necessary, once the image is in focus make minor adjustments by zooming in or out to ensure that the image size is appropriate. Minor adjustments to the image size should not change the focus. Once the GelDoc is focused properly, very little adjustment should be needed for future gels. Remove gloves for computer operation. Close the door, turn off the white light switch, and turn on the UV light which is located at the lower right hand corner of the GelDoc. (Labeled **Power**). NOTE: The UV light will not come on if the door is ajar or open. Be sure that the toggle switch on the UV light box is set for "Analytical" and not "Preparative."

   5.1.5. It is common for the Live (Initial) image to be dark. Select ***Integrate*** from the View window to visualize the image. Adjust the ***Integration Time*** until a satisfactory image is obtained. Bands on every lane should be visible without excessive brightness. NOTE: Optimize the ***Integration Time*** by selecting ***Show Saturation*** and adjusting the integration time by turning the top ring of the camera so that the strongest sample band (DNA) is just below the point of saturation (no red showing). Saturation in the gel wells may be present and is acceptable. If the image is not visible, increase the integration times or check the aperture on the camera (top ring). Adjust the aperture to the appropriate level of brightness by opening it up to the maximum setting. If the image is still not visible, the gel may have to be restained with ethidium bromide.

   5.1.6. Once the desired image has been captured, select ***Freeze*** from the ***View*** window and turn off the UV light to avoid quenching the DNA in the gel.

5.1.7. To save captured image, select ***File → Save*** and name it using the .img extension and click ***OK***. Use the modify View icon to increase or decrease the brightness of the image by clicking on the right and left arrows. When the adjustment is satisfactory, the Tools menu can be used to add a boxed text to the gel image (optional). Once a satisfactory image has been acquired, select ***View → Show Full Screen*** and print image.

5.1.8. To create a **TIFF** file, select ***File →Save As*** and change the extension to "**.tif"** and click ***OK***. This file can be either saved to your local computer (i.e. C drive) or to an external source (i.e. disk or CD).

5.1.9. Once the image has been successfully captured, remove the gel and place it in DI water until analysis is completed. Thoroughly clean the transilluminator platform with DI water and/or 70% Isopropanol and a soft, lint free towel (Kay-Dry or equivalent) making sure not to scratch the surface

5.2. GelDoc 2000 / Molecular Analyst Software or Quantity One

5.2.1. After adequate staining and de-staining of the agarose gel, a TIFF image of the gel is required for analysis using the BioNumerics software. Handle the gel with untreated gloves (no powder or aloe) at all times; remove or change gloves when using the computer keyboard or mouse.

5.2.2. Turn on the computer and printer connected with the GelDoc 2000 system. Open the Windows directory. Double click on the **Quantity One** icon with the mouse. Click on ***File→Gel Doc***. The default for the View window is ***Live***.

5.2.3. Open the drawer of the GelDoc 2000 and carefully remove gel from container with gloved hands or gel scoop. Drain excess liquid from gel and place on the transilluminator platform. Use the black gel frame (with the pegs removed) to help prevent the gel from sliding on the transilluminator platform. Close drawer and open door; turn on the white light switch (Epi-Light). Using the computer monitor to visualize the gel, center the gel on screen with the wells parallel to the top of the screen so that the wells are still visible. The camera aperture (F stop) should be almost open: a setting of 2-3 on the aperture will allow for proper exposure (adjustment is done with the **top ring on the camera**). Check the box on the screen for the alignment grid so it can be used to help align the gel.

5.2.4. Make sure that the image completely fills the window and includes the wells on the top of the screen. Slowly turn the **middle ring of the camera** to zoom in (or out) as close as possible to eliminate blank space around the top, bottom and sides of the gel, but do not cut off blank wells or the bottom of the gel. Using a flat ruler or grid, focus the image until it is sharp by turning the **bottom ring of the camera**. If necessary, once the image is in focus make minor adjustments by zooming in or out to ensure that the image size is appropriate. Minor adjustments to the image size should not change the focus. Once the GelDoc is focused properly, very little adjustment should be needed for future gels. Remove gloves for computer operation. Close the door, turn off the Epi-Light switch, and turn on the Transilluminator (UV light). NOTE: The UV light will not come on if the door is ajar or open.

5.2.5. It is common for the Live (Initial) image to be dark. Select ***Auto-Expose*** to determine an approximate exposure time. The exposure can be "fine tuned" using ***Manual Expose*** and clicking on the up and down arrows. For optimal analysis of the gel image, there should be little to no saturation in the bands. Check ***Highlight Saturated Pixels*** to determine the level of saturation, and adjust the amount of saturation by clicking the arrow icons and/or changing the camera aperture (top ring) so that the strongest sample band (DNA) is just below the point of saturation (no red showing). Saturation in the gel wells may be present and is acceptable. Inverted images can also be assessed in real time by clicking the ***Invert Display***. Refer to the manual for other options for the display or annotation of the gel. NOTE: ***Exposure Time*** is equivalent to ***Integration Time*** as seen in previous GelDoc systems.

5.2.6. Once the desired image has been captured, select ***Freeze*** from the ***View*** window and turn off the UV light to avoid quenching the DNA in the gel.

5.2.7. A picture of the image (or inverted image) can be taken anytime by clicking ***Video Print***. This also freezes the image.

5.2.8. To save the image, click ***File→Save***. A name will be suggested corresponding to the date and time of the image with a **.1sc** file extension. Change the filename to the appropriate designation, i.e., CDC11001.1sc. Print the saved image by clicking ***File→Print→Video Print.***

5.2.9. To create a **TIFF** file, open the saved file and select ***File → Export to Tiff Image→Export*** and save in appropriate drive. The file will have a corresponding name with the "**.tif"** extension.

5.2.10. Once the image has been successfully captured, remove the gel and place it in DI water until analysis is completed. Use soft, lint-free towels (Kay-Dry or equivalent) to remove excess water from surface of transilluminator and clean using DI water or 70% isopropanol making sure not to scratch the surface.

5.3. GelDoc EQ and XR (Quantity One Software – version 4.5.0 or higher).

5.3.1. After adequate staining and de-staining of the agarose gel, a TIFF image of the gel is required for analysis using the BioNumerics software. Handle the gel with untreated gloves (no powder or aloe) at all times; remove or change gloves when using the computer keyboard or mouse.

5.3.2. Turn on Universal Hood II main switch (if off); turn on the computer and printer connected with the GelDoc EQ or XR system. On the Windows desktop, double click on the Quantity One icon. Click on ***File–>Gel Doc EQ*** or ***Gel Doc XR***. The default for the View window is Live/Focus. (If "Volumes Quick Guide" box appears in upper right of screen, it can be closed by clicking the "x" in upper right hand corner of box.)

5.3.3. Open the drawer of the Universal Hood II of the GelDoc EQ or XR and carefully remove gel from container with gloved hands or gel scoop. Drain excess liquid from gel, and place it on the transilluminator platform. Use the black gel frame (with the pegs removed) to help prevent the gel from sliding on the transilluminator platform. Close drawer and open door of the hood. Press the ***Epi-Illumination*** key on the ***Light Source*** membrane touch pad control panel (upper) to turn on the Epi White lights. While watching the computer screen, position the gel so that it is centered and the wells are visible and parallel to the top of the screen. Check the box ***Show Alignment Grid*** to help align the gel. Using the ***Lens Control*** touch pad (lower) on the cabinet or the ***Iris*** **(camera aperture)**, ***Zoom***, or ***Focus*** arrows on the left side of the computer screen (Step I box), adjust the ***Iris*** so the gel image is light – medium gray and the ***Zoom*** so that the gel image completely fills the window without cutting off the wells, the bottom of the gel, or any blank wells. Place a clean, flat ruler on the top of the gel and adjust the focus until the letters or numbers are sharp and clear on the computer image; a "gel-cutter ruler" from Bio-Rad can also be used alone or placed beside the gel to help adjust the focus.

5.3.4. Once the image is focused properly, very little adjustment should be needed for future gels. Close the door, turn off the Epi White light and turn on the Trans UV light by pressing the keys of the ***Light Source*** touch pad on the Universal Hood II. Be sure the UV button is selected in the ***Image Mode*** (Step II). NOTE: The UV light will not come on if the drawer or door is ajar or open.

5.3.5. It is common for the Live/Focus image to be dark. Check ***Highlight Saturated Pixels*** (Step IV box). Select ***Auto-Expose*** (Step III box) and then make any minor adjustments to the size of the gel image with the ***Zoom*** arrow or touch pad key; use the ***Iris*** arrow or touch pad key so that the strongest sample band (DNA) is just below the point of saturation (no red showing). Saturation in the gel wells may be present and is acceptable; however, there should be no red color or saturation in any bands of the PFGE patterns. The exposure time can also be adjusted by clicking on ***Manual Expose*** and using the arrows below to adjust the iris (camera aperture) so that all saturation is removed. For most Gel Doc systems, the default image is white bands on a dark background; if dark bands on a white background (inverted image) are preferred, check the ***Invert Display*** (Step IV box). Refer to the manual for other display or analysis options and annotation of the gel images (Step V). NOTE: Exposure Time is equivalent to Integration Time as seen in previous GelDoc systems.

5.3.6. Once a satisfactory image of the gel is obtained, click ***Freeze*** (Step III). <u>Turn off the Trans UV light on the touch pad to avoid quenching the DNA in the gel</u>.

5.3.7. A picture of the image (or inverted image) can be taken anytime by clicking ***Video Print*** (Step VI). This also freezes the image. Check a print of the gel image before saving it to make sure that it is satisfactory and does not need any adjustments to the gel position or focus.

5.3.8. Save the image by clicking ***Save*** under Step VI (Select Output). A name will be suggested corresponding to the date and time of the Raw 1-D image (*.1sc file extension). The filename should be changed to the appropriate designation, i.e., CDC11001.1sc.

5.3.9. To create a TIFF file on the GelDoc EQ, open the saved image, select ***File → Export to Tiff Image.*** Check ***Export*** in lower box and export the file to your local computer (i.e. C drive) or to an external source (i.e. disk or flash drive). It will have a corresponding name with the ".tif" extension.

5.3.9.1. The camera in the GelDoc XR has much higher resolution than older models of the GelDoc systems; thus, the file size is much larger (~1MB) compared to the size of the files generated by cameras with lower resolution (~300Kb). Although the larger images can be analyzed in BioNumerics, it is not ideal for storage either at CDC or in local databases. There are limitations to the amount of data that can be stored at CDC and the tripling of file sizes would not be manageable. The default settings that are set up when the PulseNet scripts are installed (thickness of strips, resolution, etc.) are set for images that are ~300-400 Kb. After the image acquisition step is completed, the file size of the image can be reduced using the following instructions: select ***File → Export to Tiff Image***. At this screen***, Under Export Mode, Publishing***, select ***Export Views Excluding Overlays***. This automatically sets the file to an 8-bit size. Under ***Resolution*** (dpi), specify a number that when the ***Enter*** key is pressed results in the file size being converted to ~300Kb (denoted in the lower right-hand corner of the Export window). Several resolution values may need to be tested before finding the number that converts the image to the appropriate file size. This value should be used when capturing all subsequent PFGE gel images. Once the file size has been adjusted "***Save Image***." These steps convert and export TIFF files that are approximately the same size as those on the older GelDoc systems (~300Kb) so they can be emailed and/or analyzed using BioNumerics.

5.3.10. Once the image has been successfully captured and saved, remove the gel and place in DI water until analysis is completed. Use soft, lint-free towels (Kay-Dry or equivalent) to remove excess water from surface of the transilluminator and clean using DI water and/or 70% isopropanol taking care not to scratch the transilluminator platform.

5.4. GelDoc XR + (Image Lab Software – version 3.0 or higher).

5.4.1. After adequate staining and de-staining (if using ethidium bromide) of the agarose gel, a TIFF image of the gel is required for analysis using the BioNumerics software. Handle the gel with gloves (powder-free and not aloe-treated) at all times; remove or change gloves when using the computer keyboard or mouse.

5.4.2. Turn on the GelDoc XR+ imager, the computer and the printer. On the Windows desktop, double click on the **Image Lab icon**. Click on **New Protocol** or **open protocol**.

5.4.2.1. New Protocol: Step 1 – Gel imaging. Select application (nucleic acid gels, appropriate dye), enter imaging area (22cm x 16.4cm for large or 15-well gels, 18cm x 13.4cm for small or 10-well gels), select image exposure (intense or faint – may save two different protocols), select Display Options (check highlight saturate pixels, image color gray).
Step 2 – lane and band detection – leave unchecked
Step 3 – analyze molecular weight – leave unchecked
Step 4 – Specify output, customize printing and reporting options if desired or leave unchecked.
Close and save protocol as .ptl file. Run protocol.

5.4.3. Open protocol or select recent protocol.

5.4.4. Open the drawer of the Gel Doc XR+ and carefully remove gel from container with gloved hands or gel scoop. Drain excess liquid from gel and place it on the transilluminator platform.

5.4.5. Click **position gel** and hold in place using an empty frame. Close the drawer; the GelDoc XR+ will automatically focus. If desired, use **+** and **–** buttons on computer screen to zoom the camera and fine tune image size.

5.4.6. When satisfied with the image size, click **Run Protocol.** A window appears – click **OK for filter 1**.
5.4.7. Save the image as a .scn file. A name will be suggested corresponding to the date and time. The filename should be changed as desired, i.e., CDC11001.scn.
5.4.8. Under Image tools on left panel, select **Invert data→yes**.
5.4.9. In the File menu select **Export to PulseNet**. A .tiff file of the correct size with the same name as the .scn file is saved.

6. FLOW CHART:

7. BIBLIOGRAPHY:

7.1. Weinberg, Sandy. **GOOD LABORATORY PRACTICE REGULATIONS**. Second edition. Marcel Dekker, Inc. USA (1995).
7.2. Bio-Rad Laboratories, Inc**., Quantity One User Guide**, **Gel Doc 2000 Documentation Systems Hardware Instruction Manual**, and **Gel Doc EQ (ChemiDoc EQ, ChemiDoc XRS) Hardware Instruction Manual**.

8. AMENDMENTS:

8.1. 2011-07 Edited to clarify how far to zoom in on the gel during image capture. Added statements in each section about using untreated gloves (no powder or aloe) when handling gels. Section 5.4 was added.

1. PURPOSE: To describe the format and information needed for records and reports used in PulseNet.

2. SCOPE: All the records and reports that are generated from the testing and analysis of isolates by the PFGE technique.

3. DEFINITIONS/TERMS:

3.1 PFGE: Pulsed-field Gel Electrophoresis
3.2 SOP: Standard Operating Procedure
3.3 DNA: Deoxyribonucleic acid

4. RESPONSIBILITIES:

4.1 Records:

4.1.1 Establish a sample receipt record (see Appendix PNL08-1).
4.1.2 Establish a record book or log with the following information:
4.1.2.1 Sample number
4.1.2.2 Date the samples were received
4.1.2.3 Date the plugs were made
4.1.2.4 Place where the plugs are kept
4.1.2.5 Electrophoresis equipment used
4.1.2.6 Gel number and lane number establishing the sample order in the photograph or image

4.1.3 Establish a work record for each gel (for an example see Appendix PNL08-2).
4.1.4 Establish a reagent control worksheet (for an example see Appendix PNL08-3).

4.2 Reports

4.2.1 The laboratory reports will follow the standard format used for the gel's work record (see Appendix PNL08-4).
4.2.2 The analysis reports will be done according to the standard format for reporting pattern numbers (see Appendix PNL08-5).

5. PROCEDURE:

6. FLOW CHART:

7. BIBLIOGRAPHY:

8. CONTACTS:

9. AMENDMENTS:

Appendix PNL08-1

EXAMPLE OF SAMPLE RECEIPT RECORD FOR PFGE ANALYSIS

CODE	ORGANISM	DATE OF RECEIPT	ORIGIN	DISTRIBUTION	DATE OF ANALYSIS

Appendix PNL08-2

EXAMPLE OF WORK RECORD

CONDITIONS

Number of samples: _____

Cell suspension concentration (range): Lowest _____ Highest _____

Total amount of proteinase K/cell in lysis buffer: Total: _____

Lysis incubation time _____ and temperature _____

Location of standard in the gel: Lanes # _____

RESTRICTION (Master Mix Preparation)

Total amount of buffer:_____ Total amount of water:_____ Total amount of enzyme: _____

Total volume: _____

AGAROSE GEL

Gel size: _____ 10-well or _____ 15-well gel.

0.5X TBE buffer:

10X TBE amount: _____ Reagent grade water: _____ Total volume: _____

SKG agarose amount: _____ Water: _____ 0.5X TBE amount: _____

Electrophoresis run time: _____

Appendix PNL08-3

EXAMPLE OF REAGENT CONTROL WORKSHEET

Date: _____

Laboratory: _____

REAGENT	LOT #	SOURCE	DATE MADE	EXPIRATION DATE
TE Buffer				
Sea Kem Gold agarose				
SDS				
Cell suspension buffer				
Cell lysis buffer				
Proteinase K				
Reagent Grade Water				
Enzyme				
TBE (10x)				
Ethidium bromide				

_____ _____

Name Signature

Appendix PNL08-4

INTERNAL PFGE REPORT WORKSHEET

Samples Received From:
Laboratory Submitting Image:
Date Isolates Were Received:

Date PFGE Was Done:
Date Results Were Reported:
TIFF File Number:

Conditions	
Run Time	hr
Initial Switch Time	sec
Final Switch Time	sec
Voltage Gradient	6 V/cm
Included Angle	120^{o}
Ramping	Linear
Initial milliamps	

PFGE Analysis Performed by:
Reviewed by:

Information Contact Person:
Phone Number:

Lane	CDC isolate #	State isolate #	Organism	Source	Restriction enzyme	PFGE Pattern	Interpretation/Comments
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
15							

Appendix PNL08-5

Pulsed-Field Gel Electrophoresis Outbreak Report

Date:

To:

From:

Re: PFGE Molecular Subtyping Results Related to Outbreak

Isolates submitted to the CDC Outbreak Investigation Lab were analyzed by Pulsed-Field Gel Electrophoresis (PFGE) DNA fingerprinting using standardized PulseNet methods.

Isolates analyzed by PFGE subtyping were each given a unique isolate number when accessioned. Individual DNA fingerprint patterns were produced for each isolate using the restriction enzyme *Xba*I (Primary enzyme) and *Bln*I (Secondary enzyme). The outbreak DNA fingerprint pattern from this outbreak has been assigned the pattern name [ ] by the CDC PulseNet Data Administration Team.

Clinical Isolate Number	Molecular Lab Number	Date Collected	Date Received at CDC	Source	Organism Subtyped	PulseNet Pattern Name: Primary Enzyme	PulseNet Pattern Name: Secondary Enzyme	PFGE Pattern Interpretation

General Interpretation Criteria:
Isolates that have been designated with the same PFGE pattern name as the outbreak pattern may be interpreted as indistinguishable from the outbreak strain. Isolates with different PFGE fingerprint patterns have been designated as “different” in the PFGE Pattern Interpretation column. Specifically by this work, isolates that have indistinguishable DNA fingerprint patterns are more likely to have originated from a common source; isolates that have different DNA fingerprint patterns are less likely to have originated from a common source.

Pulsed-Field Gel Electrophoresis Outbreak Report

Additional Interpretation:

Disclaimer:
The results of PFGE fingerprinting analysis should always be used in conjunction with clinical, microbiologic, and epidemiologic information. PFGE analysis is a population-based assay and should not be used for individual patient diagnostic purposes. PFGE is an investigational tool and should be used for investigational purposes only. Epidemiological relatedness is considered to be the gold standard.

Report completed by: _____ Date: _____

Reviewed by: _____ Date: _____

Reference: Swaminathan B, Barrett TJ, Hunter SB, Tauxe RV, the CDC PulseNet Task Force. PulseNet: The molecular subtyping network for foodborne bacterial disease surveillance, United States. Emerg Infect Dis 2001;7:382-9

Website: www.cdc.gov/pulsenet

1. PURPOSE: To establish guidelines for the use, inspection, cleaning, maintenance, and calibration of equipment used for PulseNet-related activities.

2. SCOPE: All equipment used for the development and application of the PFGE technique, reagents, solutions, and reports related to PFGE.

3. DEFINITIONS/TERMS:

3.1 PFGE: Pulsed-field Gel Electrophoresis
3.2 SOP: Standard Operating Procedure

4. RESPONSIBILITIES/PROCEDURE:

4.1 Equipment related to this SOP and requirements:

4.1.1 Spectrophotometers should have the wavelength confirmed with each use, using a didymium filter, and calibrate using a standard solution with known absorbance on a monthly basis (linearity check) or whenever new lots of controls, reagents, or standards are obtained.
4.1.2 Colorimeters and Turbidity meters should be calibrated on a monthly basis according to the instructions provided by the manufacturer.
4.1.3 Analytical balances should be calibrated with a set of class S weights certified by the NIST or a qualified contractor on a quarterly basis.
4.1.4 Volumetric equipment, such as pipettes and autodiluters, should be checked yearly for volume of delivery.
4.1.5 Upright refrigerators and freezers should have a calibrated thermometer immersed in glycerol placed inside. Walk-in or stand-alone units may have adequate, precalibrated digital thermometers. A recording thermometer may be used if already installed in walk-in refrigerators. All temperatures should be checked daily.
4.1.6 Water bath temperatures should be checked and recorded daily with an adequate, precalibrated thermometer.
4.1.7 Thermometers used in the laboratory should be checked annually against a certified NIST thermometer over the full range of temperatures usually measured.

4.2 Develop for each item:

4.2.1 An inventory that lists all equipment, its location, model and serial number, age, description, and the name of the person responsible for the item.
4.2.2 A definition of service tasks for each piece of equipment, procedures necessary for upkeep and maintenance of equipment (calibration, proper functioning, cleaning, etc.).
4.2.3 An interval frequency during which the defined procedures should be performed.
4.2.4 A personnel list of all individuals who are available for performing the upkeep and maintenance.
4.2.5 In-service training of personnel in the use of special monitoring devices and the performance of some of the more difficult service tasks should be provided, and records of the names of those trained and the dates when the training occurred should be maintained.

4.2.6 A system so that the program may continue uninterrupted. A record sheet/book must be established for each item of equipment, in which all entries are made.

5. FLOW CHART:

6. BIBLIOGRAPHY:

7. CONTACTS:

8. AMENDMENTS:

1. PURPOSE: To describe guidelines for maintenance of CHEF electrophoresis systems.

2. SCOPE: This procedure applies to equipment used with the PulseNet Standardized PFGE Protocols.

3. DEFINITIONS/TERMS:

3.1 SOP: Standard Operating Procedure
3.2 PFGE: Pulsed-field Gel Electrophoresis
3.3 DNA: Deoxyribonucleic acid

4. RESPONSIBILITIES/PROCEDURES:

4.1 CHEF- Mapper, -DRIII, and Genepath systems

4.1.1 After each run:

4.1.1.1 Turn off cooling module, pump and main power switches of the instrument.
4.1.1.2 Install drain tube on right front port of chamber and drain buffer into sink or large flask.
4.1.1.3 Prop up back of chamber with a pipet tip box or similar object to completely drain all buffer from the chamber.

4.1.1.4 If another gel is to be run the same day:

a. Remove drain tube.
b. Add 2 liters of appropriate buffer (0.5X TBE) to the chamber.
c. Wait at least 30 minutes after the last run (4.1.1.1) to turn on the pump and chiller. If chiller is turned on and the pump is not, ice may form in the heat exchanger, which can block the flow of buffer and prevent it from reaching the desired temperature (14EC). If this occurs, turn off chiller and pump, and allow the unit to remain at room temperature for at least 30 minutes so any ice that may have formed in the heat exchanger can thaw.

4.1.1.5 If the chamber will not be reused on the same day:

a. Rinse the gel chamber with 2 liters of deionized (Type 1) water.
b. Drain by propping back of chamber with a pipet tip box or similar object as described in 4.1.1.3.
c. Wipe the inside lid and sides of chamber with a damp towel to remove any residual buffer. *Do not touch the electrodes*. Remove any remaining small pieces of agarose in chamber with a soft paper towel (Kaydry, Kimwipe, or equivalent towel).

4.1.2 Weekly

4.1.2.1 Once a week or if the PFGE unit is not to be used for several days, the gel chamber and cooling module tubing should be flushed with water.

a. Add 2 liters of deionized (Type 1) water to chamber.
b. Turn on the pump to circulate it through the tubing and electrophoresis chamber. *Do not turn on the chiller*.
c. Let the water circulate for 10-15 minutes, then turn off the pump.
d. Drain as much of the water out of the chamber as possible.

e. Disconnect tubing from the left front port, turn pump on *briefly* (30 seconds) to force any residual water from tubing into the gel chamber.
f. Drain chamber as in Step 4.1.1.3.
g. Wipe the inside lid and sides of chamber with a damp towel to remove any residual buffer. *Do not touch the electrodes*.
h. Re-connect tubing to left port of the chamber. *Note: After this step is completed, the chamber will require 2.2 liters of buffer to fill.*
i. Check that the gel chamber is level before adding buffer.

4.1.3 As necessary

4.1.3.1 If the chamber has not been used recently or if your gel is blank even after Lambda or other standards are run, the lines may be contaminated. Bacterial or fungal growth in the tubing will degrade the DNA and a blank gel will result.

a. Flush the chamber as described in 4.1.2 with 2 liters of bleach solution (5% - 10% in water) instead of deionized (Type 1) water for 15-30 minutes.
b. Drain bleach solution from chamber, add 2 liters of deionized (Type 1) water, and circulate 15-30 minutes.
c. Drain water and repeat the water wash at least two more times to be sure that all of the bleach solution is removed from the tubing.

4.1.3.2 If the problem persists (cleaning with bleach solution does not remove all of the contamination from the tubing), replace all the lines with new tubing.

4.1.4 Replacement of electrodes (if broken or damaged):

4.1.4.1 Unplug chamber from wall socket or remove electrical connector(s) from chamber.
4.1.4.2 Disconnect tubing at back and left front port of gel chamber.
4.1.4.3 Turn gel chamber upside down and remove all the screws.
4.1.4.4 Lift off the base plate.
4.1.4.5 Remove the hexagonal nut on the wire, remove the nut on the electrode to be replaced, and push down firmly on the post to remove the old electrode.
4.1.4.6 Turn the gel chamber over, insert the new electrode, pack with self-leveling silicone sealant (RTV-type sealant, which is available at most hardware stores).
4.1.4.7 Replace the nut, wire, and base plate.
4.1.4.8 If one of the pins to the serial cable bends, use tweezers to carefully straighten it.

4.1.5 Replacement of a CHEF blown fuse (available at electronic or hardware stores).

4.1.5.1 *Make sure the cord to the power module is unplugged when replacing the fuse.*
4.1.5.2 If the voltage to the electrodes exceeds 300 V, the 0.5 ampere FB (Fast Blow) fuse may blow. The high-voltage indicator light will go on and the power module will go off. Replace the fuse by unscrewing the cartridge at the front of the power module and change the blown fuse with a 0.5 ampere FB (Fast Blow) fuse.
4.1.5.3 If there is a power surge, the SB (Slow Blow) fuse may blow. The AC light on the power module will go off. The fuses are at the rear of the power module, mounted inside the power entry module. Replace the fuse with 3.0 A SB fuse if your local voltage is 120 V or 100 V, or with a 1.5 A SB fuse if your local voltage is 220 V or 240 V.

4.1.6 Replacement of a GenePath blown fuse (available at electronic or hardware stores).

4.1.6.1 *Make sure the cord to the power module (Gene Path system) is unplugged when replacing the fuse.*

4.1.6.2 If the DC current entering the gel chamber exceeds 500 mA, then the 0.5 ampere FB (Fast Blow) fuse will blow and an *error code F2 will be displayed*. Replace fuse by unscrewing the cartridge at the front of the GenePath system and change the fuse with a 0.5 ampere FB fuse.

4.1.6.3 A power surge will cause the SB (Slow Blow) line fuse to blow. The LED lights on the *GenePath system* will go off. This fuse is located at the back of the drive module. Replace the fuse with a 3.15 A SB fuse if the line voltage is 100 or 120 V, or a 1.6 A SB fuse if the line voltage is 220 or 240 V.

4.2 Cooling Module

4.2.1 Installation

4.2.1.1 See manual included with the instrument for installation instructions.

4.2.2 Operation: After the Cooling Module has been connected, the following steps should be followed for *optimal* operation:

4.2.2.1 Switch on the recirculating pump, and adjust flow rate to 1.0 liter/minute (setting of ~70-80).

4.2.2.2 Switch on the Cooling Module (main power switch is on the front panel of the unit). The temperature display will read "14.0" (or the temperature set in the previous run) after 10 beeps, indicating that the unit is on and the red light above the "SET TEMP" button will illuminate.

4.2.2.3 Enter the desired run temperature (14°C is recommended for PFGE runs) by pressing the "LOWER" or "RAISE" arrows until the desired number is reached.

4.2.2.4 After 100 seconds have elapsed, the compressor will engage (if the set temperature is below ambient temperature), and the red light above "COOLING" will illuminate. This 100-second delay allows pressure equalization in the system to avoid mechanical damage.

4.2.2.5 Press the "ACTUAL TEMP" button (the red light above the button will illuminate) to read the current temperature monitored at the internal temperature probe (located in the "OUT FLOW" port) or at the cell's internal temperature probe (if connected).

4.2.2.6 After the Cooling Module has cooled the buffer to the desired set temperature, the "COOLING" light will occasionally go on and off accompanied by a click. This is an indication of the refrigerant bypass valve cycling on and off to maintain average buffer temperature within 1° C of set temperature. [Greater temperature fluctuations may occur with longer tubing length, low buffer flow rates, and/or high amounts of input power and high ambient temperatures. However, the built-in adaptive algorithm will compensate for these conditions so that the average temperature is typically within 1°C.]

NOTE: Please allow sufficient room for ventilation at both the front and rear of the unit. The cooling fan is located in the rear.

4.2.2.7 Bio-Rad recommends that the buffer in the system be pre-cooled to the desired run temperature. The Cooling Module can quickly pre-cool the system, at an approximate rate of 0.75° C/minute in the absence of input power to the recommended operating temperature of 14° C. In other words, the cooling module can pre-cool buffer at ambient temperature to 14° C in approximately 10-15 minutes and is designed to maintain set temperature during the run. If the buffer is not pre-cooled, the presence

of input power (from the electrophoresis power supply) will greatly increase the time required to bring the system to the set temperature.

NOTE: The 0.75° C/minute pre-cooling rate decreases for set temperatures lower than 14° C.

4.3. Maintenance

4.3.1 When the Cooling Module will not be used for an extended time, rinse the lines in the heat exchanger thoroughly with distilled water to remove all traces of electrophoresis buffer. Drain the residual water from the heat exchanger, and dry it with laboratory air.

4.3.2 Main Switch Fuse Check and Replacement

a. Turn the unit around so that the back of the unit is facing forward, and locate the black plastic assembly containing the power cord receptacle on the lower left corner.
b. Turn the unit off and remove the main power cord from the receptacle (grasp the female plug, not the cord, and pull gently).
c. Turn the unit off and remove the main power cord from the receptacle (grasp the female plug, not the cord, and pull gently).
d. Using the screwdriver, gently pull the fuse holders forward (out). Remove the fuse from the holder and examine. If the fuse is obviously burned, replace it with a 6.3 Amp, 250 V SB (Slow Blow) fuse (100/120 V) or 3.0 Amp, 250 V SB fuse (220/240 V). Otherwise, check the fuse with a volt-ohmmeter.
e. Place the fuse holders back into the slots so that the arrows on the fuse holders point in the same direction as the two arrows on the inside of the front cover. Press the fuse holders firmly into place; close the front cover by pressing firmly at the top comers.
f. Replace the power cord. The unit is now ready for operation. If the unit still does not turn on, or the fuse burns again, please contact Bio-Rad instrument service.

4.4 Variable Speed Pump

4.4.1 The variable speed pump is powered from the CHEF- DRII Drive module, CHEF- DRIII power module, CHEF-Mapper power module, or Gene-Path power module.

4.4.1.1 Plug the pump into the socket marked “Pump” at the front or back of the module.

4.4.1.2 Turn the pump on using the switch marked “Pump Switch.” Adjust the flow rate by turning the dial on the variable speed pump. The setting of 100 on the dial is 100% of the pump’s maximum flow rate, though the flow is modified by varying line voltage. **A setting of 60 -70 on the dial is recommended for most pulsed-field gel electrophoresis runs with the CHEF system.** This corresponds to a flow rate of about 1 liter/minute.

4.4.1.3 If the pump does not start, check the 0.5 amp (SB) fuse on the back of the pump, located under the round gray lid. NOTE: The variable speed pump is ground isolated for safety. **Disconnect electric power to pump before changing the fuse.**

5. FLOW CHART:

6. BIBLIOGRAPHY:

Bio-Rad Laboratories. CHEF Mapper XA Pulsed-field Electrophoresis Systems, Instruction Manual and Application Guide. 1995; pp. 65.

Bio-Rad Laboratories. CHEF-DR III Pulsed-field Electrophoresis Systems, Instruction Manual and Application Guide. 1992; pp. 27.

Bio-Rad Laboratories. GenePath System, Instruction Manual. pp. 10-11.

Bio-Rad Laboratories. CHEF Variable Speed Pump Manual.

Bio-Rad Laboratories. Cooling Module Instruction Manual.

7. CONTACTS:

8. AMENDMENTS:

1. PURPOSE: To describe the guidelines for the use and maintenance of the Dade MicroScan Turbidity Meter.

2. SCOPE: This procedure applies to equipment used with the PulseNet Standardized PFGE Protocols.

3. DEFINITIONS/TERMS:

3.1 SOP: Standard Operating Procedure
3.2 PFGE: Pulsed-field Gel Electrophoresis

4. PROCEDURES:

4.1 Operational Precautions and Limitations

4.1.1 For in vitro diagnostic use only.
4.1.2 Read entire manual prior to operation.
4.1.3 Avoid spilling liquids into the instrument as this may damage sensitive components.
4.1.4 Use identical tubes for blanks and samples that are visually clean, clear and free from scratches.
4.1.5 The rechargeable NiCad batteries should last for at least 500 readings between recharging. When the battery voltage has declined to the point at which a reading error could result, a battery check circuit will extinguish the display. This is your indication that the batteries require recharging. Batteries can be recharged overnight, or the instrument can be left plugged in indefinitely without damage.

4.2 Maintenance

NOTE: Laboratorian should never attempt repair procedures on equipment such as turbidity meters, spectrophotometers, and similar electronic devices. Equipment should be sent to machine shop or back to the manufacturer for any major repair.

4.3 Operation

4.3.1 Turbidity Determination

4.3.1.1 Prepare the desired base medium in which suspensions will be made.
4.3.1.2 Dispense 2.5 ml and at least 3 ml of the medium into two clean Falcon 2054 or Falcon 2057 tubes, respectively. These will be used to confirm that the reading on the digital output is 0.00 ± 0.01.before measuring the turbidity of the test samples. One of these tubes will be referred to as the "blank."
4.3.1.3 Prepare the desired bacterial test suspensions in the same tube size and base medium as the blank. (Refer to PulseNet Standardized PFGE Protocol.)
4.3.1.4 Insert one of the blanks prepared in Step 4.3.1.2 into the "BLANK" or "1" position of the turbidity meter. Insert the test suspension into the "SAMPLE" or "2" position.
4.3.1.5 The display indicates the difference in absorbance between the blank and sample tubes. The acceptable range equivalent to a 0.5 McFarland Standard is 0.05 – 0.12.
4.3.1.6 Measure the turbidity of the test suspensions at least three times to confirm that the reading is stable.

5. FLOW CHART:

6. BIBLIOGRAPHY:

Dade MicroScan Turbidity Meter instruction pamphlet, Dade International Inc., 1996.

7. CONTACTS:

8. AMENDMENTS:

1. PURPOSE: To describe guidelines for the maintenance and cleaning of laboratory water baths.

2. SCOPE: All water baths involved with PulseNet-related activities must follow a documented schedule for maintenance and cleaning.

3. DEFINITIONS/TERMS:

3.1 SOP: Standard Operating Procedure
3.2 PFGE: Pulsed-field Gel Electrophoresis

4. RESPONSIBILITIES/PROCEDURES:

4.1 Check and record operating temperature of all water baths used in the PulseNet procedures each day prior to use (Appendix PNL13-1).

4.1.1 Accurate and reliably maintained temperature is essential for consistent results.
4.1.2 Temperature limit: desired temperature ±1.0°C.
4.1.3 If temperature is out of range, adjust control. (See instructions specific to each water bath.)

4.1.3.1 After one hour, recheck temperature and record.
4.1.3.2 If still out of range, notify party responsible for service and repair; *DO NOT USE*.
4.1.3.3 Use alternative water bath until repairs have been completed and temperature is within the accepted range.

4.2 Check water baths each day prior to use for leaks and inspect for bacterial growth/contamination.

4.2.1 If necessary, add enough distilled water to ensure there are adequate water levels to maintain the temperature(s) required for the PulseNet PFGE procedures.

4.3 Monthly drain and thoroughly clean unit per manufacturer's recommendations.

4.3.1 Turn off and unplug the water bath.
4.3.2 Use the drain (if present) or a siphon assembly for this purpose. After most of the water is drained, remove any remaining water with a sponge or soft towel.
4.3.3 Wash tank and shaker components (if present) with a solution of mild soapy water. Use a soft cloth or sponge. Make sure to wear protective gloves when cleaning water baths. Rinse all components thoroughly and dry completely. **DO NOT USE** chlorine bleach, chlorine-based cleanser, abrasives, ammonia, steel wool or scouring pads with metallic content - these will damage the water bath. Materials effective in disinfecting include glutaraldehyde or 70% alcohol.
4.3.4 Clean the low water sensor (when present) every two weeks. Scale build-up may be removed with a toothbrush while it is immersed in water.
4.3.5 Refill the tank with *DISTILLED WATER only*. Do not use tap water; it will cause mineral deposits and possible corrosion. A non-chlorine-based fungicide/bactericide may be added to the tank to inhibit fungal or bacterial contamination of the bath water.

5. FLOW CHART:

6. BIBLIOGRAPHY:

Evaluation, Verification and Maintenance Manual. College of American Pathologists. Lab-Line Instruments, Inc. Lab-Line Orbital Water Bath Shakers Operation Manual. 1998.

7. CONTACTS:

8. AMENDMENTS:

Appendix PNL13-1

Waterbath:

Location:

DATE	TEMPERATURE	INITIALS

1. PURPOSE: To describe the safe use, storage and disposal of chemicals and reagents in laboratories performing pulsed-field gel electrophoresis.

2. SCOPE: For all personnel and laboratories conducting PulseNet-related activities.

3. DEFINITIONS/TERMS:

3.1 SOP: Standard Operating Procedure
3.2 PFGE: Pulsed-field Gel Electrophoresis
3.3 PPE: Personal Protective Equipment (goggles, gloves, lab coat, etc.)
3.4 MSDS: Material Safety Data Sheets
3.5 OHS: Office of Health and Safety

4. RESPONSIBILITIES:

4.1 Use of chemicals and reagents

4.1.1 Know what chemicals and reagents are being used in any procedure.

4.1.1.1 Read chemical labels and MSDS if you are unfamiliar with any chemical or reagent.

a. All MSDS related to chemicals and reagents in the laboratory should be readily accessible to laboratory personnel.

4.1.2 Have proper safety equipment available in the laboratory.

4.1.2.1 Obtain and use all appropriate PPE.
4.1.2.2 Use the appropriate ventilation device when working with chemicals (chemical fume hood) or infectious material (biosafety cabinet).

a. *Do not use a biosafety cabinet if the work requires a chemical fume hood and vice versa.*

4.1.3 Use the smallest quantity of chemicals or reagents possible.
4.1.4 Know what to do in case of an accident.

4.1.4.1 Know the location of eye washes, showers, fire extinguishers, and exits.
4.1.4.2 Know the location and use of chemical spill kits.
4.1.4.3 Have the contact information for the OHS Environmental Program or appropriate safety office posted near the phone together with all emergency numbers.

4.2 Storage of chemicals and reagents

4.2.1 All chemicals are to be stored properly according to recognized compatibilities.

4.2.1.1 Chemicals posing special hazards or risks shall be limited to the minimum quantities required to meet short-term needs and stored under appropriate safe conditions (i.e., chemical safety cabinets).
4.2.1.2 Chemicals are not to be stored on the floor or in chemical fume hoods.
4.2.1.3 Compressed gas cylinders shall be secured at all times.

4.2.2 A chemical inventory of all hazardous chemicals will be maintained and posted on the door to each laboratory.
4.2.3 All chemicals and reagents shall be labeled with the date they were received into the laboratory.
4.2.4 Expired chemicals, reagents, and chemical waste will be disposed via the hazardous chemical waste disposal program (see http://intranet.cdc.gov/ncid/site/safety/chemhygiene/disposal.htm) or appropriate safety guidelines.
4.2.5 Each laboratory must have a written chemical hygiene plan specific for that laboratory (see http://intranet.cdc.gov/ncid/site/safety/chemhygiene/chp.htm) or appropriate safety guidelines.

4.3 Disposal of chemicals and reagents

4.3.1 Know the hazardous chemical status of your chemicals and reagents.
4.3.2 Only "certified chemical waste managers" may dispose of hazardous chemical waste. Record the name of the "certified chemical waste manager" for your laboratory and refer any questions about disposal of chemicals to him or her.
4.3.3 Keep all waste containers or expired chemicals closed tightly.

4.4 Have your "certified chemical waste manager" complete the appropriate form(s). Indicate building and room where waste is located on a "Hazardous Chemical Waste Disposal Label" (Form CDC 0.886, Rev. 10/94).

4.4.1 Send top copy to OHS Environmental Program office, Mailstop F-05, when ready for pickup.
4.4.2 Peel backing off label and attach to waste container. *Do not cover up the original container label*. Mark "XX" through label if original container is not being used.
4.4.3 For empty chemical containers, remove CDC Chemical Tracking System barcode sticker and affix to the "Chemical Disposition Form," or send "form" to OHS Environmental Program office (MS-F-05) to request a pickup.
4.4.4 For more information on hazardous or chemical waste disposal contact the Environmental Program, Office of Health and Safety, at 404-639-1464.

4.5 *DO NOT POUR HAZARDOUS CHEMICALS DOWN THE DRAIN!*

5. PROCEDURE:

6. FLOW CHART:

7. BIBLIOGRAPHY:

Laboratory Survival Skills: A Primer on Responsibilities, Safety Practices, and Emergency Response for CDC Employees.

Prudent Practices in the Laboratory: Handling and Disposal of Chemicals, Nat. Academy Press (1995).

CDC Chemical Waste Handling and Disposal Guide

CDC Chemical Hygiene Plan

8. CONTACTS:

8.1 CDC OHS Environmental Program (404) 639-1464

9. AMENDMENTS:

STANDARD OPERATING PROCEDURE FOR THE STORAGE AND USE OF EXPIRED REAGENTS	CODE: PNL15 Effective Date: 02 | 14 | 2005

1. PURPOSE: To describe under what circumstances expired reagents may be stored and used.

2. SCOPE: Expired reagents are not to be used for PulseNet-related activities except as described below.

3. DEFINITIONS/TERMS:

3.1 SOP: Standard Operating Procedure
3.2 CDC: Centers for Disease Control and Prevention
3.3 PFGE: Pulsed-field Gel Electrophoresis

4. RESPONSIBILITIES/PROCEDURES:

4.1 Expired reagents are not to be used for any PulseNet-related PFGE gels if the results are intended, or might be expected, to be included in the PulseNet national database(s).

4.1.1 Discard commercially prepared reagents on or before the expiration date or if precipitation, discoloration, or cloudiness is observed.
4.1.2 Discard laboratory-prepared reagents after six to nine months or if precipitation, discoloration, or cloudiness is observed.

4.2 Expired reagents may be retained for training, troubleshooting, and research purposes provided that they are labeled clearly with: "For training, troubleshooting, or research ONLY."

4.3 Expired reagents **must** be discarded and disposed of according to CDC's and each laboratory's protocols (see PNL14).

5. FLOW CHART:

6. BIBLIOGRAPHY:

7. CONTACTS:

8. AMENDMENTS:

1. PURPOSE: This procedure must be followed if a failure is detected in any component of the standardized PFGE protocol (bad reagents, poor standards, equipment failure, inadequately trained lab personnel) resulting in gels unacceptable for analysis and inclusion in the PulseNet National Database(s).

2. SCOPE: To be used by all laboratories involved in PulseNet-related activities.

3. DEFINITIONS/TERMS:

3.1 SOP: Standard Operating Procedure
3.2 PFGE: Pulsed-field Gel Electrophoresis
3.3 PFGE inbox: An email account that is maintained and checked by all database managers at CDC. The address is: PFGE@cdc.gov.
3.4 CDC: Centers for Disease Control and Prevention
3.5 DNA: Deoxyribonucleic acid

4. RESPONSIBILITIES/PROCEDURES:

4.1 Failure of Reagents

4.1.1 Identify what reagent(s) caused the problem. Visual inspection of the TIFF image of the problem gel by an experienced individual may lead to clues that may help narrow down potential problems.

4.1.2 Determine the nature of the problem.

4.1.2.1 Contaminated reagent
4.1.2.2 Expired reagent
4.1.2.3 Incorrect concentration, pH, etc. of one or more reagents.

4.1.3 If reagent is commercial product, open new lot/bottle and test by running protocol with a culture with a known PFGE pattern (e.g., H9812 standard strain).

4.1.4 If reagent is prepared "in-house," prepare new lot of reagent following established procedures and test by running protocol with a culture with a known PFGE pattern (e.g., H9812 standard strain).

4.2 Failure of Standards
Note: Gels with poor standard strains cannot be normalized for comparison to the national databases. In some instances the gels can be visually compared; however, all gels with poor or missing standard strains must be rerun prior to inclusion in the national databases.

4.2.1 Insufficient or too much DNA in plug

4.2.1.1 Discard and remake plug
4.2.1.2 Check equipment used to measure the cell concentration (turbidity meter, etc.) to ensure that it is operating properly.

4.2.2 Incomplete restriction

4.2.2.1 Repeat TE wash 2X, and then repeat restriction step on new slice of the same plug. Include at least one slice from a previously tested plug that gave a satisfactory pattern on another gel (+ control).

4.2.2.2 If problem persists, restrict DNA with a new vial of enzyme. Use different lot number when possible.

4.2.2.3 Confirm calculations for making enzyme/buffer mixture are correct.

4.2.3 One or more lanes with standard strain is distorted, smeared, etc.

4.2.3.1 Repeat gel

4.2.4 Inadequate number of standards run on gel.

4.2.4.1 Repeat gel with standard strain in the end lanes and after every three or four test strains (e.g., lanes 1, 5, 10 on 10-well gel; lanes 1, 5, 10, 15 on 15-well gel).

Note: Gels with an insufficient number or placement of standards may become distorted when normalized and be inadequate for analysis and comparison to the national databases.

4.3 Equipment failures may occur even with proper maintenance and service.

Note: All equipment failures, regardless of the severity, must be documented for review.

4.3.1 Temperature fluctuation in the electrophoresis chamber due to problems with the cooling module, pump, length and condition of tubing, bubbles in tubing, extreme changes in ambient temperature or voltage fluctuation with the power supply.

4.3.1.1 Temperature fluctuations of $>\pm 3^{\circ}$C may negatively impact the resolution and quality of the bands.

4.3.1.2 Each lab must monitor and document the temperature for each chamber used for PFGE for PulseNet to make sure that it operates within the parameters recommended in the standardized protocols.

4.3.2 Inadequate buffer flow due to debris or blockage in the chamber

4.3.2.1 Poor buffer flow often results in poor resolution and uneven migration of the DNA fragments through the gel.

4.3.2.2 The lines and left buffer port must be checked and cleaned periodically to ensure that the buffer can flow evenly.

4.3.2.3 The use of an agarose gel trap within the chamber can help to filter out agarose debris preventing blockage.

4.3.2.4 Always check the flow settings on the pump to make sure that it meets the rate indicated in the standardized protocol (~1L/min.).

4.3.3 Broken or failed electrodes in the chamber can cause uneven distribution of current and will distort the band movement through the gel.

4.3.3.1 Examine electrodes periodically.

4.3.3.2 Replace broken or failed electrodes as needed (see SOP PNL11).

4.3.4 Power outage or blown fuses

4.3.4.1 The most recent models of the Chef Mapper are designed to restart the program where it shuts down, but if the battery back-up is defective this may not occur.

4.3.4.2 The Chef Mapper is equipped with both Fast Blow (FB) and Slow Blow (SB) fuses, and the program may not restart where it shut down if the fuse blows and has to be replaced.

4.4 Inadequately trained lab personnel

4.4.1 Laboratory personnel who have not been certified in the PulseNet standardized protocol(s) for PFGE shall not submit TIFF or bundle files on-line to the PulseNet national database(s). Laboratories that are not certified must send their TIFF images to the PFGE inbox with the appropriate organism in the subject line of the email.

4.4.1.1 Personnel may be trained in several ways:

a. Attend a CDC-sponsored PFGE training workshop
b. Receive training through an approved PulseNet Area Laboratory
c. Receive one-on-one bench training at CDC, or in the individual's lab, by personnel from CDC or approved PulseNet Area Laboratory.

4.4.1.2 Personnel must complete their certification process prior to submitting TIFF or bundle files to the PulseNet national database(s) *unless* authorized to do so by a member of the PulseNet National Database Administration Team.

4.4.1.3 Personnel who continue to produce unsatisfactory results subsequent to an initial training *SHALL* be retrained.

5. FLOW CHART:

6. BIBLIOGRAPHY:

7. CONTACTS:

7.1 CDC PulseNet Database Administration Team
(404) 639-4558
PFGE@cdc.gov

8. AMENDMENTS:

1. **PURPOSE:** To describe the procedure for training of PulseNet personnel in PulseNet laboratory PFGE protocols.

2. **SCOPE:** This procedure applies to all PulseNet participants and hosts of PulseNet PFGE laboratory protocol training courses.

3. **DEFINITIONS/TERMS:**

3.1 Host lab: Term used to describe a PulseNet laboratory that has been approved by CDC to host a training course
3.2 Training personnel: Term used to describe PulseNet participant(s) who have been approved by CDC to train other PulseNet participants
3.3 APHL: Association of Public Health Laboratories
3.4 SOP: Standard Operating Procedure
3.5 CDC: Centers for Disease Control and Prevention
3.6 PFGE: Pulsed-Field Gel Electrophoresis
3.7 PPE: Personal Protective Equipment

4. **RESPONSIBILITIES:**

4.1 All PulseNet personnel are required to read the PulseNet QA/QC manual and all PulseNet SOPs.
4.2 At least one PulseNet participant from each participating PulseNet laboratory is required to attend annual PulseNet update meetings and regional meetings when they occur.
4.3 All PulseNet personnel performing PFGE for PulseNet pathogens and hosting PFGE training must have knowledge of aseptic techniques, pure culture isolation, principles of PFGE, and basic laboratory safety.
4.4 Host lab(s), APHL, and/or CDC will determine training needs of PulseNet participants.
4.5 All PulseNet personnel must be trained by a host lab or other approved training personnel.
4.5.1 PulseNet laboratory training covers the principles of PFGE, a detailed review of the PulseNet standardized PFGE protocol, and the use of gel image acquisition hardware and software.
4.6 Host labs and training personnel should adequately prepare participants in the PulseNet PFGE protocols. When training is finished, the trainee(s) should be able to submit PulseNet gels for gel certification (see SOP PNQ02).
4.7 Host labs and training personnel must be gel-certified.
4.8 Host labs must put together training materials for the trainees.
4.9 PulseNet participants will then be evaluated through certification (PNQ02) and proficiency testing (PNQ04).

5. **PROCEDURE:**

5.1 Adequate communication with APHL and CDC must be maintained in order to determine training needs and assure proper attendance at required meetings.
5.2 Host labs and/or training personnel should work with trainees and/or APHL to determine a feasible time and location for the course.
5.3 The following is a recommended procedure for hosting a PulseNet laboratory training course:
5.3.1 The host lab should organize an agenda committee to create an agenda and a timeline of organizational duties, and to obtain necessary laboratory training materials.

5.3.2 It is recommended that each participant be provided, but not limited to, the following items:
- 5.3.2.1 Participant and trainer contact information
- 5.3.2.2 PulseNet protocols for *E. coli*, *Campylobacter*, *Salmonella*, *Listeria monocytogenes*, and *Shigella*
- 5.3.2.3 Laboratory reagents
- 5.3.2.4 Laboratory supplies for PFGE
- 5.3.2.5 Pathogenic strains to be tested
- 5.3.2.6 Proper laboratory PPE
- 5.3.2.7 Course evaluations
- 5.3.2.8 Course certificates to indicate successful completion of training

5.3.3 The committee may also be responsible for making lodging, transportation, and meal arrangements for course participants.

5.3.4 For information regarding correct laboratory chemical handling, refer to PNL14 ("SOP for the Use, Storage, and Disposal of Chemicals and Reagents").

5.3.5 Refer to PNL01 ("SOP for Lab Equipment and Supplies") and PNL02 ("SOP for Lab Formulas and Reagents") for laboratory equipment and safety information.

5.3.6 It is recommended that training be carried out using *E. coli* or *Salmonella.*

5.3.7 Refer to Appendix PNL17-01 for a recommended sample laboratory training agenda.

5.3.8 Assign training responsibilities to trainers.

5.3.9 It is recommended that there be one trainer per five participants.

5.3.10 All reagents and strains being used during the training course should be tested before the training course.

5.3.11 At the completion of training, participants should fill out evaluations and be awarded certificates of completion.

5.3.12 Summarize evaluations and supply trainers and members of the agenda committee a summary of the conference evaluations.

6. FLOW CHART:

7. BIBLIOGRAPHY:

8. CONTACTS:

8.1 Training support at CDC: PulseNet Database Administration Team
(404) 639-4558
PFGE@cdc.gov

9. AMENDMENTS:

Appendix PNL17-01

SAMPLE Agenda for Standardized Molecular Subtyping of Foodborne Bacterial Pathogens by Pulsed-field Gel Electrophoresis (PFGE)

Day 1

Welcome, Introductions, and Remarks

General Information/Workshop Objectives

Overview of PulseNet

Epidemiology of Foodborne Diseases? or Molecular Epidemiology Using PFGE Subtyping Data? Utility of PFGE data?

Overview of PFGE Manual and Laboratory Procedures

Laboratory Safety Information

Laboratory Module IA, IB

Prepare *Salmonella* and/or *E. coli* O157:H7 agarose PFGE plugs
Lyse cells in agarose plugs

The Standardized PFGE Protocol(s) for Foodborne Enteric Pathogens
Use of Additional Restriction Enzymes for PFGE in Outbreak Situations

Laboratory Module IC

Begin washing agarose plugs with water to remove lysis reagent.
a. Wash plugs two times with 15 ml sterile Type 1 water for 10 min. at 50ºC.

Begin washing agarose plugs with TE to remove lysis reagent
a. Wash plugs three to four times with 15 ml sterile TE for 15 min. at 50ºC.
b. Discussion/demonstration of alternate ways to wash PFGE plugs.

Day 2

Laboratory Module IIA

Restriction digestion of DNA in agarose plugs

Optional Bio-Rad Product Information
Questions and Answers for Bio-Rad
Local Bio-Rad Representative

Laboratory Module IIB

Load restricted plug slices on comb and cast PFGE gel
Demonstrate PFGE equipment

Laboratory Module IIC, IID

Begin electrophoresis of PFGE gels

Break/Open Discussion

Day 3

Laboratory Module III

Begin staining the agarose gels with Ethidium Bromide
Demonstrate care of electrophoresis equipment after run is over

Begin de-staining gels

Troubleshooting gels when there are no bands (include thiourea and treatment of electrophoresis chamber with bleach)

Question/answer session about lab portion of PFGE

Demonstrate of **Gel Doc 2000**

Capture and save images of gels using the **Gel Doc 2000**

1. **PURPOSE:** To describe the standardized protocol for molecular subtyping of *Yersinia pestis* by Pulsed-field Gel Electrophoresis (PFGE).

2. **SCOPE:** To provide the PulseNet participants with the same procedure for performing PFGE of *Yersinia pestis* thus ensuring interlaboratory comparability of the generated results.

3. **DEFINITIONS/TERMS:**

   3.1 PFGE: Pulsed-field Gel Electrophoresis
   3.2 DNA: Deoxyribonucleic acid
   3.3 CDC: Centers for Disease Control and Prevention
   3.4 CLRW: Clinical Laboratory Reagent Water

4. **RESPONSIBILITIES/PROCEDURE:**

PREPARATION OF PFGE PLUGS FROM AGAR CULTURES

BIOSAFETY WARNING: *Yersinia pestis* is a human pathogen and can cause serious disease. Check with your instituation's select agent policy before handling these isolates. Biosafety Level 2 or 3 (depending on your instiution) are required when handling this agent. Always use extreme caution when transferring and handling strains in general. Work in a biological safety cabinet when handling large amounts of cells. Disinfect or dispose of all plasticware and glassware that come in contact with the cultures in a safe manner.

Please read all instructions carefully before starting protocol. Treat all plasticware, glassware, pipets, spatulas, etc. that come in contact with the cell suspensions or plugs as contaminated materials and dispose of, or disinfect according to the guidelines of your institution. Disinfect reusable plug molds before they are washed; the disposable plug molds, including the tape and the tab that is used to push the plugs out of the wells, are also contaminated and should be disinfected with 10% bleach for at least 30 minutes if they will be washed and reused.

Day 0

Streak an isolated colony from test cultures to a Blood agar plate fortified with 6% sheep blood (or comparable media) for confluent growth. Incubate cultures at 28°C (room temp) or 37°C for 48 h. If additional diagnostic tests for the presence of F1 antigen are to be done on the isolate, cultures need to be incubated at 37°C for the F1 antigen to be expressed.

Day 1

1. **Turn on shaker water bath (54ºC), stationary water baths (55-60ºC) and spectrophotometer** (or equivalent instrument such as the Dade Microscan Turbidity meter or bioMérieux Vitek colorimeter).

2. Prepare **TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0)**[1] as follows:

   10 ml of 1 M Tris, pH 8.0
   2 ml of 0.5 M EDTA, pH 8.0
   Dilute to 1000 ml with sterile Ultrapure Clinical Laboratory Reagent Water (CLRW)

[1]Additional information is found in the PulseNet PFGE Manual.

Note: The TE Buffer is used to make the plug agarose and also to wash lysed PFGE plugs.

3. Prepare 1% SeaKem Gold:1% SDS agarose in **TE Buffer** (**10 mM Tris:1 mM EDTA, pH 8.0**) for PFGE plugs as follows:

    a. Weigh 0.50 g (or 0.25 g) SeaKem Gold (SKG) into 250 ml screw-cap flask.
    b. Add 47.0 ml (or 23.5 ml) TE Buffer; swirl gently to disperse agarose.
    c. Remove cap, cover loosely with clear film, and microwave for 30-sec; mix gently and repeat for 10-sec intervals until agarose is completely dissolved. Place flask in 55-60ºC water bath for 5 minutes before adding SDS.
    d. Add 2.5 ml (or 1.25 ml) of 20% SDS (pre-heated to 55ºC) and mix well.
    e. Recap flask and return to 55- 60ºC water bath until ready to use.

SAFETY WARNING: Use heat-resistant gloves when handling hot flasks after microwaving.

Note: SeaKem Gold agarose works well for making PFGE plugs because it provides added strength to the plugs that are cast in reusable plug molds, minimizing breakage of plugs during the lysis and washing steps. The time and temperature needed to completely dissolve the agarose is dependent on the specifications of the microwave used, and will have to be determined empirically in each laboratory.

4. Label small tubes (12-mm x 75-mm Falcon tubes or equivalent) with culture numbers.

5. Prepare **Cell Suspension Buffer** (**100 mM Tris:100 mM EDTA, pH 8.0**) as follows:

    10 ml of 1 M Tris, pH 8.0
    20 ml of 0.5 M EDTA, pH 8.0
    Dilute to 100 ml with sterile Ultrapure water (CLRW)

6. Transfer ≈2 ml of **Cell Suspension Buffer** (CSB) to small labeled tubes. Use a sterile polyester-fiber or cotton swab that has been moistened with sterile CSB to remove some of the growth from agar plate; suspend cells in CSB by spinning swab gently so cells will be evenly dispersed and formation of aerosols is minimized.

Note: The minimum volume of the cell suspension needed will depend on size of the cuvettes or tubes used to measure the cell concentration and are dependent on the manufacturer's specifications for the spectrophotometer, turbidity meter, or colorimeter. Keep suspensions on ice if you have more than 6 cultures to process or refrigerate cell suspensions if you cannot adjust their concentration immediately.

7. Adjust concentration of cell suspensions to one of values given below by diluting with sterile CSB or by adding additional cells.

    a. Spectrophotometer: 610 nm wavelength, absorbance (Optical Density) of **1.35** (range of 1.3-1.4)

    b. Dade Microscan Turbidity Meter: **0.48 - 0.53** (measured in Falcon 2054 tubes)
    0.68 - 0.72 (measured in Falcon 2057 tubes)

    c. bioMérieux Vitek colorimeter: **20%** transmittance (measured in Falcon 2054 tubes)

Note: Cell suspensions need to be at room temperature when concentration is checked. The values in Steps 7a. and 7b. give satisfactory results at CDC; if different instruments or tubes are used, each laboratory may need to establish the concentration needed for satisfactory results.

CASTING PLUGS

Label wells of PFGE plug molds with culture number. When reusable plug molds are used, put strip of tape on lower part of reusable plug mold before labeling wells.

Note 1: Unused plug agarose can be kept at room temperature and reused 1-2 times. Microwave on low-medium power for 10 -15 sec and mix; repeat for 5 -10 sec intervals until agarose is completely melted. This agarose melts rapidly!

Note 2: Proteinase K solutions (20 mg/ml) are available commercially, or a stock solution of Proteinase K can be prepared from the powder in sterile Ultrapure water (CLRW), aliquoted in 300-500 µl amounts, and kept frozen. Just before use, thaw appropriate number of vials needed for the samples; keep Proteinase K solutions on ice. Discard any thawed Proteinase K stock solution that was prepared from powder by the user at end of work day. Store commercially prepared Proteinase K solutions according to directions provided by the supplier.

1. Transfer 400 µl (0.4 ml) adjusted cell suspensions to labeled 1.5-ml microcentrifuge tubes. If cell suspensions are at room temperature, agarose can be added directly without pre-warming cell suspensions. If cell suspensions are cold, place tubes containing cell suspensions in plastic holders (floats); incubate in a 37ºC water bath for a few minutes.

2. Add 20 µl of Proteinase K (20 mg/ml stock) to each tube and mix gently with pipet tip. (200 µl are needed for 10 cell suspensions.)

3. Add 400 µl (0.4 ml) melted 1% SeaKem Gold:1% SDS agarose to the 0.4-ml cell suspension; mix by gently pipetting mixture up and down a few times. Maintain temperature of melted agarose by keeping flask in beaker of warm water (55-60ºC).

4. Immediately, dispense part of mixture into appropriate well(s) of disposible plug mold.* Do not allow bubbles to form. Two plugs of each sample can be made from these amounts of cell suspension and agarose. Allow plugs to solidify at room temperature for 10-15 min. They can also be placed in the refrigerator (4ºC) for 5 minutes.

***** Disposible plug molds are recommended for BSL-3 work as the plug molds can be discarded easily.
Note: If disposable plug molds are used for making plugs with 1% SeaKem Gold:1% SDS agarose, use 200 µl cell suspension, 10 µl of Proteinase K (20 mg/ml stock) and 200 µl of agarose; up to 4 plugs can be made from these amounts of cell suspension and agarose.

LYSIS OF CELLS IN AGAROSE PLUGS

Note: Two plugs (reusable plug molds) or 3 - 4 plugs (disposable plug molds) of the same strain can be lysed in the same 50-ml tube.

1. Label 50-ml polypropylene screw-cap or 50-ml Oak Ridge tubes with culture numbers.

2. Prepare **Cell Lysis Buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl)** as follows:
   25 ml of 1 M Tris, pH 8.0
   50 ml of 0.5 M EDTA, pH 8.0

50 ml of 10 % Sarcosyl (N-Lauroylsarcosine, Sodium salt)[2]
Dilute to 500 ml with sterile Ultrapure water (CLRW)

3. Calculate the total volume of **Cell Lysis/Proteinase K Buffer** needed as follows:

   a. 5 ml **Cell Lysis Buffer** (**50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl**) is needed per tube (e. g., 5 ml x 10 tubes = 50 ml).

   b. 25 µl Proteinase K stock solution (20 mg/ml) is needed per tube of the cell lysis buffer (e. g., 25 µl x 10 tubes = 250 µl).

   c. Measure correct volumes into appropriate size test tube or flask and mix well.

Note: The final concentration of Proteinase K in the lysis buffer is 0.1 mg/ml, and is different from the concentration that was added to the cell suspension (0.5 mg/ml).

4. Add 5 ml of Proteinase K/Cell Lysis Buffer to each labeled 50 ml tube.

5. Trim excess agarose from top of plugs with scalpel or razor blade (optional). Open reusable plug mold and transfer plugs from mold with a 6-mm wide spatula to appropriately labeled tube.
   If disposable plug molds are used, remove white tape from bottom of mold and push out plug(s) into appropriately labeled tube. Be sure plugs are under buffer and not on side of tube.

Note: The excess agarose, plug mold, spatula, etc. are contaminated. Discard or disinfect appropriately.

6. **Remove tape from reusable mold.** Place both sections of plug mold, spatulas, and scalpel in 70% isopropanol (IPA) or other suitable disinfectant. **Soak them for 15 minutes before washing them.** Discard disposable plug molds or disinfect them in 10% bleach for 30-60 minutes if they will be washed and reused.

7. Place tubes in rack and incubate in a 54ºC shaker water bath for 2 h with constant and vigorous agitation (175-200 rpm). Be sure water level in water bath is above level of lysis buffer in tubes.

8. Pre-heat enough sterile Ultrapure water (CLRW) to 50ºC so that plugs can be washed two times with 10-15 ml water (200-250 ml for 10 tubes).

WASHING OF AGAROSE PLUGS AFTER CELL LYSIS

Lower the temperature of the shaker water bath to 50ºC.

1. Remove tubes from water bath, and carefully pour off lysis buffer into an appropriate discard container; plugs can be held in tubes with a screened cap or spatula.

Note: It is important to remove all of the liquid during this and subsequent wash steps by touching edge of tube or screened cap on an absorbent paper towel.

[2]The N-Lauroylsarcosine, Sodium salt can be added directly to the other ingredients and allowed to dissolve.

2. Add at 10-15 ml sterile Ultrapure water (CLRW) that has been pre-heated to 50ºC to each tube and shake the tubes vigorously in a 50ºC water bath for 10-15 min.

3. Pour off water from the plugs and repeat wash step with pre-heated water (Step 2) one more time.
   a. Pre-heat enough sterile **TE Buffer** (**10 mM Tris:1 mM EDTA, pH 8.0**) in a 50ºC water bath so that plugs can be washed four times with 10-15 ml TE (300-350 ml for 10 tubes) after beginning last water wash.

4. Pour off water, add 10-15 ml pre-heated (50ºC) sterile TE Buffer, and shake the tubes vigorously in 50ºC water bath for 10-15 min.

5. Pour off TE and repeat wash step with pre-heated TE three more times.

6. Decant last wash and add 5-10 ml sterile TE. Continue with step 1 in "Restriction Digestion" section or store plugs in TE Buffer at 4ºC until needed. Plugs can be transferred to smaller tubes for storage.

Note: If restriction digestion is to be done the same day, complete Steps 1-3 of next section (**RESTRICTION DIGESTION OF DNA IN AGAROSE PLUGS WITH *Asc*I & *Xba*I**) during last TE wash step for optimal use of time.

RESTRICTION DIGESTION OF DNA IN AGAROSE PLUGS WITH *Asc*I & *Xba*I

Note: A small slice of the plug or the entire plug (made in disposable plug molds) can be digested with the restriction enzyme. Restriction digestion of a small slice of the plug is recommended because less enzyme is required and other slices of the plug can be subjected to restriction analysis with other enzymes, such as *FseI*, etc. This is important when the PFGE patterns obtained with the primary enzyme from two or more isolates are indistinguishable, and confirmation is needed to determine that the PFGE patterns of these isolates are also indistinguishable with additional enzymes.

1. Label 1.5-ml microcentrifuge tubes with *Yersinia pestis* culture numbers; label 3 (10-well gel) or 4 (15-well gel) tubes for *Salmonella* ser. Braenderup H9812 standards.
   a. **Optional Pre-Restriction Incubation Step**: Dilute 10X H buffer (Roche Molecular Biochemicals or equivalent) and 10X Buffer 4 (New England Biolabs or equivalent) 1:10 with sterile Ultrapure water (CLRW) according to the following table.

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices
Sterile Clinical Laboratory Reagent Water (CLRW)	180 µl	1800 µl	2700 µl
H Buffer	20 µl	200 µl	300 µl
Total Volume	200 µl	2000 µl	3000 µl

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices
Sterile Clinical Laboratory Reagent Water (CLRW)	180 µl	1800 µl	2700 µl
Buffer 4	20 µl	200 µl	300 µl
Total Volume	200 µl	2000 µl	3000 µl

b. Add 200 µl diluted H buffer (1X) to *S.* ser. Braenderup labeled 1.5-ml microcentrifuge tubes.

c. Add 200 ul diluted buffer 4 (1X) to *Yersinia pestis* labeled 1.5 ml microcentriufge tubes.

d. Carefully remove plug from TE with spatula and place in a sterile disposable Petri dish or on large glass slide.

e. Cut a 2.0- to 2.5-mm-wide slice from test samples with a single edge razor blade (or scalpel, cover slip, etc.) and transfer to tube containing diluted buffer 4. Be sure plug slice is under buffer. Replace rest of plug in original tube that contains 5 ml TE buffer. Store at 4ºC.

Note: The shape and size of the plug slice that is cut will depend on the size of the comb teeth that are used for casting the gel. PulseNet recommends that the combs with larger teeth (10-mm-wide teeth) be used to cast the gels because computer analysis of the gel lanes is more accurate and less tedious than analysis of gel lanes cast with combs with the smaller teeth (5.5-mm). The number of slices that can be cut from the plugs will depend on the skill and experience of the operator, integrity of the plug, and whether the slices are cut vertically or horizontally (plugs made in disposable molds).

f. Cut three or four 2.0-mm-wide slices from plug of the *S.* ser. Braenderup H9812 standard and transfer to tubes with diluted H buffer. Be sure plug slices are under buffer. Replace rest of plug in original tube that contains 5 ml TE buffer. Store at 4ºC.

g. Incubate sample and control plug slices in 37ºC water bath for 5-10 min or at room temperature for 10-15 min.

h. After incubation, remove buffer from plug slice using a pipet fitted with 200-250 µl tip all the way to bottom of tube and aspirate buffer. Be careful not to cut plug slice with pipet tip and that plug slice is not discarded with pipet tip.

2. Dilute 10X H buffer 1:10 with sterile Ultrapure water (CLRW) and add restriction enzyme[3] (50 U/sample for *S. ser.* Braenderup and 40U/sample for *Y. pestis*) according to the following table. Mix in the same tube that was used for the diluted H buffer.

Reagent	µl/Plug Slice	Ml/10 Plug Slices	µl/15 Plug Slices
Sterile Clinical Laboratory Reagent Water (CLRW)	175 µl	1750 µl	2625 µl
H Buffer	20 µl	200 µl	300 µl
Enzyme *XbaI* (10 U/µl)	5 µl	50 µl	75 µl
Total Volume	200 µl	2000 µl	3000 µl

Reagent	µl/Plug Slice	Ml/10 Plug Slices	µl/15 Plug Slices
Sterile Clinical Laboratory Reagent Water (CLRW)	174 µl	1740 µl	2610 µl
Buffer 4	20 µl	200 µl	300 µl
Bovine Serum Albumin (BSA)	2 ul	20 ul	30 ul
Enzyme *AscI* (10 U/µl)	4 µl	40 µl	60 µl
Total Volume	200 µl	2000 µl	3000 µl

Note: Keep vial of restriction enzyme on ice or in insulated storage box (-20˚C) at all times.

3. Add 200 µl restriction enzyme mixture to each tube. Close tube and mix by tapping gently; be sure plug slices are under enzyme mixture.

4. Incubate sample and control plug slices in 37˚C water bath for 4 h.

5. If plug slices will be loaded into the wells, continue with Steps 1-4 of the next section (**CASTING AGAROSE GEL**) approximately 1 h before restriction digest reaction is finished so the gel can solidify for at least 30 minutes before loading the restricted PFGE plugs.

CASTING AGAROSE GEL

A. Loading Restricted Plug Slices on the Comb:

1. **Confirm that water bath is equilibrated to 55- 60ºC.**

2. Make volume of 0.5X Tris-Borate EDTA Buffer (TBE) that is needed for both the gel and electrophoresis running buffer according to one of the following tables.

5X TBE:

Reagent	Volume in milliliters (ml)					
5X TBE	200	210	220	230	240	250
Sterile Clinical Laboratory Reagent Water (CLRW)	1800	1890	1980	2070	2160	2250
Total Volume of 0.5X TBE	2000	2100	2200	2300	2400	2500

10X TBE:

Reagent	Volume in milliliters (ml)					
10X TBE	100	105	110	115	120	125
Sterile Clinical Laboratory Reagent Water (CLRW)	1900	1995	2090	2185	2280	2375
Total Volume of 0.5X TBE	2000	2100	2200	2300	2400	2500

3. Make 1% SeaKem Gold (SKG) Agarose in 0.5X TBE as follows:

   a. Weigh appropriate amount of SKG into 500 ml screw-cap flask.
   b. Add appropriate amount of 0.5X TBE; swirl gently to disperse agarose.
   c. Remove cap, cover loosely with clear film, and microwave for 60-sec; mix gently and repeat for 15-sec intervals until agarose is completely dissolved.
   d. Recap flask and place in 55-60ºC water bath.

   Mix 1.0 g agarose with 100 ml 0.5X TBE for 14-cm-wide gel form (10 wells)
   Mix 1.5 g agarose with 150 ml 0.5X TBE for 21-cm-wide gel form (≥15 wells)

SAFETY WARNING: Use heat-resistant gloves when handling hot flasks after microwaving.

4. A small volume (2-5 ml) of melted and cooled (50-60ºC) 1% SKG 1% SKG agarose may be wanted to seal wells after plugs are loaded. Prepare 50 ml by melting 0.5 g agarose with 50 ml 0.5X TBE in 250 ml screw-cap flask as described above. Unused SKG agarose can be kept at room temperature, melted, and reused several times. Microwave for 15-20 sec and mix; repeat for 10-sec intervals until agarose is completely melted. Place in 55-60ºC water bath until ready to use. Alternatively, save approximately 5 ml of the melted agarose used to cast the gel in a pre-heated (55-60ºC) 50 ml flask and place in 55-60ºC water bath until used.

Note: Confirm that gel form is level on leveling table, that **front** of comb holder and teeth face the bottom of gel, and that the comb teeth touch the gel platform.

5. Remove restricted plug slices from 37ºC water bath. Remove enzyme/buffer mixture and add 200 µl 0.5X TBE. Incubate at room temperature for 5 min.

6. Remove plug slices from tubes; put comb on bench top and load plug slices on the bottom of the comb teeth as follows:

a. Load *S.* ser. Braenderup H9812 standards on teeth (lanes) 1, 5, 10 (10-well gel) or on teeth 1, 6, 10, 15 (15-well gel)
b. Load samples on remaining teeth

7. Remove excess buffer with tissue. Allow plug slices to air dry on the comb for ≈5 minutes or seal them to the comb with 1% SKG agarose (55-60ºC).

8. Position comb in gel form and confirm that the plugs slices are correctly aligned on the bottom of the comb teeth, that the lower edge of the plug slice is flush against the black platform, and there are no bubbles (if allowed to air dry).

9. Carefully pour the agarose (cooled to 55-60ºC) into the gel form.

10. Put black gel frame in electrophoresis chamber. Add 2 -2.2 L freshly prepared 0.5X TBE. Close cover of unit. (The amount of buffer needed depends on whether residual buffer was left in tubing or if unit was flushed with water after the last gel was run.)

11. Turn on power supply to CHEF Mapper, pump (setting of ≈70 for a flow of 1 liter/minute) and cooling module (14°C).

12. Remove comb after gel solidifies for 20 minutes.

13. Fill in wells of gel with melted and cooled (55- 60ºC) 1% SKG Agarose (optional). Unscrew and remove end gates from gel form; remove excess agarose from sides and bottom of casting platform with a tissue. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber. Close cover of chamber.

B. Loading Restricted Plug Slices into the Wells:

1. Follow steps 1-4 in Option A on pages 7 and 8 (**Loading Restricted Plug Slices on the Comb**).

Note: Confirm that gel form is level on gel-leveling table before pouring gel, that front of comb holder and teeth face bottom of gel, and the bottom of the comb is 2 -mm above the surface of the gel platform.

2. Cool melted SKG agarose in 55-60ºC water bath for 15-20 min; carefully pour agarose into gel form (casting stand) fitted with comb. Be sure there are no bubbles.

3. Put black gel frame in electrophoresis chamber. Add 2-2.2 L freshly prepared 0.5X TBE. Close cover of unit. (The amount of buffer depends on whether residual buffer was left in tubing, or if unit was flushed with water after the last gel was run.)

4. Turn on power supply to CHEF Mapper, pump (setting of ≈70 for a flow of 1 liter/minute) and cooling module (14°C).

5. Remove restricted plug slices from 37ºC water bath. Remove enzyme/buffer mixture and add 200 µl 0.5X TBE. Incubate at room temperature for 5 minutes.

6. Remove comb after gel solidifies for at least 20 minutes.

7. Remove restricted plug slices from tubes with tapered end of spatula and load into appropriate wells. Gently push plugs to bottom and front of wells with wide end of spatula. Manipulate position with spatula and be sure that are no bubbles.

   a. Load *S.* ser. Braenderup H9812 standards in wells (lanes) 1, 5, 10 (10-well gel) or in wells 1, 6, 10, 15 (15-well gel).

   b. Load samples in remaining wells.

Note: Loading the plug slices can be tedious; each person has to develop his/her own technique for consistently placing the plug slices in the wells so the lanes will be straight and the bands sharp.

8. Fill in wells of gel with melted 1% SKG Agarose (equilibrated to 55- 60ºC). Allow to harden for 3-5 min. Unscrew and remove end gates from gel form; remove excess agarose from sides and bottom of casting platform with a tissue. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber. Close cover of chamber.

ELECTROPHORESIS CONDITIONS

1a. Select following conditions on Chef Mapper for *Yersinia pestis* restricted with *AscI*
Auto Algorithm
25 kb - low MW
215 kb - high MW
Select default values except where noted by pressing "enter".
Change run time to 17:30 h (See note below)
(Default values: Initial switch time = 1.79 s; Final switch time = 18.66 s)
linear ramping factor

1b. Select following conditions on CHEF DR II or III.
Initial A time: 1.79 s
Final A time: 18.66 s
Start ratio: 1.0
Voltage: 200 V
Run time: 20-22 h (DR II); 18-20 h (DR III)

Note: The electrophoresis running times recommended above are based on the equipment and reagents used at the CDC. Run times may be different in your laboratory and will have to be optimized for your gels so that the lowest band in the *S.* ser. Braenderup H9812 standard migrates 1.0 - 1.5 cm from the bottom of the gel.

Day 2

STAINING AND DOCUMENTATION OF PFGE AGAROSE GEL

1. When electrophoresis run is over, turn off equipment (cooling module **FIRST** then pump); remove and stain gel with ethidium bromide. Dilute 40 µl of ethidium bromide stock solution (10 mg/ml) with 400 ml of Ultrapure water (CLRW) (this volume is for a staining box that is approximately 14-cm x 24-cm; a larger container may require a larger amount of staining solution). Stain gel for 20 - 30 min in covered container.

Note: Ethidium bromide is toxic and a mutagen; the solution can be kept in dark bottle and reused 4 - 5 times before discarding according to your institution's guidelines for hazardous waste or use the destaining bags recommended for disposal of ethidium bromide (**Section 10**).

2. Destain gel in approximately 500 ml Ultrapure water (CLRW) for 60 - 90 min; change water every 20 minutes. Capture image on Gel Doc 1000, Gel Doc 2000, or equivalent documentation system. If background interferes with resolution, destain for an additional 30-60 min.

Note: If both a digital image and conventional photograph are wanted, photograph gel first before capturing digital image.

3. Follow directions given with the imaging equipment to save gel image as an ***.img** or ***.1sc** file; convert this file to ***.tif** file for analysis with the BioNumerics software program (Additional information is in **Section 11** of the PFGE Manual).

4. Drain buffer from electrophoresis chamber and discard. Rinse chamber with 2 L Ultrapure water (CLRW) or, if unit is not going to be used for several days, flush lines with water by letting pump run for 5-10 min before draining water from chamber.

Please note the following if PFGE results do not have to be available within 24-28 hours:

1. Plugs can be lysed for longer periods of time (3-16 hours).

2. The washing steps with TE to remove the lysis buffer from the PFGE plugs can be done for longer periods of time (30-45 min) and at lower temperatures (37°C or room temperature). They can be started on Day 1 and finished on Day 2 after overnight refrigeration of the plugs in TE.

3. The restriction digestion can be done for longer periods of time (3-16 hours).

4. If the lowest band in the H9812 standard does not migrate within 1 -1.5 cm of the bottom of the gel, the run time will need to be determined empirically for the conditions in each laboratory.

Use of trade names and commercial sources is for identification purposes only and does not imply endorsement by CDC or the U.S. Department of Health and Human Services.

NOTE: CLIA LABORATORY PROCEDURE MANUAL REQUIREMENTS

Efforts have been made to assure that the procedures described in this protocol have been written in accordance with the 1988 Clinical Laboratory Improvement Amendments (CLIA) requirements for a procedure manual (42 CFR 493.1211). However, due to the format required for training, the procedures will require some modifications and additions to customize them for your particular laboratory operation.

Any questions regarding the CLIA requirements for a procedure manual, quality control, quality assurance, etc., should be directed to the agency or accreditation organization responsible for performing your laboratory's CLIA inspection. In addition, some states and accreditation organizations may have more stringent requirements that will need to be addressed.

Formulas of Selected Reagents used in PulseNet Standardized Laboratory Protocol for PFGE

Tris:EDTA Buffer, pH 8.0 (TE, 10 mM Tris:1 mM EDTA, pH 8.0)[4]

10 ml of 1 M Tris, pH 8.0
2 ml of 0.5 M EDTA, pH 8.0
Dilute to 1000 ml with sterile Ultrapure water (CLRW)

Cell Suspension Buffer (100 mM Tris:100 mM EDTA, pH 8.0)

10 ml of 1 M Tris, pH 8.0
20 ml of 0.5 M EDTA, pH 8.0
Dilute to 100 ml with sterile Ultrapure water (CLRW)

Cell Lysis Buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosine + 0.1 mg/ml Proteinase K)

25 ml (50 ml) of 1 M Tris, pH 8.0
50 ml (100 ml) of 0.5 M EDTA, pH 8.0
50 ml (100 ml) 10% N-Lauroylsarcosine, Sodium salt (Sarcosyl)
or
5 g (10 g) of N-Lauroylsarcosine, Sodium salt (Sarcosyl)[5]
Dilute to 500 ml (1000 ml) with sterile Ultrapure water (CLRW)

Add 25 µl Proteinase K stock solution (20 mg/ml) per 5 ml of cell lysis buffer just before use for a final concentration in the lysis buffer of 0.1 mg/ml Proteinase K.

Additional enzyme for *Y pestis* PFGE

Use the following calculations for *Fse*I (40 Units/plug slice):

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices
Sterile Clinical Laboratory Reagent Water (CLRW)	163 µl	1630 µl	2445 µl
Buffer 4	20 µl	200 µl	300 µl
BSA	2 µl	20 µl	30 µl
Enzyme *FseI* (10 U/µl)	15 µl	150 µl	225 µl
Total Volume	200 µl	2000 µl	3000 µl

[4]This formula for TE is from Molecular Cloning - A Laboratory Manual by J. Sambrook and E. Russell, 3rd edition. TE Buffer from Life Technologies (CP0558; 0126A) used at CDC is 0.01M (10 mM) for both ingredients. To duplicate this commercial formula, increase the amount of 0.5 M EDTA to 20 ml per liter.

[5]If Sarcosyl powder is added directly to the other components of this reagent, warm the solution to 50- 60ºC for 30-60 minutes, or leave at room temperature for ≈2 hours to completely dissolve the Sarcosyl; adjust to the final volume with sterile Ultrapure Water.

Note: Keep vial of restriction enzyme on ice or in insulated storage box (-70ºC) during storage. Keep *Fse*I on ice or in an insulated storage box at all times when in use.

ELECTROPHORESIS CONDITIONS

1a. Select following conditions on Chef Mapper for *Yersinia pestis* restricted with *FseI*
Auto Algorithm
30 kb - low MW
286 kb - high MW
Select default values except where noted by pressing "enter".
Change run time to 17:30 h (See note below)
(Default values: Initial switch time = 2.16 s; Final switch time = 25.0 s)
linear ramping factor

1b. Select following conditions on CHEF DR II or III.
Initial A time: 2.16 s
Final A time: 25.0 s
Start ratio: 1.0
Voltage: 200 V
Run time: 20-22 h (DR II); 18-20 h (DR III)

5. FLOW CHART:

6. BIBLIOGRAPHY:

7. CONTACTS:

8. AMENDMENTS:

8.1 The phrase “Type I Water” has been changed to “Ultrapure Clinical Laboratory Reagent Water (CLRW).” The water composition is the same, but this reflects a change in the terminology used by the Clinical Laboratory Standards Institute (CLSI).

1. **PURPOSE:** to describe the standardized laboratory protocol for molecular subtyping of Shiga toxin-producing *Escherichia coli* O157 (STEC O157) and *Salmonella enterica* serotypes Typhimurium and Enteritidis.

2. **SCOPE**: to provide the PulseNet participants with a single protocol for performing MLVA of STEC O157 and *Salmonella* serotypes Typhimurium and Enteritidis, thus ensuring inter-laboratory comparability of the generated results using the Beckman Coulter CEQ 8000/8800/GeXP platform.

3. **DEFINITIONS:**
   - **3.1.** MLVA: Multiple-locus variable-number tandem repeat analysis
   - **3.2.** VNTR: Variable-number tandem repeat
   - **3.3.** DNA: Deoxyribonucleic acid
   - **3.4.** DNase: Deoxyribonuclenase
   - **3.5.** PCR: Polymerase chain reaction
   - **3.6.** HPLC: High purity liquid chromatography
   - **3.7.** dNTP: Deoxyribonucleotide triphosphate
   - **3.8.** CDC: Centers for Disease Control and Prevention

4. **RESPONSIBILITIES/PROCEDURE**
   - **4.1. Biosafety warning**: STEC O157 and *Salmonella* serotypes Typhimurium and Enteritidis with an infectious dose as low as 100 cells are human pathogens capable of causing serious disease. Always use a minimum of Biosafety level 2 practices and extreme caution when transferring and handling strains of these serotypes. Work in a biological safety cabinet when handling large amounts of cells. Disinfect or dispose of all plastic ware and glassware that come in contact with the cultures in a safe manner.
   - **4.2. Reagents, supplies and equipment needed for DNA template preparation**
     - 4.2.1. Trypticase soy agar with 5 % sheep blood (TSA-SB) or comparable media
     - 4.2.2. 1 µl inoculation loops
     - 4.2.3. 0.5 ml microcentrifuge tubes
     - 4.2.4. DNase-free, molecular biology -grade water
     - 4.2.5. Vortex
     - 4.2.6. Boiling water bath or thermal block / thermocycler accommodating 0.5 ml tubes
     - 4.2.7. Tabletop centrifuge for high rpm (up to 13,000-14,000 rpm) spinning
   - **4.3. Reagents, supplies and equipment needed for PCR**
     - 4.3.1. DNA templates from isolates (store at -20°C or -80°C freezer for long term)
     - 4.3.2. PCR primers (see appendix PNL19-1)
       - 4.3.2.1. Fluorescent-labeled forward primers
         - 4.3.2.1.1. HPLC-purified
       - 4.3.2.2. Unlabeled reverse primers
         - 4.3.2.2.1. Regular gel filtration purification
       - 4.3.2.3. Integrated Technologies (Skokie, IL; www.idtdna.com, 1-800-328-2661) and Proligo (Boulder, CO; www.proligo.com, 1-800-234-5362) are currently the suppliers for the Wellred dye-labeled (D2)

primers recommended by Beckman Coulter. Biosearch Technologies (Novato, CA; www.biosearchtech.com; 1-800-436-6631) is the recommended supplier for the Quasar dye –labeled (Quas670 and 705) primers.

4.3.2.4. Divide the concentrated stocks (100 µM) in portions and store at -80ºC freezer

4.3.2.4.1. One vial should contain enough to prepare 25-50 µl of working solution. Avoid repetitive freeze-thaw cycles of concentrated primer stocks.

4.3.2.5. The 1, 2.5, 5, 12.5 and 25 µM working solutions can be stored at either -20ºC or -80ºC freezer

4.3.2.6. Prepare new working solutions every month or if a significant drop in the fluorescence level is observed (for instructions refer to PNQ05_MLVA CEQ certification, appendix PNQ05-5)

4.3.3. 96-well polypropylene PCR plates (Fisher, Cat. No. 07-200-613) or Microamp PCR tubes without caps (Life Technologies, Cat. No. N8010533)

4.3.4. 8-well strip caps for polypropylene plate (Fisher, Cat. No. 07-200-639) or MicroAmp strip caps for individual tubes (Life Technologies, Cat. No. N8010535)

4.3.5. DNase-free, molecular biology -grade water

4.3.6. 1.5 ml Eppendorf microcentrifuge tubes

4.3.7. PCR Nucleotide mix (ready-to-use dNTP mix containing all four nucleotides; Roche, Cat. No. 11 814 362 001)

4.3.8. Platinum Taq Polymerase with 50 mM $MgCl_2$ and 10X buffer (Life Technologies, Cat. No. 10966-034)

4.3.9. PCR cooling block (VWR International, Cat. No. 62111-762)

4.3.10. DNA Engine (Biorad), GeneAmp (Life Techologies) or similar thermocycler with a heated lid option and 96-well block format

4.3.11. Parafilm M, 4" width (VWR, Cat. No. 52858-032)

4.3.12. Complete set (1000 µl, 200 µl, 100 µl, 20 µ, 10 µl, and 2 µl) of single channel pipettors for mastermix set-up ("clean set")

4.3.13. A 1-10 µl single channel pipettor for adding DNA templates

4.3.14. Filtered tips for pipettors

4.3.15. Microfuge for low (up to 6,000 rpm) rpm spinning

4.4. Reagents, supplies and equipment needed for Beckman CEQ™ 8000/ 8800/GeXP)

4.4.1. DNase-free, molecular biology -grade water

4.4.2. PCR cooling block (VWR International, Cat. No. 62111-762)

4.4.3. 10 µl, 100 µl, and 1000 µl single channel pipettors

4.4.4. 1-10 µl and 20-200 µl multichannel pipettors

4.4.5. Filtered pipette tips

4.4.6. Sterile solution basins

4.4.7. 1.5 ml Eppendorf microcentrifuge tubes

4.4.8. 5 ml polystyrene round bottom tubes (for example Falcon tube 352058)

4.4.9. 96-well polypropylene (non-PCR) V-bottom plate (for dilutions; Fisher Scientific, Cat. No. 07-200-698)
4.4.10. 96-well Beckman sample plates (Beckman Coulter, Cat. No. 609801; alternative: Fisher Scientific, Cat. No. 07-200-613)
4.4.11. 96-well Beckman CEQ buffer plate (Beckman Coulter, Cat. No. 609844; alternative: Fisher Scientific, Cat. No. 07-200-98)
4.4.12. Mineral oil (molecular grade) (Sigma, Cat. No. M5904)
4.4.13. GenomeLab™ Sample Loading Solution (Beckman Coulter, Cat. No. 608082)
4.4.13.1. After first thawing, divide in portions of 1.0 ml / vial in order to avoid repeated freeze-thaw cycles
4.4.14. GenomeLab™ DNA size standard kit 600 bp (Beckman Coulter, Cat. No. 608095)
4.4.14.1. After first thawing, divide in portions of 8 µl / vial in order to avoid repeated freeze-thaw cycles
4.4.15. GenomeLab™ Fragment Analysis Test Sample (Beckman Coulter, Cat. No. 608105)
4.4.15.1. Needed only once to establish the system dye color spectra for the instrument
4.4.15.2. Useful also for troubleshooting
4.4.16. GenomeLab™ Separation Capillary Array (Beckman Coulter, Cat. No. 608087)
4.4.17. GenomeLab™ Separation Buffer, 4x30 ml (Beckman Coulter, Cat. No. 608012)
4.4.18. GenomeLab™ Separation Gel LPAI (Beckman Coulter, Cat. No. 608010 for CEQ 8000/single rail GeXP; Cat. No. 391438 for CEQ 8800/double rail GeXP)
4.4.19. Parafilm M, 4" width (VWR, Cat. No. 52858-032)
4.4.20. A rinse bottle containing distilled water
4.4.21. Centrifuge with a microtiter plate rotor
4.4.22. Microfuge for low (up to 6,000 rpm) rpm spinning

4.5. Isolate preparation

4.5.1. **Day 0**
4.5.1.1. Streak an isolated colony from pure test cultures to TSA-SB plate (or comparable media). Incubate cultures at 37ºC for 14-18 hrs.

4.5.2. **Day 1**
4.5.2.1. For each isolate to be typed, aliquot 100 µl of sterile, molecular biology-grade water into 0.5 ml microfuge tubes. Use a sterile, disposable 1 µl loop to pick 2-3 colonies (about half of a loopfull); rotate the loop in the microfuge tube to release the bacteria into the water. Cap and vortex for 10-15 seconds to disperse any clumps.
4.5.2.2. Place the tubes in a 99-100ºC water bath or heat block for 10-15 minutes. Cool briefly on ice or in fridge and centrifuge for 10 minutes at 10,000 rpm. Place on ice or in fridge while preparing PCR reactions. These DNA templates can be stored at -20ºC or -80ºC for several years.

4.6. PCR procedure

4.6.1. **Day 1**

4.6.1.1. Fill out, save with the run name, and print a MLVA Fragment Analysis CEQ Worksheet (see appendix PNL19-2) with appropriately labeled samples (a maximum of 43 isolates/plate; 40 unknowns + two positive controls and a negative control; two wells are reserved for the internal ladder; the first column of the plate is reserved for conditioning).

4.6.1.1.1. For each isolate, two wells must be labeled as follows: BNkeyR1 where "BNkey" represents the isolate-specific state laboratory identification number (be sure to use the exact same isolate ID that is used in the PFGE gels uploaded to the national database) and "R1" represents one of the two specific multiplex PCR reactions (R1, R2).

4.6.1.1.2. The CEQ™8000/ 8800/GeXP machine has an 8-capillary array and must always be run with a complete column (n=8 wells). For example, 54 wells (6.75 8-well columns) are required to test 26 samples (including controls). The remaining two wells for the seventh column must be filled and run with 20 µl of water or left-over sample loading solution (blanks).

4.6.1.1.3. Also save the MLVA Fragment Analysis CEQ Worksheet for the run on a flash drive (memory stick). That way you can easily import the sample plate set-up in the CEQ without having to re-type the sample keys.

4.6.1.2. Fill out, and print a PCR Mastermix Calculation Worksheet (see appendices PNL19-3a, PNL19-3b, PNL19-3c) by typing the number of isolates to be tested (plus 2-3 extra) in the PCR mastermix calculators labeled R1 and R2. This number is highlighted in red and is next to "number of samples to be analyzed". The mastermixes for reactions 1 and 2 (R1, R2) for one sample are as follows:

STEC O157:H7 (Appendix PNL19-3a)

R1	Volume (µl)	Final conc.
PCR water	5.16	
PCR buffer (10x)	1.00	1x
$MgCl_2$ (50 mM)	0.40	2.00 mM
dNTPs (10 mM)	0.20	0.20 mM
VNTR-3F (25 µM)	0.18	0.45 µM
VNTR-3R (25 µM)	0.18	0.45 µM
VNTR-34F (5 µM)	0.28	0.14 µM
VNTR-34R (5 µM)	0.28	0.14 µM
VNTR-9F (5 µM)	0.28	0.14 µM
VNTR-9R (5 µM)	0.28	0.14 µM
VNTR-25F (2.5 µM)	0.28	0.07µM

VNTR-25R (2.5 µM)	0.28	0.07 µM
Taq (5 U/µl)	0.20	1 U
	= 9.00	

R2	Volume (µl)	Final conc.
PCR water	5.96	
PCR buffer (10x)	1.00	1x
$MgCl_2$ (50 mM)	0.40	2.00 mM
dNTPs (10 mM)	0.20	0.20 mM
VNTR-17F (5 µM)	0.18	0.09 µM
VNTR-17R (5 µM)	0.18	0.09 µM
VNTR-19F (1 µM)	0.20	0.02µM
VNTR-19R (1 µM)	0.20	0.02 µM
VNTR-36F (1 µM)	0.12	0.012 µM
VNTR-36R (1 µM)	0.12	0.012 µM
VNTR-37F (2.5 µM)	0.12	0.03µM
VNTR-37R (2.5 µM)	0.12	0.03µM
Taq (5 U/µl)	0.20	1 U
	= 9.00	

Salmonella serotype Typhimurium (Appendix PNL19-3b)

R1	Volume (µl)	Final conc.
PCR water	2.45	
PCR buffer (10x)	1.00	1x
$MgCl_2$ (50 mM)	0.45	2.25 mM
dNTPs (10 mM)	0.20	0.20 mM
ST3-F (5 µM)	0.26	0.13 µM
ST3-R (5 µM)	0.26	0.13 µM
ST5-F (5 µM)	1.30	0.65 µM
ST5-R (5 µM)	1.30	0.65 µM
ST7-F (5 µM)	0.46	0.23 µM
ST7-R (5 µM)	0.46	0.23 µM
ST10-F (2.5 µM)	0.28	0.07 µM
ST10-R (2.5 µM)	0.28	0.07 µM
Taq (5 U/µl)	0.30	1.50 U
	= 9.00	

R2	Volume (µl)	Final conc.
PCR water	0.06	
PCR buffer (10x)	1.00	1x
$MgCl_2$ (50 mM)	0.32	1.60 mM
dNTPs (10 mM)	0.20	0.20 mM
ST2-F (5 µM)	1.60	0.80 µM

ST2-R (5 µM)	1.60	0.80 µM
ST6-F4 (5 µM)	0.22	0.11 µM
ST6-R2 (5 µM)	0.22	0.11 µM
ST8-F3 (5 µM)	1.74	0.87 µM
ST8-R2 (5 µM)	1.74	0.87 µM
Taq (5 U/µl)	0.30	1.50 U
	= 9.00	

<u>*Salmonella* serotype Enteritidis (Appendix PNL19-3c)</u>

<u>R1</u>	Volume (µl)	Final conc.
PCR water	4.41	
PCR buffer (10x)	1.00	1x
$MgCl_2$ (50 mM)	0.40	2.0 mM
dNTPs (10 mM)	0.20	0.20 mM
SE1-F (1.0 µM)	0.30	0.03 µM
SE1-R (1.0 µM)	0.30	0.03 µM
SE2-F (12.5 µM)	0.14	0.17 µM
SE2-R (12.5 µM)	0.14	0.17 µM
SE8-F (2.5 µM)	0.28	0.07 µM
SE8-R (2.5 µM)	0.28	0.07 µM
SE6-F (2.5 µM)	0.68	0.17 µM
SE6-R (2.5 µM)	0.68	0.17 µM
Taq (5 U/µl)	0.20	1.00 U
	= 9.00	

<u>R2</u>	Volume (µl)	Final conc.
PCR water	6.03	
PCR buffer (10x)	1.00	1x
$MgCl_2$ (50 mM)	0.40	2.00 mM
dNTPs (10 mM)	0.20	0.20 mM
SE5-F (2.5 µM)	0.32	0.08 µM
SE5-R (2.5 µM)	0.32	0.08 µM
SE3-F (12.5 µM)	0.14	0.18 µM
SE3-R (12.5 µM)	0.14	0.18 µM
SE9-F (2.5 µM)	0.12	0.03 µM
SE9-R (2.5 µM)	0.12	0.03 µM
Taq (5 U/µl)	0.20	1.00 U
	= 9.00	

4.6.1.2.1. NOTE: **NOTE:** these primer concentrations serve as a starting point. Since laboratory-specific factors, such as the age of the primer stocks, calibration status of the thermocyclers and pipettes, etc. affect amplification efficiency, each laboratory will have to re-optimize the primer concentrations for optimal

detection of all targets. However, any other parameters stated in the SOP should not be changed.

4.6.1.3. Thaw all reagents and supplies needed for PCR reactions and place on ice; keep primers light protected as much as possible.

4.6.1.3.1. PCR mastermixes should be set up in a clean hood that is dedicated just for this purpose and where no cultures or DNA are handled.

4.6.1.4. Prepare the two separate PCR mastermixes in 1.5 ml Eppendorf tubes following the instructions in the PCR Mastermix Calculation Worksheet (see appendix PNL19-3a-c) and place on ice. Add the mastermix components in the following order: water, 10x PCR buffer, Mg_2Cl, dNTPs, primers, and then finally Taq polymerase. Mix the reaction mixture by vortexing briefly.

4.6.1.4.1. Vortex all reagents except Taq polymerase before adding to the mastermix. Taq may be briefly centrifuged with low rpm, if necessary, to pull the enzyme down to the bottom of the tube.

4.6.1.5. Place a 96-well PCR plate or required number of PCR tubes in a PCR cooling block.

4.6.1.6. Dispense 9.0 µl of each mastermix into the appropriate columns of the 96-well polypropylene plate / PCR tubes as noted in the MLVA Fragment Analysis CEQ Worksheet (see appendix PNL19-2)

4.6.1.7. Add 1 µl of PCR water to each of the two different wells representing the negative controls of the two reactions.

4.6.1.8. Add 1.0 µl of DNA template to each of the two different wells representing the two PCR reactions for each isolate to be tested.

4.6.1.9. Add the positive controls (it is recommended to run the positive control in duplicate).

4.6.1.9.1. Use STEC O157 strain EDL933 (ATCC 43895) as a positive control. The internal ladder to be used will be comprised of pooled PCR products of the isolates EC04PN0139 and EC04PN0570 (see appendix PNL19-4 for instructions for ladder preparation).

4.6.1.9.2. Use *S. enterica* serotype Typhimurium strain LT2 (ATCC 29946) as a positive control. The internal ladder to be used will be comprised of pooled PCR products of the isolates CDC_2009K0825 and CDC_2009K0826 (see appendix PNL19-4 for instructions for ladder preparation).

4.6.1.9.3. Use *S. enterica* serotype Enteritidis strain K1891 (ATCC 25928) as a positive control. The internal ladder to be used will be comprised of pooled PCR products of the isolates H9560 and 2010K0017 (see appendix PNL19-4 for instructions for ladder preparation).

4.6.1.10. Cover all wells / tubes with 8-well strip caps and firmly clamp down to avoid any evaporation during PCR amplification.

4.6.1.10.1. Recommendation: briefly spin down the plate / tubes to remove any air bubbles.

4.6.1.11. Program and save the following two PCR cycling conditions:

STEC O157:H7 and *Salmonella* serotype Enteritidis

"O157-SEMLVA"

* 95ºC for 5 min	Step 1
* 94ºC for 20 sec	Step 2
* 65ºC for 20 sec	Step 3
* 72ºC for 20 sec	Step 4
* Go to step 2, 34x	Step 5
* 72ºC for 5 min	Step 6
* Indefinite hold at 4ºC	Step 7

Salmonella serotype Typhimurium

"STMLVA"

* 95ºC for 5 min	Step 1
* 94ºC for 20 sec	Step 2
* 63ºC for 20 sec	Step 3
* 72ºC for 20 sec	Step 4
* Go to step 2, 34x	Step 5
* 72ºC for 5 min	Step 6
* Indefinite hold at 4ºC	Step 7

4.6.1.11.1. Make sure to use the heated lid option on the PCR block and "calculated" or tube control (instead of block or probe control) as a temperature control method.

4.6.1.12. When the PCR is complete, store the amplification products light-protected at 4ºC until ready to run on CEQ/GeXP. If the fragment analysis is not performed the same day, the plate should be stored at -20ºC or -80ºC. The PCR products are stable for approximately one month, when stored frozen.

4.7. Initial setup of CEQ™8000/ 8800/GeXP instrument

4.7.1. **NOTE**: Steps 4.7.2.-4.7.8. only need to be performed before the very first run and every time a new working database is created.

4.7.2. In the main page of the CEQ software version 8.0 or 9.0 or the GeXP software version 10.2, click on the "Fragments" icon. Select "Analysis" in the bar at the top of the window to open a window called "Analysis Parameters".

4.7.3. Select "DefaultFragmentAnalysisParameters" in the top drop-down menu and click "Edit". In the appearing "Edit Fragment Analysis Parameters" window, select the tab "Analysis Method" and select "Size Standard 600" for size standard and "Quartic" for model. Do not make any other changes.

4.7.4. Click on "Save As" and name the analysis parameter as "MLVA600".

4.7.5. Go back to the main page of the CEQ program and click on the "Set Up" icon. Select "Create a New Sample Plate" and click "OK".

4.7.6. In the "Sample Setup" window, check to be sure that the methods "Condition" and "Frag-test" are present in the drop-down menu below each column. These should be the instrument default methods.

4.7.7. If they are not present or to create the frag-test_pause method, highlight any of the default methods and select "Method" tab from below. This will open a "Method" window. Click on the "Edit" button in this open window and put in the following parameters in the "CEQ Methods" table below.

CEQ Methods:

		Condition	Frag-test_pause
Capillary	Temperature	35	35
	Wait	0	Yes
Denature	Temperature	90	90
	Time	0	120 seconds
Pause	Time	NA	10 min
Inject	Voltage	2.0 kV	2.0 kV
	Time	30 seconds	15 seconds
Separation	Voltage	7.5 kV	6.0 kV
	Time	35 minutes	60 minutes
Pause		0 minutes	0 minutes

4.7.8. Be sure to save the new method with new file names. Each time a new CEQ database is generated, these methods are not copied. However, if these methods were in the machine as a default (Frag-test_pause is not), they are copied when a new database is created.

4.8. CEQ™8000/ 8800/GeXP instrument preparation before each run

4.8.1. **Day 1**

4.8.1.1. Make sure a capillary array is installed in the instrument. For installation, follow the instructions in the appendix PNL19-5.

4.8.1.1.1. If the instrument is used frequently, there is no need to remove the array after every run. Beckman Coulter guarantees the shelf life of the array for up to 30 days (or up to 100 runs) at room temperature. However, as long as the positive control and the internal ladder fall within the sizing range indicated in the appendices PNL19-2 and PNL19-4 and the fragment analysis failure rate is less than 5 %, the same capillary array can be used up to 45 to 50 days.

4.8.1.2. To setup the plate run on the CEQ\GeXP, click on the "Set Up" icon on the CEQ\GeXP main page and select "Create a New Sample Plate" and click "OK".

4.8.1.3. Using a USB flash drive, open the MLVA Fragment Analysis CEQ Worksheet (PNL19-2) saved for this plate run and copy only the 96-well format and paste it directly into the blank sample setup window.

4.8.1.4. Under the first column of the plate format, select the method "Condition" from the drop-down menu. Select the method "Frag-test_pause" for the columns containing the samples.

4.8.1.5. Highlight all columns containing samples. At the bottom of the screen, select the "Analysis" tab, check the box for "Perform analysis", and select the "MLVA600" parameter set from the drop-down menu.

4.8.1.5.1. **NOTE:** The export tab does not contain the correct format (CSV) and should be left unchecked.

4.8.1.6. Save the plate with a run name following the standardized PulseNet naming system: Use the unique identifier code that was assigned to your laboratory by PulseNet for the first two to four letters of the file name. The next two spaces indicate the year and the next four spaces indicate the month and the date the run was performed. For example GA070426 is a run made at the GA Public Health Laboratory on April 26th 2007. If several runs are performed the same day, separate the file names by using sequential numbers, for example GA070426-1, GA070426-2.

4.8.1.7. Close "Set Up" module.

4.8.1.8. Click on the "Run" icon in the main CEQ\GeXP window. The "Run Control – CEQ System" window will appear.

4.8.1.9. If the instrument does not have a gel cartridge installed, the "Instrument Warning Message" window will appear stating "Instrument Status: Loading Gel Cartridge". Click "OK".

4.8.1.10. If the instrument already has a gel cartridge installed, the above message does not appear. In this case, you can check the amount of gel available by clicking on the "Life" tab on the left side of the "Run Control – CEQ System" window. If a new gel cartridge needs to be installed, select "Release Gel Cartridge" from the "Replenish" drop-down menu or from the toolbar and wait for the CEQ\GeXP to display a green "Go" signal box.

4.8.1.10.1. **NOTE:** A full plate uses approximately 9.0 ml of gel.

4.8.1.11. Open the gel access door, pull out the yellow plug or the old gel cartridge and install the new gel cartridge. Select "Install Gel Cartridge" from the "Replenish" drop-down menu or from the tool bar. The "Install Gel Cartridge" dialog box will open. Type in the lot number for the gel, click on "Set To New" to zero out the time on the

instrument (if the cartridge is new) or type in the number of hours the gel cartridge (previously used) has spent in the instrument. For the GeXP, designate whether the gel cartridge is 'New' or 'Used' by selecting the corresponding radial button. If installing a previously used cartridge, enter the number of hours on instrument. When finished click on "Done".

4.8.1.12. Perform a manual purge by selecting "Manifold Purge" from the "Direct Control" drop-down menu or by clicking on the funnel on the direct control screen. A "Manifold Purge" dialog box will open. Set 0.1 ml as the purge volume and 2 as the number of purge cycles and click on "Purge".

4.8.1.12.1. **NOTE:** a manifold purge is only necessary when a new/used gel cartridge is inserted. If using a cartridge already installed in the instrument, a manifold purge is not necessary.

4.9. Fragment analysis sample preparation

4.9.1. **Day 1**

4.9.1.1. **NOTE:** The fragment analysis method is not organism specific therefore; STEC O157 and *Salmonella* serotypes Typhiumurium and Entertidis may be run on a single fragment analysis plate.

4.9.1.2. Thaw the CEQ sample loading solution (SLS), 600 bp DNA size standard and internal ladder (see appendix PNL19-4 for preparation instructions) and place on ice.

4.9.1.3. While the reagents are thawing, prepare a Beckman buffer plate by filling a required amount of wells about ¾ full (~250 µl) with CEQ Separation Buffer.

4.9.1.4. Prepare a 96-well V-bottom plate for diluting the PCR reactions. Using a 200 µl multichannel pipettor and a solution basin, dispense 59 µl of molecular-grade water in the required number of wells.

4.9.1.5. Remove the plate / tubes with the PCR reactions from the thermocycler. Briefly spin down the plate / tubes, if necessary. Use a 10 µl multichannel pipettor to transfer 1 µl of each PCR reaction directly across to the corresponding set of wells in the dilution plate. In order to avoid cross-contamination, remove the strip cap from just one column at a time and recap the column before opening the next one.

4.9.1.6. For the internal ladder, combine R1 and R2 PCR products from the four PCR reactions of both internal ladder isolates into one tube to end with a total of 40 µl. Mix well by pipetting up and down a few times and add 3 µl of internal ladder in two wells.

4.9.1.7. Using a 200 µl multichannel pipettor, mix the dilutions by pipetting up and down a few times. Cover the plate with Parafilm and put in the fridge or on ice.

4.9.1.8. Prepare a fragment analysis master mix containing DNA size standard and SLS buffer for the samples following the calculations indicated in the table below. The fragment analysis mastermix calculations can also be performed using the autocalculate box at the bottom of the MLVA Fragment Analysis CEQ Worksheet (see appendix PNL19-2). Vortex briefly and place on ice.

Reagents	**Frag. anal. mastermix**
CEQ SLS buffer	20 µl x (# samples +3)=
CEQ 600 bp size standard	0.08 µl x (# samples +3)=

4.9.1.9. Place a Beckman 96-well sample plate in a cold block. Add two drops of separation buffer to each well of the column 1 (conditioning lane). Next, aliquot 20 µl of the prepared fragment analysis mastermix to the required number of wells. Cover the plate loosely with Parafilm.

4.9.1.10. Using the 10 µl multichannel pipettor, add 1 µl of 1:60 diluted PCR reactions to the appropriate columns in the Beckman sample plate. Keep sliding the Parafilm sheet from column to column to keep track of the sample order.

4.9.1.11. Briefly (15 s) spin down the CEQ Beckman sample plate to remove any air bubbles.

4.9.1.12. Overlay the wells containing samples with 1 drop of mineral oil.

4.9.1.13. Select "Start Sample Plate" from the "Run" pull-down menu or click on the "Run" button in the toolbar.

4.9.1.13.1. For the CEQ 8000, a "Select the Sample Plate to Run" window will open. Select the plate layout that was saved above. The "Confirm Configuration-(*Sample Plate Name*)" window will pop up. Confirm that the plate layout matches with the lanes highlighted in the plate configuration window and click on "Load Plates". When the "Access Plates" dialog box appears, click on "Start". Wait for the CEQ to display the green "Go" signal box.

4.9.1.13.2. For the GeXP, a "Sample Plate Run Confirmation" window will open. This offers the option to designate operator's name. At the bottom of the window, select "Sample Plate Name" and select the plate layout that was previously saved above. Confirm that the plate layout matches with the lanes highlighted in the plate configuration window and that the plate location is correct. If running two plates, designate which plate to run first by selecting the corresponding radial button. (This window also offers the option to replenish the gel cartridge at this time by selecting the "Install Gel Cartridge" button at the bottom of the window and follow instructions as previously described in

4.8.1.11). Click on "Load Plates". When the "Access Plates" dialog box appears, click on "Start". Wait for the GeXP to display the green "Go" signal box.

4.9.1.14. Lift the sample access cover. Rinse and refill wetting tray(s) with dH_2O. (If setting up on the GeXP the corresponding wetting tray for each rail must be filled if running two plates.) Dry off outer tray, clean off any dried deposits of gel from the lid and place the wetting tray(s) back on machine.

4.9.1.15. Load the buffer plate(s) on the front tray and the sample plate(s) on the back tray with notched corners top right. Close the sample access cover. Click on "Load". This will take you back to the "Sample Plate Run Confirmation" window.

4.9.1.16. Once the instrument has loaded the plates, the "Start" button will be highlighted and can be clicked to start the run.

4.9.1.17. Running one column (8 wells) takes about 105 min. A full plate run takes 19½ h. At end of the run, the data will be automatically analyzed and saved.

4.10. Data export from the Beckman Coulter CEQ™ 8000/ 8800/GeXP

4.10.1. **Day 2**

4.10.1.1. To view the analyzed data, click on the "Fragments" icon in the main CEQ window and a window labeled "Study" will open up.

4.10.1.1.1. If the "Study" window does not pop up, select "New Study" from the "File" drop-down menu.

4.10.1.2. Select the "Analyzed Results" button and then select the desired plate run listed in the window and click "OK". The "Select Result Data" window will open.

4.10.1.3. In the "Select Result Data" window, select the "Plate View" tab and the plate format will pop up. Select the desired wells for viewing from the plate by either clicking on them individually or select a whole column by clicking on the column number. Selected wells will be highlighted. Complete the sample selection by clicking on the "Finish" button. "Fragment Analysis – New Study" window will open with all the selected wells listed under the "Results" section.

4.10.1.4. Check the fragment result data (the fluorescent peaks) for each well by clicking on the well IDs individually.

4.10.1.4.1. Make sure that all VNTRs amplified in the positive control and that the fragment sizes are within the range specified in the appendix PNL19-2 and record the fragment sizes on the MLVA Fragment Analysis CEQ Worksheet.

4.10.1.4.2. The size calling for the internal ladder should also be within the range specified in the appendix PNL19-2 (or PNL19-4).

4.10.1.4.3. Write down any failed reactions on the MLVA Fragment Analysis CEQ Worksheet. Make a note of non-specific peaks,

primer-dimers, and atypical appearance of the DNA size standard peaks.

4.10.1.4.4. If the molecular size standard does not run all the way till the end (640 bp) or if it skips fragments, a reaction is considered "a fragment analysis failure" and should be re-run.

4.10.1.4.5. Low fluorescence level background peaks can sometimes interfere with data analysis in BioNumerics. They can be filtered out by using the following procedure:

4.10.1.4.5.1 From the "Study explorer" field on the top left corner, click on "Fragment list". "Exclusion filter set" is located in the right top corner.

4.10.1.4.5.2From the "Name" drop-down menu, select the "pk height (rfu)" option.

4.10.1.4.5.3From the "Operators" drop-down menu select the "<" option. Type in "1000" (or the fluorescence unit value you have determined is adequate to filter out the background without eliminating the true positive peaks) in the "Value(s)" field.

4.10.1.4.5.4Click on "Apply".

4.10.1.5. Save the study: from "File" drop-down menu, select the option "Save Study As". "Save Study As" dialog box will appear. Name the study with the standardized run name (for example, "GA070426") and click "OK". This ensures that you don't have to re-analyze the samples if you want to go to check the raw data again later.

4.10.1.6. From the "File" drop-down menu, select the option "Export Fragments/Genotypes". "Export Fragments/Genotypes" dialog box will appear. Choose the location you would like the file to be saved to (examples: E:/Compact Disk or F:/Removable Disk). Name the peak file containing the fragment list with the run name (see section 4.8.1.6. for instructions; for example "GA070426"), make sure that the file type is "CSV (comma limited)" and save it by clicking on "Save" button. "Export Status" dialog box will appear. Once the export is completed, "OK" button will be highlighted and can be clicked.

4.10.1.7. The remaining gel can stay in the instrument if it is going to be used within 48 hours. The shelf-life of the gel at the room temperature is 72 hours. Using an expired gel may cause inaccurate sizing of fragments in some loci.

5. FLOW CHART:

6. BIBLIOGRAPHY:

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number tandem repeat analysis (MLVA) of Shiga toxin-producing *Escherichia coli* O157 (STEC O157). Foodborne Pathog. Dis. 3, 118-131.

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7. CONTACTS:

7.1 Eija Trees, D.V.M., Ph.D.
PulseNet Next Generation Subtyping Methods Unit, EDLB, DFWED, CDC
(404) 639-3672
EHyytia-Trees@cdc.gov

7.2 Patti Lafon
PulseNet Next Generation Subtyping Methods Unit, EDLB, DFWED, CDC
(404) 639-2828
PLafon@cdc.gov

7.3 Ashley Sabol
PulseNet Next Generation Subtyping Methods Unit, EDLB, DFWED, CDC
(404) 639-2947
ASabol@cdc.gov

8. AMENDMENTS:

1/27/2009: Separation buffer is to be used for conditioning instead of distilled water (step 4.9.1.8)
10/6/2009: statement was added to the step 4.10.1.7. that using expired gel may cause inaccurate fragment sizing.
6/8/2011: Instructions for operating the new GeXP version of the Beckman Coulter Genetic Analysis system were added.
4/10/2012: "Frag-test" CEQ running conditions were replaced with "Frag-test_pause" conditions
4/4/2013: former appendix PNL19-4 (BioNumerics specifications for the *E. coli* O157 VNTR loci) was moved to SOP PND14 (PulseNet standard operating procedure for analysis of MLVA data of Shiga toxin-producing *Escherichia coli* in BioNumerics – Beckman Coulter CEQ 8000/8800/GeXP data). Former appendices PNL19-5 and PNL19-6 were renamed PNL19-4 and PNL19-5, respectively.
2/26/14: the three laboratory SOPs for STECO157 (PNL19), and *Salmonella* serotypes Typhimurium (PNL21) and Enteritidis (PNL27) using the beckman Coulter CEQ8000/8800/GeXP platform were combined into a single SOP (PNL19).

Appendix PNL19-1
MLVA PCR Primer sequences for STEC O157:H7 and *Salmonella* serotypes Typhimurium and Enteritidis

Locus	Dye[1, 2]	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
STEC**O157:H7**			
VNTR-3	D2	GG CGG TAA GGA CAA CGG GGT GTT TGA ATT G	GAA CAA CCT AAA ACC CGC CTC GCC ATC G
VNTR-34	Quas670	GA CAA GGT TCT GGC GTG TTA CCA ACG G	GTT ACA ACT CAC CTG CGA ATT TTT TAA GTC CC
VNTR-9	Quas670	GC GCT GGT TTA GCC ATC GCC TTC TTC C	GTG TCA GGT GAG CTA CAG CCC GCT TAC GCT C
VNTR-25	Quas705	GC CGG AGG AGG GTG ATG AGC GGT TAT ATT TAG TG	GCG CTG AAA AGA CAT TCT CTG TTT GGT TTA CAC GAC
VNTR-17	D2	GC AGT TGC TCG GTT TTA ACA TTG CAG TGA TGA	GGA AAT GGT TTA CAT GAG TTT GAC GAT GGC GAT C
VNTR-19	Quas670	GC AGT GAT CAT TAT TAG CAC CGC TTT CTG GAT GTT C	GGG GCA GGG AAT AAG GCC ACC TGT TAA GC
VNTR-36	Quas670	GG CGT CCT TCA TCG GCC TGT CCG TTA AAC	GCC GCT GAA AGC CCA CAC CAT GC
VNTR-37	Quas705	GC CGC CCC TTA CAT TAC GCG GAC ATT C	GCA GGA GAA CAA CAA AAC AGA CAG TAA TCA GAG CAG C
***Salmonella* Typhimurium**			
ST3	Quas705	GT TCT TCT GCA ACG CAG GCA	GAT GGC ATG ACG CTG CAA CG
ST5	Quas670	TT TTC GCT CAA CAA ACT T	ACA GCA CCA GAA GCA AT
ST7	D2	CG ATT GAC GAT ATC TAT GAC TT	GTT TTT CAC GTT TGC CTT TC
ST10	Quas705	CG GGC GCG GCT GGA GTA TTT G	GAA GGG GCC GGG CAG AGA CAG C
ST2	Quas670	CA ACG CCT GTT CAG CAA C	ATC AAC AGC GGG TGG AT
ST6	D2	AG CAG TGG CTG GCG GGA AAC C	GCA GCC GGA CAG GGG ATA AGC C
ST8	Quas705	GC AGG TGT GGC TAT TGG CGT TGA AA	GAT GGT GAC GCC GTT GCT GAA GG
***Salmonella* Enteritidis**			
SE-1	Quas670	TGT GGG ACT GCT TCA ACC TTT GGG C	CCA GCC ATC CAT ACC AAG ACC AAC ACT CTA TGA
SE-2	D2	GTG CTT CCT CAG GTT GCT TTT AGC CTT GTT CG	GGG GAA TGG ACG GAG GCG ATA GAC G
SE-8	Quas705	GGT AGC TTG CCG CAT AGC AGC AGA AGT	GGC GGC AAG CGA GCG AAT CC
SE-6	Quas670	CTG GTC GCA GGT GTG GC	GGT GAC GCC GTT GCT GAA GGT AAT AAC AGA GTC
SE-5	Quas705	GGC TGG CGG GAA ACC ACC ATC	GCC GAA CAG CAG GAT CTG TCC ATT AGT CAC TG
SE-3	D2	CGG GAT AAG TGC CAC ATA ACA CAG TCG CTA AGC	CGC CAG TGT TAA AGG AAT GAA TGA ACC TGC TGA TG
SE-9	Quas670	CCA CCT CTT TAC GGA TAC TGT CCA CCA GC	GGC GTT ACT GGC GGC GTT CG

[1] Only the 5' of the forward primer is fluorescently labeled
[2] Wellred dye used for D2 (black); Quas705 and Quas670 used as more inexpensive replacements for Wellred D3 (green); and D4 (blue). Only the 5' end of the forward primer is labeled.

Appendix PNL19-2

PCR run date:
PCR instrument:
CEQ run date/Initials:
CEQ instrument:

MLVA Fragment Analysis CEQ Worksheet

	1	2	3	4	5	6	7	8	9	10	11	12
A	blank											
B	blank											
C	blank											
D	blank											
E	blank											
F	blank											
G	blank											
H	blank											

	1 [put in # of samples only]
SLS	80
600 bp	0.32

SLS lot no. **Exp. Date**
600 bp lot no. **Exp. Date**
Gel lot no. **Exp. Date**
Buffer lot no. **Exp Date**

Organism - Positive Control			
Multiplex Reaction fragment sizes		Multiplex Reaction fragment sizes	
VNTR (size range)		VNTR (size range)	
VNTR (size range)		VNTR (size range)	
VNTR (size range)		VNTR (size range)	
VNTR (size range)			

Organism - Internal Ladder fragment sizes				
	Expected Size Ranges		Fragment Sizes	
VNTR				
VNTR				
VNTR				
VNTR				
VNTR				
VNTR				
VNTR				

1. For each isolate, fill in an appropriate BioNumerics key number for PCR reactions R1 and R2.
   (example: for an isolate CDC_K1720 the BN key numbers are CDC_K1720R1 and CDC_K1720R2).
2. Add 2 drops of separation buffer in each well of the first column.
3. Fill in the exact number of fragment analysis reactions (2 per isolate) in the red box (extra already included in calculation).
Mix the Sample Loading Solution (SLS) and 600 bp ladder in an appropriate tube. Add 20 µl of mixture to each well.
4. Using a multichannel pipettor, add 1 ul of 1:60 diluted PCR reaction to wells containing 20 ul of SLS/600bp ladder mixture.
5. Briefly spin down the plate.
6. Overlay all wells, except blank, with 1 drop mineral oil. Immediately place on CEQ or store at -80C.

Note: this appendix has been posted on the SharePoint site as an Excel file so that it can be saved locally for data entry and autocalculation

Appendix PNL19-2 cont'd

Expected sizes for positive controls and internal ladders

E. coli O157 - Positive Control (EDL933)			
R1 fragment sizes		R2 fragment sizes	
VNTR_25 (134-136)		VNTR_17 (156-159)	
VNTR_34 (278-280)		VNTR_36 (158-161)	
VNTR_3 (373-377)		VNTR_37 (187-191)	
VNTR_9 (530-534)		VNTR_19 (307-311)	

E. coli O157 - Internal Ladder fragment sizes				
	Expected Size Ranges		Fragment Sizes	
VNTR_25	122-124	134-136		
VNTR_17	149-152	175-178		
VNTR_36	172-175			
VNTR_37	194-197	200-203		
VNTR_34	224-227	260-262		
VNTR_19	296-299	320-322		
VNTR_3	392-396	429-431		
VNTR_9	518-521	566-570		

Salmonella Typhimurium - Positive Control (LT2)			
R1 fragment sizes		R2 fragment sizes	
ST7 (151-154)		ST6 (264-268)	
ST5 (220-223)		ST2 (358-363)	
ST3 (174-182)		ST8 (553-558)	
ST10 (373-375)			

Salmonella Typhimurium - Internal Ladder fragment sizes				
	Expected Size Ranges		Fragment Sizes	
ST7	130-136	139-145		
ST3	164-171	174-182		
ST5	186-188	232-234		
ST6	247-250	278-280		
ST2	358-363	387-392		
ST10	379-381	411-413		
ST8	580-584	587-592		

Salmonella Enteritidis - Positive Control (K1891)			
R1 fragment sizes		R2 fragment sizes	
SE1 (193 - 196)		SE9 (183-186)	
SE2 (325-329)		SE5 (201-203)	
SE8 (433-435)		SE3 (209-214)	
SE6 (476-481)			

Salmonella Enteritidis - Internal Ladder fragment sizes				
	Expected Size Ranges		Fragment Sizes	
SE9	183-186			
SE1	193-196	214-217		
SE3	197-201	209-214		
SE5	201-203	219-221		
SE2	312-316	353-354		
SE8	344-347	433-435		
SE6	443-445	476-481		

PCR Mastermix calculations

Appendix PNL19-3a

Type in the number of reactions (+ 2 extra), currently a red one, next to "Number to analyze" for autocalculations.

Date:
Technician:
PCR instrument:

E_coli multiplex PCR **R1**

1 Number of samples to be analyzed
10 ul Total Reaction Volume

		Start		Final		Add	
	PCR Buffer	10	X	1	X	1.00	ul
	MgCl2	50	mM	2	mM	0.40	ul
	dNTPs	10	mM	0.2	mM	0.20	ul
black	VNTR-3F	25	uM	0.45	uM	0.18	ul
	VNTR-3R	25	uM	0.45	uM	0.18	ul
blue	VNTR-34F	5	uM	0.14	uM	0.28	ul
	VNTR-34R	5	uM	0.14	uM	0.28	ul
blue	VNTR-9F	5	uM	0.14	uM	0.28	ul
	VNTR-9R	5	uM	0.14	uM	0.28	ul
green	VNTR-25F	2.5	uM	0.07	uM	0.28	ul
	VNTR-25R	2.5	uM	0.07	uM	0.28	ul
	Platinum taq	5	U/ul	1	U	0.20	ul
	PCR Water					5.16	ul

	Lane 1	Lane 2	Lane 3
A			
B			
C			
D			
E			
F			
G			
H			

	Date	PCR Reagents	Lot#	Exp.
VNTR-3F		10X Buffer		
VNTR-3R		MgCl2		
VNTR-34F		dNTPs		
VNTR-34R		Platinum Taq		
VNTR-9F				
VNTR-9R				
VNTR-25F				
VNTR-25R				

E_coli multiplex PCR **R2**

1 Number of samples to be analyzed
10 ul Total Reaction Volume

		Start		Final		Add	
	PCR Buffer	10	X	1	X	1.00	ul
	MgCl2	50	mM	2	mM	0.40	ul
	dNTPs	10	mM	0.2	mM	0.20	ul
black	VNTR-17F	5	uM	0.09	uM	0.18	ul
	VNTR-17R	5	uM	0.09	uM	0.18	ul
blue	VNTR-19F	1	uM	0.02	uM	0.20	ul
	VNTR-19R	1	uM	0.02	uM	0.20	ul
blue	VNTR-36F	1	uM	0.012	uM	0.12	ul
	VNTR-36R	1	uM	0.012	uM	0.12	ul
green	VNTR-37F	2.5	uM	0.03	uM	0.12	ul
	VNTR-37R	2.5	uM	0.03	uM	0.12	ul
	Platinum taq	5	U/ul	1	U	0.20	ul
	PCR Water					5.96	ul

	Lane 3	Lane 4	Lane 5
A			
B			
C			
D			
E			
F			
G			
H			

VNTR-17F	
VNTR-17R	
VNTR-19F	
VNTR-19R	
VNTR-36F	
VNTR-36R	
VNTR-37F	
VNTR-37R	

Note: this appendix has been posted on the SharePoint site as an Excel file so that it can be saved locally for data entry and autocalculation

Appendix PNL19-3b

Date:
Technician
PCR Instrument:

S. Typhimurium multiplex **R1**

1 Number of samples to be analyzed
10 ul Total Reaction Volume

		Start		Final		Add	
	PCR Buffer	10	X	1	X	1.00	ul
	MgCl2	50	mM	2.25	mM	0.45	ul
	dNTPs	10	mM	0.2	mM	0.20	ul
green	ST3-F	5	uM	0.13	uM	0.26	ul
	ST3-R	5	uM	0.13	uM	0.26	ul
blue	ST5-F	5	uM	0.65	uM	1.30	ul
	ST5-R	5	uM	0.65	uM	1.30	ul
black	ST7-F	5	uM	0.23	uM	0.46	ul
	ST7-R	5	uM	0.23	uM	0.46	ul
green	ST10-F	2.5	uM	0.07	uM	0.28	ul
	ST10-R	2.5	uM	0.07	uM	0.28	ul
	Platinum taq	5	U/ul	1.5	U	0.30	ul
	PCR Water					2.45	ul

Lane 1	Lane 2	Lane 3	

Primer	Date prepared	PCR Reagents	Lot#	Exp.
ST3-F		10X Buffer		
ST3-R		MgCl2		
ST5-F		dNTPs		
ST5-R		Platinum Taq		
ST7-F				
ST7-R				
ST10-F				
ST10-R				

S. Typhimurium multiplex **R2**

1 Number of samples to be analyzed
10 ul Total Reaction Volume

		Start		Final		Add	
	PCR Buffer	10	X	1	X	1.00	ul
	MgCl2	50	mM	1.6	mM	0.32	ul
	dNTPs	10	mM	0.2	mM	0.20	ul
blue	ST2-F	5	uM	0.8	uM	1.60	ul
	ST2-R	5	uM	0.8	uM	1.60	ul
black	ST6-F4	5	uM	0.11	uM	0.22	ul
	ST6-R2	5	uM	0.11	uM	0.22	ul
green	ST8-F3	5	uM	0.87	uM	1.74	ul
	ST8-R2	5	uM	0.87	uM	1.74	ul
	Platinum taq	5	U/ul	1.5	U	0.30	ul
	PCR Water					0.06	ul

Lane 2	Lane 3	Lane 4		

Primers	
ST2-F	
ST2-R	
ST6-F4	
ST6-R2	
ST8-F3	
ST8-R2	

Note: this appendix has been posted on the SharePoint site as an Excel file so that it can be saved locally for data entry and autocalculation

Appendix PNL19-3c

Date:
Technician:
Pcr Instrument:

S. Enteritidis multiplex PCR	R1						
1	Number of samples to be analyzed						
10	ul Total Reaction Volume						
		Start		Final		Add	
	PCR Buffer	10	X	1	X	1.00	ul
	MgCl2	50	mM	2	mM	0.40	ul
	dNTPs	10	mM	0.2	mM	0.20	ul
Blue	SE1F	1	uM	0.03	uM	0.30	ul
	SE1R	1	uM	0.03	uM	0.30	ul
Black	SE2F	12.5	uM	0.17	uM	0.14	ul
	SE2R	12.5	uM	0.17	uM	0.14	ul
Green	SE8F	2.5	uM	0.07	uM	0.28	ul
	SE8R	2.5	uM	0.07	uM	0.28	ul
Blue	SE6F	2.5	uM	0.17	uM	0.68	ul
	SE6R	2.5	uM	0.17	uM	0.68	ul
	Platinum taq	5	U/ul	1	U	0.20	ul
	PCR Water					4.41	ul

A B C D E F G H

Row 1	Row 2	

Primer	Date prepared	PCR Reagents	Lot#	Exp.
SE1F		10X Buffer		
SE1R		MgCl2		
SE2F		dNTPs		
SE2R		Platinum Taq		
SE8F				
SE8R				
SE6F				
SE6R				

S. Enteritidis multiplex PCR	R2						
1	Number of samples to be analyzed						
10	ul Total Reaction Volume						
		Start		Final		Add	
	PCR Buffer	10	X	1	X	1.00	ul
	MgCl2	50	mM	2	mM	0.40	ul
	dNTPs	10	mM	0.2	mM	0.20	ul
Green	SE5F	2.5	uM	0.08	uM	0.32	ul
	SE5R	2.5	uM	0.08	uM	0.32	ul
Black	SE3F	12.5	uM	0.18	uM	0.14	ul
	SE3R	12.5	uM	0.18	uM	0.14	ul
Blue	SE9F	2.5	uM	0.03	uM	0.12	ul
	SE9R	2.5	uM	0.03	uM	0.12	ul
	Platinum taq	5	U/ul	1	U	0.20	ul
	PCR Water					6.03	ul

A B C D E F G H

Row 2	Row 3	Row 4	

Primers	Date prepared
SE5F	
SE5R	
SE3F	
SE3R	
SE9F	
SE9R	

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Appendix PNL19-4

Instructions to prepare the internal ladders

1. Prepare DNA templates from isolates as described in the protocol step 4.5. Store the templates at -20°C or -80°C freezer.
   1.1 For STEC O157:H7, use the strains EC04PN0139 and EC04PN0570
   1.2 For *Salmonella* serotype Typhimurium, use strains 2009K0825 and 2009K0826
   1.3 For *Salmonella* serotype Enteritidis, use strains H9560 and 2010K0017
2. Use the DNA templates to set up and run the PCR reactions R1 and R2 as described in the protocol step 4.6.
3. After PCR amplification, pool the R1 and R2 reactions for the two strains into one single PCR tube to end up with a final volume of 40 µl. Mix by pipetting up and down a few times.
4. A new lot of internal ladder must be tested against the old ladder lot by running them in the same fragment analysis run.
5. Store the ladder in -20°C or -80°C freezer. It should remain stable at least 5-6 freeze-thaw cycles for a period of one month.

Expected fragment sizes (bp) of the fifteen fragments (locus VNTR_36 is a null allele in EC04PN0139) present in the internal ladder as seen in the electropherogram from left to right:
(**NOTE:** fragment size ranges for the internal ladders are based on multiple independent runs at CDC and PulseNet Participating Laboratories)

VNTR-25 (Quas705 = D3):	122-124	134-136
VNTR-17 (D2):	149-152	175-178
VNTR-36 (Quas670 = D4):	172-175	NA
VNTR-37 (Quas705 = D3):	194-197	200-203
VNTR-34 (Quas670 = D4):	224-227	260-262
VNTR-19 (Quas670 = D4):	296-299	320-322
VNTR-3 (D2):	392-396	429-431
VNTR-9 (Quas670 = D4):	518-521	566-570

Expected fragment sizes (bp) of the fifteen fragments (locus VNTR_36 is a null allele in EC04PN0139) present in the internal ladder as listed in the peak file:

VNTR-17 (D2):	149-152	175-178
VNTR-3 (D2):	392-396	429-431
VNTR-25 (Quas705 = D3):	122-124	134-136
VNTR-37 (Quas705 = D3):	194-197	200-203
VNTR-36 (Quas670 = D4):	172-175	NA
VNTR-34 (Quas670 = D4):	224-227	260-262
VNTR-19 (Quas670 = D4):	296-299	320-322
VNTR-9 (Quas670 = D4):	518-521	566-570

Expected fragment sizes (bp) of the fourteen fragments present in the internal ladder from the smallest to the largest in the order they appear in the chromatogram:

ST7 (D2):	130-136	139-145
ST3 (Quas705 = D3):	164-171	174-182
ST5 (Quas670 = D4):	186-188	232-234
ST6 (D2):	247-250	278-280
ST2 (Quas670 = D4):	358-363	387-392
ST10 (Quas705 = D3):	379-381	411-413
ST8 (Quas705 = D3):	580-584	587-592

Expected fragment sizes (bp) of the fourteen fragments present in the internal ladder by the dye in the order they appear in the peak file:

ST7 (D2):	130-136	139-145
ST6 (D2):	247-250	278-280
ST3 (Quas705 = D3):	164-171	174-182
ST10 (Quas705 = D3):	379-381	411-413
ST8 (Quas705 = D3):	580-584	587-592
ST5 (Quas670 = D4):	186-188	232-234
ST2 (Quas670 = D4):	358-363	387-392

Expected fragment sizes (bp) of the thirteen fragments (SE9 has the same allele in both ladder strains) present in the internal ladder from the smallest to the largest in the order they appear in the chromatogram:

SE9 (Quas 670 = D4):	183-186	183-186
SE1 (Quas 670 = D4):	193-196	214-217
SE3 (D2):	197-201	209-214
SE5 (Quas 705 = D3):	201-203	219-221
SE2 (D2):	312-316	353-354
SE8 (Quas 705 = D3):	344-347	433-435
SE6 (Quas 670 = D4):	443-445	476-481

Expected fragment sizes (bp) of the thirteen fragments (SE9 has the same allele in both ladder strains) present in the internal ladder by the dye in the order they appear in the peak file:

SE3 (D2):	197-201	209-214
SE2 (D2):	312-316	353-354
SE5 (Quas 705 = D3):	201-203	219-221
SE8 (Quas 705 = D3):	344-347	433-435
SE9 (Quas 670 = D4):	183-186	183-186
SE1 (Quas 670 = D4):	193-196	214-217
SE6 (Quas 670 = D4):	443-445	476-481

Appendix PNL19-5

Maintenance of the Beckman Coulter CEQ™ 8000/ 8800/GeXP

After each run

1. Clean the wetting tray thoroughly by first rinsing it with warm tap water and then with distilled water. Remove any dried gel from the tray and the lid with a paper towel. Refill the tray with distilled water.
2. The remaining gel can stay in the instrument if it is going to be used within 48 hours. The shelf-life of the gel at the room temperature is 72 hours.

Instrument is not used for a week or more

1. Refill the wetting tray at least twice a week with distilled water to keep the capillaries moist at all times.
2. It is recommended to keep a gel in the injection pump at all times to keep the pump moist. Use leftover gels (< 3.0 ml) that cannot be used for a run anymore.
3. Perform a "Capillary Fill" and a "Manual Purge" (0.1 ml 4 times) once a week to keep the lines open and the capillaries unblocked.
4. Remove the capillary array if the instrument is not used for more than two weeks to extend the shelf-life of the array. Replace with an old array and keep performing capillary fills and manual purges once a week as described above.

Instructions to change the Beckman CEQ capillary array

1. Open the new capillary array package and carefully work loose the plastic covers from the electrode block, but leave the covers still attached.
2. To release the old capillary array, select the "Release Capillary Array" from the "Run" pull down menu or click on the capillary array picture in the direct control screen → the "Remove Capillary Array" dialog box will appear
3. Open the sample access cover
4. Open the capillary access cover
5. To open the capillary temperature control cover, unlatch the two rubber latches
6. To open the manifold access cover and the plenum assembly, loosen the captive screws
7. Lift the red "eject lever" to release the array fitting
8. Pull out the array fitting with your right hand and the electrode block with your left hand and set aside
9. Remove the plastic covers from the electrode block and the array fitting of the new array. Be careful not to break the capillaries!
10. Remove the yellow lens cover from the array fitting. Be careful not to touch the lens!
11. Align the array fitting and the electrode block with the guide pins.
12. Replace the manifold access cover and the plenum assembly. Carefully route the capillaries through the whole in the plenum assembly.
13. Close the capillary temperature control cover.

14. Lower the capillary access cover and the sample access cover to their locking positions
15. Click on "OK" in the "Remove Capillary Array" dialog box → the "Install Capillary Array" dialog box will appear
16. Change the serial number and click first on "Set to New" and then on "Done" → the "Confirm Capillary Array Selection" dialog box will appear → select "Yes"
17. Make sure that the instrument has a gel cartridge and a wetting tray in place. Select "Capillary Fill" from the "Direct Control" drop-down menu. Let the instrument fill the capillaries (take's about 3.7 min).
18. Repeat the capillary fill step.
19. Perform three manual purge cycles with 0.1 to 0.4 ml of gel by selecting "Manifold Purge" from the "Direct Control" drop-down menu or by clicking on the funnel on the direct control screen.
20. To perform the optical alignment, either select "Optical Alignment" from the "Direct Control" drop-down menu or click on the lens picture in the direct control screen
21. After the optical alignment has been completed, monitor the fluorescence baseline: select "Monitor Baseline" from the "Run" drop-down menu → "Monitor Baseline" dialog box will appear → check the "Enable Monitor Baseline" box and click "OK"
22. To view the baseline trace, switch to data monitor window and view the baseline for each capillary. Baseline should be less than 5000 units. If it is higher, perform the following procedure:
    a. Release the capillary array and open the sample access cover, the capillary access cover, the temperature control cover and the manifold access cover
    b. Remove the array fitting
    c. Carefully wipe clean both sides of the optical window in the array fitting by using a cotton swab moistened with distilled water. Wipe to one direction only.
    d. Dry the optical window using a second swab by wiping to the same direction as above
    e. Clean the manifold access area first by using a moist swab and then with a dry swab
    f. Replace the array fitting in the manifold
    g. Click on "OK" in the "Remove Capillary Array" dialog box → the "Install Capillary Array" dialog box will appear
    h. Select "Clean Capillaries" button, and click on "Done"
    i. Perform two capillary fills and three manual purges
    j. Repeat the optical alignment step. If the baseline is still above 5000 units after the repeated optical alignment, the new capillary array is most likely defective and must be replaced with another array.
23. To stop the baseline monitoring, go back to the "Monitor Baseline" dialog box and uncheck the "Enable Monitor Baseline" box and click on "OK"
24. To check the optical scan data, go the Main menu and click on the "Sequencing" icon. From the "File" drop-down menu, select "Open" → the "Open" dialog box will appear → select the "Optical scan data" tab. Find today's optical scan file by using the filter (type in today's date as a start and an end date and click on "Refresh") → highlight today's scan file and click on "OK". The red and blue peaks in the optical scan data should start from the galvanometer position 45-50 and end at about 200-210. If the peaks start much earlier or later, the optical alignment of the instrument is off and may contribute to increased fragment analysis failure rate. The red and blue peaks should be superimposed at each galvanometer reading. If they are clearly separated, the optical

alignment is not optimal. In either case, a service call to the Beckman technical support is needed.

Instructions to make a new working database

1. Close all open modules.
2. In the main menu of the CEQ software, click on the "Database" icon.
3. From the "File" drop-down menu, select "New Database" → The "New Database" dialog box will appear
4. Enter a name for the new database by indicating the date on which it was created. For example: Oct_6_2006.
5. Check the box "Set as a Working Database" and click "OK".

STANDARD OPERATING PROCEDURE FOR ADDING THIOUREA TO 0.5X TBE BUFFER FOR STRAINS OF *E.COLI* O157:H7, *SALMONELLA, VIBRIO,* AND OTHER SPECIES OR GENERA THAT ARE "UNTYPEABLE" BY PFGE	CODE: PNL20 Effective Date: 05 18 07

1. PURPOSE: To describe the guidelines for the addition of Thiourea to 0.5X TBE Buffer for strains of *E. coli* O157:H7, *Salmonella*, *Vibrio*, and other species or genera that are "untypeable" by PFGE.

2. SCOPE: This procedure applies to all PulseNet procedures written in relation to the standardized PFGE laboratory protocols.

3. DEFINITIONS/TERMS:

3.1 PFGE: Pulsed-field Gel Electrophoresis

3.2 CDC: Centers for Disease Control and Prevention

4. RESPONSIBILITIES/PROCEDURE:

4.1 Make aqueous stock solution containing 10 mg Thiourea/ml (10 µg/µl):

4.1.1 Dissolve 1 gram thiourea in 100 ml **sterile** Reagent Grade water in clear sterile screw-cap glass bottle or flask (final concentration is 10 mg/ml). Cover bottle of stock solution with aluminum foil to protect from light.[1]

4.1.2 **Safety Caution**: Thiourea is a **toxic** chemical. Weigh thiourea in a chemical fume hood; use gloves, eye protection, and disposable spatula (e.g., wood tongue depressor) when handling this chemical. Clean up any spills, and wipe down balance and surrounding area with a moistened towel. Discard gloves, spatula, weighing paper, etc. as hazardous waste, according to the guidelines of your institution. Re-cap bottle tightly after use.

4.2 Restrict *Salmonella ser.* Braenderup H9812 PulseNet standard strain with *Xba*I and the test samples with the appropriate restriction enzyme (*Xba*I, *Bln*I, *Not*I, etc.); load restricted plug slices on comb or in wells of 1% agarose gel. Include a "positive control" on the gel (a slice from a plug that was "untypeable" without thiourea, but gave a typical PFGE pattern when thiourea was added to the running buffer).

4.2.1 **Note: Do not add thiourea to the melted agarose used for the gel.** It will contaminate the gel form, platform, comb and glassware.

4.3 Make 0.5X TBE with Reagent Grade water; add buffer to electrophoresis chamber and circulate and cool to 14°C. Put gel in chamber.

4.4 Add the following amount of the Thiourea Stock Solution (final concentration of thiourea is 50 µM) to the running buffer close to the back of the chamber, near the wells of the gel:
836 µl thiourea stock solution when 2.2 liters of 0.5X TBE is in the chamber and lines
760 µl thiourea stock solution when 2.0 liters of 0.5X TBE is in chamber and lines

4.5 Allow buffer to circulate for 2-3 minutes before starting electrophoresis using PulseNet standard electrophoresis conditions.

4.6 Stain, de-stain, and document gel according to the appropriate (organism) PulseNet standardized PFGE protocol.

4.7 When electrophoresis run is over, remove as much of the buffer as possible; pour down sink with tap water running. Add 2 liters of DI water to the gel chamber and circulate for 15-20 minutes through the chamber and lines before draining water, adding fresh buffer and using chamber for another gel.

[1] Effect of light has not been determined empirically. Do not store solution in a dark bottle because it will not be possible to tell if solution is discolored or has precipitated.

4.8 **Note:** If possible, use the same electrophoresis unit for all thiourea experiments until the long-term effects (if any) of this chemical on the electrophoresis chamber and tubing have been determined.[2]

5. FLOW CHART:

6. BIBLIOGRAPHY:

7. CONTACTS:

7.1 Efrain Ribot, Ph.D.
CDC
PulseNet Methods Development and Research Unit
(404) 639-3521
ERibot@cdc.gov

8. AMENDMENTS:

[2] At CDC, no negative effects have been observed on the gel chambers or tubing after adding thiourea to the 0.5X TBE running buffer for an average of 2 – 3 gels per month for over five years.

1. PURPOSE: to describe the standardized laboratory protocol for molecular subtyping of Shiga toxin-producing *Escherichia coli* O157 (STEC O157) and *Salmonella enterica* serotypes Typhimurium and Enteritidis.

2. SCOPE: to provide the PulseNet participants with a single protocol for performing MLVA of STEC O157 and *Salmonella* serotypes Typhimurium and Enteritidis, thus ensuring inter-laboratory comparability of the generated results.

3. DEFINITIONS:

3.1 MLVA: Multiple-locus variable-number tandem repeat analysis
3.2 VNTR: Variable-number tandem repeat
3.3 DNA: Deoxyribonucleic acid
3.4 DNase: Deoxyribonuclenase
3.5 PCR: Polymerase chain reaction
3.6 HPLC: High purity liquid chromatography
3.7 dNTP: Deoxyribonucleotide triphosphate
3.8 CDC: Centers for Disease Control and Prevention
3.9 SOP: Standard Operating Procedure

4. RESPONSIBILITIES/PROCEDURE

4.1. Biosafety warning: STEC O157 and *Salmonella* serotypes Typhimurium and Enteritidis with an infectious dose as low as 100 cells are human pathogens capable of causing serious disease. Always use a minimum of Biosafety level 2 practices and extreme caution when transferring and handling strains of these serotypes. Work in a biological safety cabinet when handling large amounts of cells. Disinfect or dispose of all plastic ware and glassware that come in contact with the cultures in a safe manner.

4.2. Reagents, supplies and equipment needed for DNA template preparation

4.2.1 Trypticase soy agar with 5 % sheep blood (TSA-SB) or comparable media
4.2.2 1 µl inoculation loops
4.2.3 0.5 ml microcentrifuge tubes
4.2.4 DNase-free, molecular biology -grade water
4.2.5 Vortex
4.2.6 Boiling water bath or thermocycler/thermal block accommodating 0.5 ml tubes
4.2.7 Tabletop centrifuge for high rpm (up to 13,000-14,000 rpm) spinning
4.2.8 Pipets (200 µl) for aliquoting 100 µl of DNase-free, molecular biology-grade water
4.2.9 Filtered Sterile Pipet tips

4.3. Reagents, supplies and equipment needed for PCR

4.3.1 DNA templates from isolates (keep at -20^{o}C or -80^{o}C freezer for long term)
4.3.2 PCR primers (see appendix PNL23-1)
4.3.2.1 Fluorescent-labeled forward primers
4.3.2.1.1 HPLC-purified
4.3.2.2 Unlabeled reverse primers

4.3.2.2.1 Regular gel filtration purification

4.3.2.3 Biosearch Technologies (Novato, CA; www.biosearchtech.com; 1-800-436-6631) synthesizes primers labeled with the three dyes needed for the protocol

4.3.2.4 Divide the concentrated stocks (100 µM) in portions and store at -80ºC freezer

4.3.2.4.1 One vial should contain enough to prepare 25-50 µl of working solution. Avoid repetitive freeze-thaw cycles of concentrated primer stocks.

4.3.2.5 The 1.0, 2.5, 5.0, 12.5 and 25.0 µM working solutions can be stored at either -20ºC or -80ºC freezer

4.3.2.6 Prepare new working solutions every month or if a significant drop in the fluorescence level is observed (for instructions refer to PNQ06_MLVA ABI certification, appendix PNQ06-5)

4.3.3 96-well polypropylene PCR plates (Fisher, Cat. No. 07-200-613) or Microamp PCR tubes without caps (Life Technologies, Cat. No. N8010533)

4.3.4 8-well strip caps for the polypropylene plate (Fisher, Cat. No. 07-200-639) or MicroAmp strip caps for the individual tubes (Life technologies, Cat. No. N8010535)

4.3.5 DNase-free, molecular biology -grade water

4.3.6 1.5 ml Eppendorf microcentrifuge tubes

4.3.7 PCR Nucleotide Mix (ready-to-use dNTP mix containing all four nucleotides; Roche, Cat. No. 11 814 362 001)

4.3.8 Platinum Taq Polymerase with 50 mM $MgCl_2$ and 10X buffer (Life Technologies, Cat. No. 10966-034)

4.3.9 PCR Cooling block (VWR International, Cat. No. 62111-762)

4.3.10 DNA Engine (Biorad), GeneAmp (Life Technologies) or similar thermocycler with a heated lid option and a 96-well block format

4.3.11 Parafilm M, 4" width (VWR, Cat. No. 52858-032)

4.3.12 Complete set (1000 µl, 200 µl, 100 µl, 20 µl, 10 µl and 2 µl) of single channel pipettors for mastermix set-up ("clean set")

4.3.13 1-10 µl single channel pipettor for adding DNA templates

4.3.14 Filtered tips for pipettors

4.3.15 Microfuge for low (up to 6,000 rpm) rpm spinning

4.4. Reagents, supplies and equipment needed for Genetic Analyzer 3130

4.4.1 DNase-free, molecular biology -grade water

4.4.2 PCR Cooling block (VWR International, Cat. No. 62111-762)

4.4.3 10 µl, 100 µl, and 1000 µl single channel pipettors

4.4.4 1-10 µl and 20-200 µl multichannel pipettors

4.4.5 Filtered pipette tips

4.4.6 Sterile solution basins

4.4.7 1.5 ml Eppendorf microcentrifuge tubes

4.4.8 96-well polypropylene (non-PCR) V-bottom plate (for dilutions; Fisher Scientific, Cat. No. 07-200-698)

4.4.9 MicroAmp Optical 96-well reaction plates (Life Technologies, Cat. No. 4306737)

4.4.10 96-well plate base (Life Technologies, Cat. No. 4317237)

4.4.11 Rubber septa for 96-well reaction plates (Life Technologies, Cat. No. 4315933)
4.4.12 96-well plate retainer (Life Technologies, Cat. No. 4317241)
4.4.13 Hi-Di Formamide (Life Technologies, 25 ml Cat. No. 4311320)
4.4.14 GeneFlo 625 DNA size standard – ROX, 800 µl (Chimerx, Cat. No. 3125-02)
4.4.15 Multi-Capillary DS-30 (Dye Set D) Matrix Standard Kit (Life Technologies, Cat. No. 4345827)
4.4.15.1 Needed to establish the system dye color spectra for the instrument. Required when analyzing fragments labeled with FAM, HEX, NED, and ROX.
4.4.15.2 **NOTE:** DS-33 Matrix Standard (Dye set G5, required for analyzing fragments labeled with FAM, VIC, NED, PET, and LIZ) is typically installed as default as part of the instrument installation process
4.4.15.3 In order to install the DS-30 Matrix, follow the instructions of the kit insert and the "Getting Started Guide", chapter "Performing a Spectral Calibration"
4.4.16 3130 & 3100 Capillary Array 50 cm (Life Technologies, Cat. No. 4315930)
4.4.17 Genetic Analyzer Buffer (10x) with EDTA, 25 ml (Life Technologies, Cat. No. 402824)
4.4.18 3130 POP7 Performance Optimized Polymer, 3.5 ml (Life Technologies, Cat. No. 4363785)
4.4.19 Parafilm M, 4" width (VWR, Cat. No. 52858-032)
4.4.20 50 ml conical tube
4.4.21 A rinse bottle containing distilled water
4.4.22 Centrifuge with a microtiter plate rotor
4.4.23 Heating block or thermal cycler accommodating a 96-well plate for denaturation
4.4.24 Microfuge for low (up to 6,000 rpm) rpm spinning

4.5. DNA template preparation

4.5.1 **Day 0:**
4.5.1.1 Streak an isolated colony from test cultures to TSA-SB plate (or comparable media). Incubate cultures at 37ºC for 14-18 hrs.

4.5.2 **Day 1:**
4.5.2.1 For each isolate to be typed, aliquot 100 µl of sterile, molecular biology-grade water into 0.5 ml microfuge tubes. Use a sterile, disposable 1 µl loop to pick 2-3 colonies (about half of a loopful); rotate the loop in the microfuge tube to release the bacteria into the water. Cap and vortex for 10-15 seconds to disperse any clumps.
4.5.2.2 Place the tubes in a 99-100ºC water bath or heat block for 10-15 minutes. Cool briefly on ice or in fridge and centrifuge for 10 minutes at 10,000 rpm. Place on ice or in fridge while preparing PCR reactions. These DNA templates can be stored at -20ºC or -80ºC for several years.

4.6. PCR procedure

4.6.1 **Day 1:**
4.6.1.1 Fill out, save with the run name, and print an organism specific (copy and paste the appropriate sizing tables for the controls) MLVA Fragment Analysis ABI

Worksheet (see appendix PNL23-2) with appropriately labeled samples (a maximum of 46 isolates/plate; 44 unknowns + two positive controls and a negative control; two wells are reserved for the internal ladder).

4.6.1.1.1 For each isolate, two wells must be labeled as follows: "BNkeyR1" where "BNkey" represents the isolate-specific state laboratory identification number (be sure to use the exact same isolate ID that is used in the PFGE gels uploaded to the national database) and "R1" represents one of the two specific multiplex PCR reactions (R1, R2).

4.6.1.2 Fill out, and print a PCR mastermix calculation worksheet (see appendices PNL23-3a, PNL23-3b and PNL23-3c) by typing the number of isolates to be tested (plus 2-3 extra) in the PCR mastermix calculators labeled R1 and R2. This number is highlighted in RED and is next to "number of samples to be analyzed". The mastermixes for reactions 1 and 2 (R1, R2) for one sample are as follows:

STEC O157:H7 (appendix PNL23-3a)

R1	Volume (µl)	Final conc.
PCR water	5.30	
PCR buffer (10x)	1.00	1x
$MgCl_2$ (50 mM)	0.40	2.00 mM
dNTPs (10 mM)	0.20	0.20 mM
VNTR-3F (25 µM)	0.27	0.67 µM
VNTR-3R (25 µM)	0.27	0.67 µM
VNTR-34F (5 µM)	0.24	0.12 µM
VNTR-34R (5 µM)	0.24	0.12 µM
VNTR-9F (5 µM)	0.24	0.12 µM
VNTR-9R (5 µM)	0.24	0.12 µM
VNTR-25F (2.5 µM)	0.20	0.05 µM
VNTR-25R (2.5 µM)	0.20	0.05 µM
Taq (5 U/µl)	0.20	1 U
	= 9.00	

R2	Volume (µl)	Final conc.
PCR water	5.92	
PCR buffer (10x)	1.00	1x
$MgCl_2$ (50 mM)	0.40	2.00 mM
dNTPs (10 mM)	0.20	0.20 mM
VNTR-17F (5 µM)	0.30	0.15 µM
VNTR-17R (5 µM)	0.30	0.15 µM
VNTR-19F (1 µM)	0.16	0.016 µM
VNTR-19R (1 µM)	0.16	0.016 µM
VNTR-36F (1 µM)	0.11	0.011 µM

VNTR-36R (1 µM)	0.11	0.011 µM
VNTR-37F (2.5 µM)	0.07	0.017µM
VNTR-37R (2.5 µM)	0.07	0.017µM
Taq (5 U/µl)	0.20	1 U
	= 9.00	

Salmonella serotype Typhimurium (appendix PNL23-3b)

R1	Volume (µl)	Final conc.
PCR water	3.81	
PCR buffer (10x)	1.00	1x
$MgCl_2$ (50 mM)	0.45	2.25 mM
dNTPs (10 mM)	0.20	0.20 mM
ST3-F (5 µM)	0.10	0.05 µM
ST3-R (5 µM)	0.10	0.05 µM
ST5-F (25 µM)	0.60	1.50 µM
ST5-R (25 µM)	0.60	1.50 µM
ST7-F (5 µM)	0.80	0.40 µM
ST7-R (5 µM)	0.80	0.40 µM
ST10-F (2.5 µM)	0.12	0.03 µM
ST10-R (2.5 µM)	0.12	0.03 µM
Taq (5 U/µl)	0.30	1.50 U
	= 9.00	

R2	Volume (µl)	Final conc.
PCR water	4.38	
PCR buffer (10x)	1.00	1x
$MgCl_2$ (50 mM)	0.32	1.60 mM
dNTPs (10 mM)	0.20	0.20 mM
ST2-F (25 µM)	0.36	0.90 µM
ST2-R (25 µM)	0.36	0.90 µM
ST6-F4 (5 µM)	0.56	0.28 µM
ST6-R2 (5 µM)	0.56	0.28 µM
ST8-F3 (5 µM)	0.48	0.24 µM
ST8-R2 (5 µM)	0.48	0.24 µM
Taq (5 U/µl)	0.30	1.50 U
	= 9.00	

Salmonella serotype Enteritidis (appendix PNL23-3c)

R1	Volume (µl)	Final conc.
PCR water	4.91	
PCR buffer (10x)	1.00	1x
$MgCl_2$ (50 mM)	0.40	2.00 mM
dNTPs (10 mM)	0.20	0.20 mM
SE1-F (2.5 µM)	0.20	0.05 µM
SE1-R (2.5 µM)	0.20	0.05 µM
SE2-F (12.5 µM)	0.32	0.40 µM
SE2-R (12.5 µM)	0.32	0.40 µM
SE8-F (2.5 µM)	0.28	0.07 µM
SE8-R (2.5 µM)	0.28	0.07 µM
SE6-F (12.5 µM)	0.34	0.43 µM
SE6-R (12.5 µM)	0.34	0.43 µM
Taq (5 U/µl)	0.20	1.00 U
	= 9.00	

R2	Volume (µl)	Final conc.
PCR water	5.52	
PCR buffer (10x)	1.00	1x
$MgCl_2$ (50 mM)	0.40	2.00 mM
dNTPs (10 mM)	0.20	0.20 mM
SE5-F (2.5 µM)	0.20	0.05 µM
SE5-R (2.5 µM)	0.20	0.05 µM
SE3-F (12.5 µM)	0.40	0.50 µM
SE3-R (12.5 µM)	0.40	0.50 µM
SE9-F (2.5 µM)	0.08	0.02 µM
SE9-R (2.5 µM)	0.08	0.02 µM
Taq (5 U/µl)	0.20	1.00 U
	= 9.00	

4.6.1.2.1. **NOTE:** these primer concentrations serve as a starting point. Since laboratory-specific factors, such as the age of the primer stocks, calibration status of the thermocyclers and pipettes, etc. affect amplification efficiency, each laboratory will have to re-optimize the primer concentrations for optimal detection of all targets. However, any other parameters stated in the SOP should not be changed.

4.6.1.3 Thaw all reagents and supplies needed for PCR reactions and place on ice; keep primers light protected as much as possible

4.6.1.3.1 **NOTE:** PCR mastermixes should be set up in a clean hood that is dedicated just for this purpose and where no cultures or DNA are handled.

4.6.1.4 Prepare the two separate PCR mastermixes in 1.5 ml Eppendorf tubes following the instructions in the PCR mastermix calculation worksheet (see appendices PNL23-3a, PNL23-3b and PNL23-3c). Keep the mastermix on ice while preparing. Add the mastermix components in the following order: water, 10x PCR buffer, Mg_2Cl, dNTPs, primers, and then finally Taq polymerase. Mix the reaction mixture by vortexing briefly.

4.6.1.4.1 **NOTE**: All components except Taq polymerase should be vortexed thoroughly before adding to the mastermix. Taq may be briefly centrifuged with low rpm, if necessary, to pull the enzyme down to the bottom of the tube.

4.6.1.5 Place a 96-well PCR plate or required number of PCR tubes in a PCR cooling block.

4.6.1.6 Dispense 9.0 µl of each mastermix into the appropriate rows of the 96-well polypropylene plate / PCR tubes as noted in the PCR template worksheet (see appendix PNL23-2).

4.6.1.7 Add 1 µl of PCR water to each of the two different wells representing the negative controls of the two reactions.

4.6.1.8 Add 1.0 µl of DNA template to each of the two different wells representing the two PCR reactions for each isolate to be tested

4.6.1.9 Add the positive controls (it is recommended to run the positive control in duplicate).

4.6.1.9.1 Use STEC O157 strain EDL933 (ATCC 43895) as a positive control. The internal ladder to be used will be comprised of pooled PCR products of the isolates EC04PN0139 and EC04PN0570 (see appendix PNL23-4 for instructions for ladder preparation).

4.6.1.9.2 Use *S. enterica* serotype Typhimurium strain LT2 (ATCC 29946) as a positive control. The internal ladder to be used will be comprised of pooled PCR products of the isolates CDC_2009K0825 and CDC_2009K0826 (see appendix PNL23-4 for instructions for ladder preparation).

4.6.1.9.3 Use *S. enterica* serotype Enteritidis strain K1891 (ATCC 25928) as a positive control. The internal ladder to be used will be comprised of pooled PCR products of the isolates H9560 and 2010K0017 (see appendix PNL23-4 for instructions for ladder preparation).

4.6.1.10 Cover all wells / tubes with 8-well strip caps and firmly clamp down to avoid any evaporation during PCR amplification.

4.6.1.10.1 Recommendation: briefly spin down the plate / tubes to remove any air bubbles.

4.6.1.11 Program and save the following two PCR cycling conditions:

STEC O157:H7 and *Salmonella* serotype Enteritidis

"O157-SEMLVA"

* 95ºC for 5 min	Step 1
* 94ºC for 20 sec	Step 2
* 65ºC for 20 sec	Step 3
* 72ºC for 20 sec	Step 4
* Go to step 2, 34x	Step 5
* 72ºC for 5 min	Step 6
* Indefinite hold at 4ºC	Step 7

Salmonella serotype Typhimurium

"STMLVA"

* 95ºC for 5 min	Step 1
* 94ºC for 20 sec	Step 2
* 63ºC for 20 sec	Step 3
* 72ºC for 20 sec	Step 4
* Go to step 2, 34x	Step 5
* 72ºC for 5 min	Step 6
* Indefinite hold at 4ºC	Step 7

4.6.1.12.1 **NOTE:** Make sure to use the heated lid option on the PCR block and tube (calculated) temperature control.

4.6.1.13 When the PCR is complete store the amplification products light-protected at 4ºC until ready to run on the sequencer. If the fragment analysis is not performed the same day, the plate should be stored at -20ºC or -80ºC. The PCR products are stable for approximately one month, when stored frozen.

4.7. Initial setup of Genetic Analyzer 3130 instrument:

4.7.1 **NOTE**: steps 4.7.3 and 4.7.4 only need to be performed before the very first run

4.7.2 Click on the "Run 3130 Data Collection v 3.0" icon. The "Service Console" window will appear. If the connections are functioning properly, the "Messaging Service", "Data Service", "Instrument Service" and "Viewer" icons will change from red circles to green squares. The main window of the "Foundation Data Collection" software will open. Check to make sure system status is green.

4.7.3 Set up a results group:

4.7.3.1 On the left side of the window under "GA Instruments", highlight "Results group" and click on "New".

4.7.3.2 The "Results Group Editor" window will appear.

4.7.3.3 Under "General" tab, name the new results group as "MLVA1".

4.7.3.3.1 When the results group reaches its upper limit set up a new results group with a different name (MLVA2, MLVA3…).

4.7.3.4 Under "Analysis" tab, select "GeneMapper-Generic" from the "Analysis type" drop-down menu. Leave "Analysis actions" unchecked.

4.7.3.5 Under "Destination" tab, keep the default root destination (E:\AppliedBiosystems\udc\DataCollection\Data).

4.7.3.6 Under "Naming" Tab, select "Sample Name" from the first "Sample File Name Format" drop-down menu, and select "Plate Name" from the first "Run Folder Name Format" drop-down menu. Leave all other fields blank.

4.7.3.7 Under "Automated Processing" tab, leave the default "Only when the results group is complete" checked.

4.7.4 Set up running conditions:

4.7.4.1 Under "GA Instruments", click on + to expand "ga3130" subfolders.

4.7.4.2 Highlight "Protocol Manager". The instrument protocols will be listed on the right side of the window.

4.7.4.3 Click on "New", and the "Protocol Editor" window will appear.

4.7.4.3.1 Name the new protocol "FragTest".

4.7.4.3.2 Leave the protocol type as "Regular".

4.7.4.3.3 From the "Run Module" drop-down menu, select "Fragment Analysis 50_POP7_1" as running conditions.

4.7.4.3.3.1 **NOTE:** These are the instrument default running conditions for 50 cm capillary array and POP7 polymer. You can check the running conditions and modify them by highlighting "Module Manager" subfolder under ga3130 and by double clicking on the protocol name. The default conditions are:

* Oven_Temperature: 60°C
* Poly Fill Vol: 7300 Steps
* Current Stability: 5.0 μAmps
* PreRun_Voltage: 15.0 kVolts
* Pre-Run-Time: 180 sec.
* Injection_Voltage: 1.6 kVolts
* Injection_Time: 15 sec.
* Voltage_Number_Of_Steps: 30 nk
* Voltage_Step_Interval: 15 sec.
* Data_Delay_Time: 200 sec.
* Run_Voltage: 15.0 kVolts
* Run time: 1800 sec.

4.7.4.3.4 From the "Dye Set" drop-down menu, select "D" as dye set.

4.8. Genetic Analyzer 3130 instrument preparation before each run

4.8.1. **Day 1**

4.8.1.1 Make sure the service console is open and all components are green. Make sure a capillary array is installed in the instrument. For installation, follow the instructions of the "Install Capillary Wizard". You can find the wizards by

expanding the 3130 subfolder and by highlighting "Manual Control". The "Wizards" drop-down menu will appear on top of the window.

4.8.1.2 Set up the plate run on the 3130 manually, as detailed below, or follow the steps in the appendix PNL23-5 to create a template that can be used to import the plate set up from a separate Excel file.

4.8.1.2.1 Under the ga3130, highlight "Plate Manager".

4.8.1.2.2 Click on "New" and a "New Plate Dialog Window" will appear.

4.8.1.2.3 Name the run following the standardized PulseNet naming system: use the unique identifier code that was assigned to your laboratory by PulseNet for the first two to four letters of the file name. The next two spaces will indicate the year and the next four spaces will indicate the month and the date the run was performed. For example GA070426 is a run made at the GA Public Health Laboratory on April 26th 2007. If several runs are performed the same day, separate the file names by using sequential numbers, for example GA070426-1, GA070426-2.

4.8.1.2.4 From the "Application" drop-down menu, select "GeneMapper-Generic"

4.8.1.2.5 Type in the Owner Name and the Operator Name.

4.8.1.2.6 Click "OK" and the "GeneMapper Plate Editor" window will appear.

4.8.1.2.6.1 Type in the sample IDs.

4.8.1.2.6.2 Select "MLVA1" from the "Results Group 1" drop-down menu for the first sample, highlight the results group column, and select "Fill Down" from the "Edit" drop-down menu.

4.8.1.2.6.3 Select "FragTest" from the "Instrument Protocol 1" drop-down menu, highlight the instrument protocol column, and select "Fill Down" from the "Edit" drop-down menu.

4.8.1.2.6.4 Click "OK".

4.8.1.3 Install the POP7 polymer in the instrument

4.8.1.3.1 Expand the 3130 subfolder and highlight "Manual Control". The "Wizards" drop-down menu will appear on top of the window.

4.8.1.3.2 Follow the instructions of the "Replenish Polymer Wizard"

4.8.1.3.2.1 **NOTE1:** if an old polymer (been on the instrument > 7 days) is switched to a new one follow the instructions of the "Water Wash Wizard" until you reach the step in which the array port should be flushed. At this point, if you don't see an air bubble in the port, cancel out the water wash wizard and perform a spatial calibration with a capillary fill. If you see an air bubble in the port perform the flush as instructed by the wizard and then continue to capillary fill as instructed.

4.8.1.3.2.2 **NOTE2:** Use distilled water that has been heated to 37-40^{o}C for water wash.

4.8.1.3.2.3 **NOTE3:** Water wash should be performed once a week.

4.9. Fragment analysis sample preparation

4.9.1 **Day 1**

4.9.1.1 **NOTE:** The fragment analysis method is not organism specific therefore; STEC O157 and *Salmonella* serotypes Typhiumurium and Entertidis may be run on a single fragment analysis plate.

4.9.1.2 Thaw the Hi-Di Formamide, the GeneFlo 625 DNA size standard and the internal ladder (see appendix PNL23-4) and place on ice.

4.9.1.2.1 **NOTE:** aliquot Hi-Di Formamide (500 µl / tube) and the size standard (50 µl / tube) in order to avoid frequent freeze-thaw cycles.

4.9.1.3 Prepare a 96-well V-bottom plate for diluting the PCR reactions. Using a 200 µl multichannel pipettor and a solution basin, dispense 19 µl of molecular-grade water in the required number of wells.

4.9.1.4 Remove the plate / tubes with the PCR reactions from the thermocycler. Briefly spin down the plate / tubes, if necessary. Use a 10 µl multichannel pipettor to transfer 1 µl of each PCR reaction directly across to the corresponding set of wells in the dilution plate. In order to avoid cross-contamination, remove the strip cap from just one column at a time and recap the column before opening the next one.

4.9.1.5 For the internal ladder, combine R1 and R2 PCR products from the four PCR reactions of both internal ladder isolates into one tube to end with a total of 40 µl. Mix well by pipetting up and down a few times and add 3 µl of internal ladder in two wells.

4.9.1.6 Using a 200 µl multichannel pipettor, mix the dilutions by pipetting up and down a few times. Cover the plate with parafilm and put in the fridge or on ice.

4.9.1.7 Prepare a fragment analysis master mix containing DNA size standard and Hi-Di Formamide for the samples following the calculations indicated in the table below. The fragment analysis mastermix calculations can also be performed using the autocalculate box at the bottom of the MLVA Fragment Analysis ABI Worksheet (see appendix PNL23-2). Vortex briefly and place on ice.

Reagents	Frag. anal. mastermix
Hi-Di Formamide	8 µl x (# samples +3)=
GeneFlo 625 bp size standard	1 µl x (# samples +3)=

4.9.1.8 Place a MicroAmp Optical 96-well sample plate in a cold block. Aliquot 9 µl of the prepared fragment analysis mastermix to the required number of wells. Cover the plate loosely with Parafilm.

4.9.1.9 Using the 10 µl multichannel pipettor, add 1 µl of 1:20 diluted PCR reactions to the appropriate columns in the sample plate. Keep sliding the Parafilm sheet from column to column to keep track of the sample order.

4.9.1.10 Denaturate templates by heating the reaction plate uncovered at 95^{o}C for 3 min

4.9.1.11 Manually turn on the oven of the 3130, by using the Command options under the Manual Control submenu to change the set point to 60°C.

4.9.1.12 While the templates denaturate, prepare 30 ml 1X running buffer.

4.9.1.12.1 Add 3 ml of 10X Genetic Analyzer buffer in a 50 ml conical tube.

4.9.1.12.2 Add purified water to bring the total volume up to 30 ml.

4.9.1.12.3 Mix well.

4.9.1.13 Briefly spin down the sample plate to remove any air bubbles.

4.9.1.14 Seal the plate with the rubber septa and place the sample plate in a plate base. Snap the plate retainer onto the plate and the plate base.

4.9.1.15 Place the plate assembly and the buffer into the Genetic Analyzer 3130.

4.9.1.15.1 Push on the Tray button at the front of the Genetic Analyzer 3130 to bring the autosampler to the forward position. Open the instrument doors.

4.9.1.15.2 Place the plate assembly on the autosampler in position A or B with the notched end of the plate base away from you.

4.9.1.15.3 Add 1X running buffer to the anode and cathode reservoirs.

4.9.1.15.4 Add distilled water to the waste and rinse reservoirs.

4.9.1.15.5 Close the instrument doors and wait for the green light to illuminate.

4.9.1.16 Start the run after making sure the system status is green.

4.9.1.16.1 Expand 3130 computer name icon.

4.9.1.16.2 Highlight "Run Scheduler".

4.9.1.16.3 Click on "Find All".

4.9.1.16.4 Highlight the plate name for the run.

4.9.1.16.5 Link the plate by clicking on the yellow squares on the right side of the window that correspond to the position of the plate.

4.9.1.16.6 The "Start Run" button will turn green indicating that the run can be started. Click on this button, and then click "OK" on the alert window. The run will start.

4.10. Viewing and exporting data from the Genetic Analyzer 3130

4.10.1 **Day 2**

4.10.1.1 **NOTE**: Steps 4.10.1.3 and 4.10.1.4 only need to be performed before the very first analysis.

4.10.1.2 Double-click on the shortcut icon for GeneMapper v.4.0 and enter the appropriate password to access the software. The main menu window will open.

4.10.1.3 Set up the size standard:

4.10.1.3.1 From the "Tools" drop-down menu, select "GeneMapper Manager". A "GeneMapper Manager" window will open.

4.10.1.3.2 Select the "Size Standards" tab.

4.10.1.3.3 Click on "New", leave the default option "Basic or Advanced" checked and click "OK". The "Size Standard Editor" window will appear.

4.10.1.3.4 Name the new size standard "GeneFlo 625".

4.10.1.3.5 Leave the default option "Red" as "Size Standard Dye".

4.10.1.3.6 Enter sizes for each peak in the table (refer to the GeneFlo 625 product insert for the peak sizes).

4.10.1.3.7 When finished, click "OK".

4.10.1.4 Set up analysis method:

4.10.1.4.1 From the "Tools" drop-down menu, select "GeneMapper Manager". A "GeneMapper Manager" window will open.

4.10.1.4.2 Select the "Analysis Methods" tab.

4.10.1.4.3 Click on "New", leave the default option "Microsatellite" checked and click "OK". The "Analysis Methods Editor" window will appear.

4.10.1.4.4 Name the new method "PNMLVA" and click "OK".

4.10.1.4.5 Highlight the new method name "PNMLVA" and click "Open".

4.10.1.4.6 Select the "Peak Detector" tab and change "Peak Detection Algorithm" to "Advanced" from the drop-down menu.

4.10.1.4.7 Input the following analysis settings and click "OK" when finished:

* Analysis: Full Range
* Sizing: Partial Sizing
    * Start Size: 50
    * Stop Size 625
* Smoothing: none
* Baseline window: 51 pts
* Size Calling Method: Local Southern Method
* Peak Amplitude Thresholds
    * B: 600
    * G: 600
    * Y: 600
    * R: 20
    * O: 50
* Min. Peak Half Width: 2 pts
* Polynomial Degree: 2
* Peak Window Size: 21 pts
* Slope Threshold
    * Peak Start: 0.0
    * Peak End: 0.0

4.10.1.5 From the "File" drop-down menu select "Add Samples to Project".

4.10.1.6 Find the folder containing the data file to be analyzed: My Computer → E: → Applied Biosystems → UDC → Data Collection → Data.

4.10.1.7 Highlight the desired file(s) and click on "Add to List". File(s) will appear in the window on the right. Click "Add" below the file list to return to the original screen.

4.10.1.8 Samples in the selected file(s) will be listed in a new window and the "Analyze" (play) button appears in green color in the toolbar indicating that the files are ready to be analyzed.

4.10.1.9 Select the size standard GeneFlo 625 for the first sample, highlight the size standard column, and select "Fill Down" from the "Edit" drop-down menu.

4.10.1.10 Select the analysis method PNMLVA for the first sample, highlight the analysis method column, and select "Fill Down" from the "Edit" drop-down menu.

4.10.1.11 Click on the "Play" icon.

4.10.1.12 Name the project with the run name (for example, "GA070426") and click "OK"

4.10.1.13 A successful analysis is indicated by green squares. Yellow triangles indicate problematic components (i.e. missing size standard peaks). Red circles indicate that results fell below acceptable quality values. Samples with yellow or red circles in the SQ column should be selected for re-analysis.

4.10.1.13.1 To resolve failed analyses due to sub-optimal molecular marker peak profile (i.e. miscalling of peaks), select a row with a yellow triangle or red circle in the SQ column and click on the "Size Match Editor" icon on the toolbar. The "Size Match Editor" view will appear.

4.10.1.13.2 Place the cursor near the X-axis to activate the magnifying lens, and then pull up (mouse left-click and hold) to zoom in a specific area to facilitate editing

4.10.1.13.3 Left-click at the base of a peak to select. Right-click and select "Add', "Delete", or "Change".

4.10.1.13.4 Select the correct molecular weight for the selected peak from the drop-down menu. Repeat this process for all other miscalled peaks. Click "OK" when finished.

4.10.1.13.5 After the size standard has been adjusted, click the "Play" button to re-analyze the data. After a successful analysis, the samples will have green squares under the SQ column. If the size standard cannot be adjusted, the reaction is considered a fragment analysis failure and must be re-run

4.10.1.14 Check the fragment result data (the fluorescent peaks) for each well by highlighting the well ID and by clicking on the "Display Plots" icon on the toolbar.

4.10.1.14.1 Make sure that all VNTRs amplified in the positive control and that the fragment sizes are within the range specified in the appendix PNL23-2 and record the fragment sizes on the MLVA Fragment Analysis ABI Worksheet.

4.10.1.14.2 The size calling for the internal ladder should also be within the range specified in the appendix PNL23-2 (or PNL23-4).

4.10.1.14.3 Write down any failed reactions in the MLVA Fragment Analysis ABI Worksheet. Make a note of non-specific bands and primer-dimers.

4.10.1.15 Export the peak file:

4.10.1.15.1 **NOTE:** the following columns should appear in the exported table in the following order from left to right: "Dye/Sample Peak", "Sample File Name", "Marker", "Size", "Height", "Area", "Data Point". You can modify the format of the table by selecting "Table setting editor" from the "Tools"

drop-down menu. Select the "Genotype" tab and make sure that the boxes for the above mentioned columns are checked and no additional boxes are checked.

4.10.1.15.2 Highlight the samples for which you want to export peak data.

4.10.1.15.3 Click on the "Display Plots" icon on the toolbar.

4.10.1.15.4 Click on the "Sizing Table" icon on the toolbar and a table will appear below the electropherograms.

4.10.1.15.5 From the "File" drop-down menu, select "Export Table".

4.10.1.15.6 Select the location (for example a flash drive) where you want to export the data.

4.10.1.15.7 Name the export file with the run name (for example GA070426) and make sure the file type is a tab-delimited text (.txt) file.

4.10.1.16 The remaining gel can stay in the instrument if it is going to be used within 7 days.

4.10.1.16.1 **NOTE:** To extend life of polymer, remove after run, place in refrigerator, and replace with an old polymer (on instrument longer than 7 days) or water bottle until next use. Polymer should not be on instrument for more than a total of 7 days.

5. FLOW CHART:

6. REFERENCES:

6.1 Hyytiä-Trees, E., Smole, S. C., Fields, P. I.., Swaminathan, B., and Ribot, E. M. (2006) Second generation subtyping: a proposed PulseNet protocol for multiple-locus variable-number tandem repeat analysis (MLVA) of Shiga toxin-producing *Escherichia coli* O157 (STEC O157). Foodborne Pathog. Dis. 3, 118-131.

6.2 Hyytia-Trees, E., Lafon, P., Vauterin, P., and Ribot, E. (2010) Multi-laboratory validation study of standardized multiple-locus VNTR analysis (MLVA) protocol for Shiga toxin-producing E*scherichia coli* O157 (STEC O157): a novel approach to normalize fragment size data between capillary electrophoresis platforms. Foodborne Path. Dis. 7, 129-136.

7. CONTACTS:

7.1 Eija Trees, D.V.M., Ph.D.
PulseNet Next Generation Subtyping Methods Unit, CDC
(404) 639-3672
EHyytia-Trees@cdc.gov

7.2 Patti Lafon
PulseNet Next Generation Subtyping Methods Unit, CDC
(404) 639-2828
PLafon@cdc.gov

7.3 Ashley Sabol
PulseNet Next Generation Subtyping Methods Unit, CDC

(404) 639-2947
ASabol@cdc.gov

8. AMENDMENTS:

7/19/2011: appendix PNL23-5 was added. This document gives instructions on how to export a plate set up from the plate manager in order to create a template that can be used to import plate set ups from a separate Excel file.

4/10/2013: instructions to perform the water wash at step 4.8.1.3.2.1 were changed to reflect the fact that performing the array port flush after loosening up the ferule, as instructed by the instrument water wash wizard, may damage the array when performed multiple times.

4/10/2013: instructions to prepare the 1X running buffer were changed in step 4.9.1.11. The buffer should be prepared in a conical tube instead of a graduated cylinder to minimize possible contamination and ensure adequate mixing.

4/10/2013: former appendix PNL23-4 (BioNumerics specifications for the *E. coli* O157 VNTR loci) was moved to SOP PND16 (PulseNet standard operating procedure for analysis of MLVA data of Shiga toxin-producing *Escherichia coli* in BioNumerics – Applied Biosystems Genetic Analyzer 3130/3500 data). Former appendices PNL23-5 and PNL23-6 were renamed PNL23-4 and PNL23-5, respectively.

2/26/2014: the three laboratory SOPs for STECO157 (PNL23), and *Salmonella* serotypes Typhimurium (PNL24) and Enteritidis (PNL26) using the ABI 3130 platform were combined into a single SOP (PNL23).

Appendix PNL23-1
MLVA PCR Primer sequences for STEC O157:H7 and *Salmonella* serotypes Typhimurium and Enteritidis

Locus	Dye[1]	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
STEC O157:H7			
VNTR-3	CalRed590	GG CGG TAA GGA CAA CGG GGT GTT TGA ATT G	GAA CAA CCT AAA ACC CGC CTC GCC ATC G
VNTR-34	FAM	GA CAA GGT TCT GGC GTG TTA CCA ACG G	GTT ACA ACT CAC CTG CGA ATT TTT TAA GTC CC
VNTR-9	FAM	GC GCT GGT TTA GCC ATC GCC TTC TTC C	GTG TCA GGT GAG CTA CAG CCC GCT TAC GCT C
VNTR-25	HEX	GC CGG AGG AGG GTG ATG AGC GGT TAT ATT TAG TG	GCG CTG AAA AGA CAT TCT CTG TTT GGT TTA CAC GAC
VNTR-17	CalRed590	GC AGT TGC TCG GTT TTA ACA TTG CAG TGA TGA	GGA AAT GGT TTA CAT GAG TTT GAC GAT GGC GAT C
VNTR-19	FAM	GC AGT GAT CAT TAT TAG CAC CGC TTT CTG GAT GTT C	GGG GCA GGG AAT AAG GCC ACC TGT TAA GC
VNTR-36	FAM	GG CGT CCT TCA TCG GCC TGT CCG TTA AAC	GCC GCT GAA AGC CCA CAC CAT GC
VNTR-37	HEX	GC CGC CCC TTA CAT TAC GCG GAC ATT C	GCA GGA GAA CAA CAA AAC AGA CAG TAA TCA GAG CAG C
***Salmonella* Typhimurium**			
ST3	HEX	GT TCT TCT GCA ACG CAG GCA	GAT GGC ATG ACG CTG CAA CG
ST5	FAM	TT TTC GCT CAA CAA ACT T	ACA GCA CCA GAA GCA AT
ST7	CalRed590	CG ATT GAC GAT ATC TAT GAC TT	GTT TTT CAC GTT TGC CTT TC
ST10	HEX	CG GGC GCG GCT GGA GTA TTT G	GAA GGG GCC GGG CAG AGA CAG C
ST2	FAM	CA ACG CCT GTT CAG CAA C	ATC AAC AGC GGG TGG AT
ST6	CalRed590	AG CAG TGG CTG GCG GGA AAC C	GCA GCC GGA CAG GGG ATA AGC C
ST8	HEX	GC AGG TGT GGC TAT TGG CGT TGA AA	GAT GGT GAC GCC GTT GCT GAA GG
***Salmonella* Enteritidis**			
SE-1	FAM	TGT GGG ACT GCT TCA ACC TTT GGG C	CCA GCC ATC CAT ACC AAG ACC AAC ACT CTA TGA
SE-2	CallRed590	GTG CTT CCT CAG GTT GCT TTT AGC CTT GTT CG	GGG GAA TGG ACG GAG GCG ATA GAC G
SE-8	HEX	GGT AGC TTG CCG CAT AGC AGC AGA AGT	GGC GGC AAG CGA GCG AAT CC
SE-6	FAM	CTG GTC GCA GGT GTG GC	GGT GAC GCC GTT GCT GAA GGT AAT AAC AGA GTC
SE-5	HEX	GGC TGG CGG GAA ACC ACC ATC	GCC GAA CAG CAG GAT CTG TCC ATT AGT CAC TG
SE-3	CallRed590	CGG GAT AAG TGC CAC ATA ACA CAG TCG CTA AGC	CGC CAG TGT TAA AGG AAT GAA TGA ACC TGC TGA TG
SE-9	FAM	CCA CCT CTT TAC GGA TAC TGT CCA CCA GC	GGC GTT ACT GGC GGC GTT CG

[1] Only the 5' of the forward primer is fluorescently labeled

Appendix PNL23-2

PCR run date:
PCR instrument:
ABI run date/Initials:
ABI instrument:

MLVA Fragment Analysis ABI Worksheet

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

	1 [put in # of samples only]
Hi-Di	32
625 bp	4.00

Hi-Di lot no. **Exp. Date**
625 bp lot no. **Exp. Date**
Polymer lot no. **Exp. Date**
Buffer lot no. **Exp Date**

1. For each isolate, fill in an appropriate BioNumerics key number for PCR reactions R1 and R2 (for example: for an isolate CDC_K1720 the BN key numbers are CDC_K1720R1 and CDC_K1720R2
2. Fill in the exact number of fragment analysis reactions (2 per isolate + controls) in the red box (extra already included in calculation). Mix the Hi-Di Formamide and 625 bp size standard in an appropriate tube. Add 9 µl of mixture to each well.
3. Using a multichannel pipettor, add 1 µl of 1:20 diluted PCR reaction to wells containing the Formamide / 625 bp mixture.
4. Denaturate the plate at 95°C for 3 min.
5. Briefly spin down the plate.
6. Place the plate in the plate base; cover wells with a rubber septa; finish the plate assembly by snapping on the plate retainer.

Organism – Positive Control			
Multiplex Reaction fragment sizes		Multiplex Reaction fragment sizes	
VNTR (size range)		VNTR (size range)	
VNTR (size range)		VNTR (size range)	
VNTR (size range)		VNTR (size range)	
VNTR (size range)			

Organism – Internal Ladder fragment sizes				
	Expected Size Ranges		Fragment Sizes	
VNTR				
VNTR				
VNTR				
VNTR				
VNTR				
VNTR				
VNTR				

Note: this appendix has been posted on the SharePoint site as an Excel file so that it can be saved locally for data entry and autocalculation

Appendix PNL23-2 continued

Expected fragment sizes for positive controls and internal ladders

E. coli O157 - Positive Control (EDL933)			
R1 fragment sizes		R2 fragment sizes	
VNTR_25 (138 - 140)		VNTR_17 (159 - 161)	
VNTR_34 (278 - 280)		VNTR_36 (157 - 158)	
VNTR_3 (379 - 382)		VNTR_37 (187 - 189)	
VNTR_9 (530 - 534)		VNTR_19 (308 - 310)	

E. coli O157 - Internal Ladder fragment sizes				
	Expected Size Ranges		Fragment Sizes	
VNTR_25	127 - 128	138 - 140		
VNTR_17	153 - 155	177 - 179		
VNTR_36	170 - 172			
VNTR_37	192 - 194	198 - 200		
VNTR_34	222 - 224	260 - 262		
VNTR_19	296 - 298	320 - 322		
VNTR_3	397 - 401	433 - 435		
VNTR_9	518 - 522	565 - 569		

Salmonella Enteritidis - Positive Control (K1891)			
R1 fragment sizes		R2 fragment sizes	
SE1 (190 - 193)		SE9 (181 - 184)	
SE2 (335 - 339)		SE5 (201 - 203)	
SE8 (433 - 436)		SE3 (211 - 215)	
SE6 (479 - 482)			

Salmonella Enteritidis - Internal Ladder fragment sizes				
	Expected Size Ranges		Fragment Sizes	
SE9	181 - 184			
SE1	190 - 193	211 - 213		
SE3	199 - 203	211 - 215		
SE5	201 - 203	218 - 221		
SE2	317.5 - 324	363 - 364		
SE8	346 - 350	433 - 436		
SE6	446 - 447	479 - 482		

Salmonella Typhimurium - Positive Control (LT2)			
R1 fragment sizes		R2 fragment sizes	
ST7 (157 - 158)		ST6 (271 - 273)	
ST3 (188 - 192)		ST2 (370 - 373)	
ST5 (219 - 220)		ST8 (556 - 558)	
ST10 (376 - 378)			

Salmonella Typhimurium - Internal Ladder fragment sizes				
	Expected Size Ranges		Fragment Sizes	
ST7	137 - 139	147 - 151		
ST3	177 - 180	188 - 192		
ST5	184 - 185	231 - 233		
ST6	253 - 255	283 - 285		
ST2	370 - 373	399 - 401		
ST10	383 - 384	413 - 415		
ST8	582 - 584	589- 591		

Note: this appendix has been posted on the SharePoint site as an Excel file so that it can be saved locally for data entry and autocalculation

Appendix PNL23-3a

PCR Mastermix Calculations

Type in the number of reactions (+2 extra), currently a red one next to "Number to analyze" for autocalculations

Date:

Technician:

Thermocycler:

E. coli multiplex PCR **R1**

1 Number of samples to be analyzed

10 ul Total Reaction Volume

		Start		Final		Add	
	PCR Buffer	10	X	1	X	**1.00**	ul
	MgCl2	50	mM	2	mM	**0.40**	ul
	dNTPs	10	mM	0.2	mM	**0.20**	ul
black	VNTR-3F	25	uM	0.67	uM	**0.27**	ul
	VNTR-3R	25	uM	0.67	uM	**0.27**	ul
blue	VNTR-34F	5	uM	0.12	uM	**0.24**	ul
	VNTR-34R	5	uM	0.12	uM	**0.24**	ul
blue	VNTR-9F	5	uM	0.12	uM	**0.24**	ul
	VNTR-9R	5	uM	0.12	uM	**0.24**	ul
green	VNTR-25F	2.5	uM	0.05	uM	**0.20**	ul
	VNTR-25R	2.5	uM	0.05	uM	**0.20**	ul
	Platinum taq	5	U/ul	1	U	**0.20**	ul
	PCR Water					**5.30**	ul

	Lane 1	Lane 2	Lane 3	Lane 4
A				
B				
C				
D				
E				
F				
G				
H				

	Date	PCR Reagents	Lot#	Exp.
VNTR-3F		10X Buffer		
VNTR-3R		MgCl2		
VNTR-34F		dNTPs		
VNTR-34R		Platinum Taq		
VNTR-9F				
VNTR-9R				
VNTR-25F				
VNTR-25R				

E. coli multiplex PCR **R2**

1 Number of samples to be analyzed

10 ul Total Reaction Volume

		Start		Final		Add	
	PCR Buffer	10	X	1	X	**1.00**	ul
	MgCl2	50	mM	2	mM	**0.40**	ul
	dNTPs	10	mM	0.2	mM	**0.20**	ul
black	VNTR-17F	5	uM	0.15	uM	**0.30**	ul
	VNTR-17R	5	uM	0.15	uM	**0.30**	ul
blue	VNTR-19F	1	uM	0.016	uM	**0.16**	ul
	VNTR-19R	1	uM	0.016	uM	**0.16**	ul
blue	VNTR-36F	1	uM	0.011	uM	**0.11**	ul
	VNTR-36R	1	uM	0.011	uM	**0.11**	ul
green	VNTR-37F	2.5	uM	0.017	uM	**0.07**	ul
	VNTR-37R	2.5	uM	0.017	uM	**0.07**	ul
	Platinum taq	5	U/ul	1	U	**0.20**	ul
	PCR Water					**5.92**	ul

	Lane 4	Lane 5	Lane 6	Lane 7
A				
B				
C				
D				
E				
F				
G				
H				

VNTR-17F	
VNTR-17R	
VNTR-19F	
VNTR-19R	
VNTR-36F	
VNTR-36R	
VNTR-37F	
VNTR-37R	
VNTR-37F	
VNTR-37R	

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PCR Mastermix calculations **Appendix PNL23-3b**

Type in the number of reactions (+ 2 extra), currently a red zero, next to "Number to analyze" for autocalculations.

Date:
Technician:
Thermocycler

S. Typhimurium multiplex PCR **R1**

1 Number of samples to be analyzed
10 ul Total Reaction Volume

		Start		Final		Add	
	PCR Buffer	10	X	1	X	1.00	ul
	MgCl2	50	mM	2.25	mM	0.45	ul
	dNTPs	10	mM	0.2	mM	0.20	ul
green	ST3-F	5	uM	0.05	uM	0.10	ul
	ST3-R	5	uM	0.05	uM	0.10	ul
blue	ST5-F	25	uM	1.5	uM	0.60	ul
	ST5-R	25	uM	1.5	uM	0.60	ul
yellow	ST7-F	5	uM	0.4	uM	0.80	ul
	ST7-R	5	uM	0.4	uM	0.80	ul
green	ST10-F	2.5	uM	0.03	uM	0.12	ul
	ST10-R	2.5	uM	0.03	uM	0.12	ul
	Platinum taq	5	U/ul	1.5	U	0.30	ul
	PCR Water					3.81	ul

Increased ST5 from 1.2 to 1.5
Decreased ST3 from .07 to .05

Row 1	Row 2	Row 3	Row 4	Row 5	Row 6
A					
B					
C					
D					
E					
F					
G					
H					

Primer	Dateprepared
ST3-F	
ST3-R	
ST5-F	
ST5-R	
ST7-F	
ST7-R	
ST10-F	
ST10-R	

PCR Reagents	Lot	Exp.
10X Buffer		
MgCl2		
dNTPs		
Platinum Taq		

S. Typhimurium multiplex PCR **R2**

1 Number of samples to be analyzed
10 ul Total Reaction Volume

		Start		Final		Add	
	PCR Buffer	10	X	1	X	1.00	ul
	MgCl2	50	mM	1.6	mM	0.32	ul
	dNTPs	10	mM	0.2	mM	0.20	ul
blue	ST2-F	25	uM	0.9	uM	0.36	ul
	ST2-R	25	uM	0.9	uM	0.36	ul
yellow	ST6-F4	5	uM	0.28	uM	0.56	ul
	ST6-R2	5	uM	0.28	uM	0.56	ul
green	ST8-F3	5	uM	0.24	uM	0.48	ul
	ST8-R2	5	uM	0.24	uM	0.48	ul
	Platinum taq	5	U/ul	1.5	U	0.30	ul
	PCR Water					4.38	ul

Row 7	Row 8	Row 9	Row 10	Row 11	Row 12
A					
B					
C					
D					
E					
F					
G					
H					

Primers	Dateprepared
ST2-F	
ST2-R	
ST6-F4	
ST6-R2	
ST8-F3	
ST8-R2	

Note: this appendix has been posted on the SharePoint site as an Excel file so that it can be saved locally for data entry and autocalculation

PCR Mastermix calculations **Appendix PNL23-3c**

Type in the number of reactions (+ 2 extra), currently a red zero, next to "Number to analyze" for autocalculations.

PCR run by:
Date:
Thermocycler:

S. Enteritidis multiplex PCR **R1**

1	Number of samples to be analyzed							
10	ul Total Reaction Volume							
		Start		Final		Add		A
	PCR Buffer	10	X	1	X	1.00	ul	B
	MgCl2	50	mM	2	mM	0.40	ul	C
	dNTPs	10	mM	0.2	mM	0.20	ul	D
Blue	SE1F	2.5	uM	0.05	uM	0.20	ul	E
	SE1R	2.5	uM	0.05	uM	0.20	ul	F
Black	SE2F	12.5	uM	0.4	uM	0.32	ul	G
	SE2R	12.5	uM	0.4	uM	0.32	ul	H
Green	SE8F	2.5	uM	0.07	uM	0.28	ul	
	SE8R	2.5	uM	0.07	uM	0.28	ul	
Blue	SE6F	12.5	uM	0.43	uM	0.34	ul	
	SE6R	12.5	uM	0.43	uM	0.34	ul	
	Platinum taq	5	U/ul	1	U	0.20	ul	
	PCR Water					4.91	ul	

Row 1	Row 2	Row 3	Row 4	Row 5	Row 6

Primer	Date prepared	PCR Reagents		
SE1F			Lot#	Exp.
SE1R		**10X Buffer**		
SE2F		**MgCl2**		
SE2R		**dNTPs**		
SE8F		**Platinum Taq**		
SE8R				
SE6F				
SE6R				

S. Enteritidis multiplex PCR **R2**

1	Number of samples to be analyzed							
10	ul Total Reaction Volume							
		Start		Final		Add		A
	PCR Buffer	10	X	1	X	1.00	ul	B
	MgCl2	50	mM	2	mM	0.40	ul	C
	dNTPs	10	mM	0.2	mM	0.20	ul	D
Green	SE5F	2.5	uM	0.05	uM	0.20	ul	E
	SE5R	2.5	uM	0.05	uM	0.20	ul	F
Black	SE3F	12.5	uM	0.5	uM	0.40	ul	G
	SE3R	12.5	uM	0.5	uM	0.40	ul	H
Blue	SE9F	2.5	uM	0.02	uM	0.08	ul	
	SE9R	2.5	uM	0.02	uM	0.08	ul	
	Platinum taq	5	U/ul	1	U	0.20	ul	
	PCR Water					5.84	ul	

Row 1	Row 2	Row 3	Row 4	Row 5	Row 6

Primers	Date prepared
SE5F	
SE5R	
SE3F	
SE3R	
SE9F	
SE9R	

Appendix PNL23-4

Instructions to prepare the internal ladders

1. Prepare DNA templates from isolates as described in the protocol step 4.5. Store the templates at -20°C or -80°C freezer.
   1.1 For STEC O157:H7, use the strains EC04PN0139 and EC04PN0570
   1.2 For *Salmonella* serotype Typhimurium, use strains 2009K0825 and 2009K0826
   1.3 For *Salmonella* serotype Enteritidis, use strains H9560 and 2010K0017
2. Use the DNA templates to set up and run the PCR reactions R1 and R2 as described in the protocol step 4.6.
3. After PCR amplification, pool the R1 and R2 reactions for the two strains into one single PCR tube to end up with a final volume of 40 µl. Mix by pipetting up and down a few times.
4. A new lot of internal ladder must be tested against the old ladder lot by running them in the same fragment analysis run.
5. Store the ladder in -20°C or -80°C freezer. It should remain stable at least 5-6 freeze-thaw cycles for a period of one month.

Expected fragment sizes (bp) of the fifteen fragments (locus VNTR_36 is a null allele in EC04PN0139) present in the STEC O157:H7 internal ladder as listed in the peak file:

VNTR-36 (B):	170 - 172	NA
VNTR-34 (B):	222 - 224	260 - 262
VNTR-19 (B):	296 - 298	320 - 322
VNTR-9 (B):	518 - 522	565 - 569
VNTR-25 (G):	127 - 128	138 - 140
VNTR-37 (G):	192 - 194	198 - 200
VNTR-17 (Y):	153 - 155	177 - 179
VNTR-3 (Y):	397 - 401	433 - 435

Expected fragment sizes (bp) of the fifteen fragments (locus VNTR_36 is a null allele in EC04PN0139) present in the STEC O157:H7 internal ladder as they appear in the electropherogram:

VNTR-25 (G):	127 - 128	138 - 140
VNTR-17 (Y):	153 - 155	177 - 179
VNTR-36 (B):	170 - 172	NA
VNTR-37 (G):	192 - 194	198 - 200
VNTR-34 (B):	222 - 224	260 - 262
VNTR-19 (B):	296 - 298	320 - 322
VNTR-3 (Y):	397 - 401	433 - 435
VNTR-9 (B):	518 - 522	565 - 569

Expected fragment sizes (bp) of the fourteen fragments present in the *Salmonella* serotype Typhimurium internal ladder as listed in the peak file:

ST5 (B):	184 - 185	231 - 233
ST2 (B):	370 - 373	399 - 401
ST3 (G):	177 - 180	188 - 192
ST10 (G):	383 - 384	413 - 415
ST8 (G):	582 - 584	589 - 591
ST7 (Y):	137 - 139	147 - 151
ST6 (Y):	253 - 255	283 - 285

Expected fragment sizes (bp) of the fourteen fragment present in the *Salmonella* serotype Typhimurium internal ladder as they appear in the electropherogram:

ST7 (Y):	137 - 139	147 - 151
ST3 (G):	177 - 180	188 - 192
ST5 (B):	184 - 185	231 - 233
ST6 (Y):	253 - 255	283 - 285
ST2 (B):	370 - 373	399 - 401
ST10 (G):	383 - 384	413 - 415
ST8 (G):	582 - 584	589 - 591

Expected fragment sizes (bp) of the thirteen fragments (SE9 has the same allele in both ladder strains) present in the *Salmonella* serotype Enteritidis internal ladder as listed in the peak file:

SE9 (B)	181 - 184	181 - 184
SE6 (B):	446 - 447	479 - 482
SE1 (B)	190 - 193	211 - 213
SE5 (G):	201 - 203	218 - 221
SE8 (G):	346 - 350	433 - 436
SE3 (Y):	199 – 203	211 - 215
SE2 (Y):	317.5 - 324	363 - 364

Expected fragment sizes (bp) of the fifteen fragments (SE9 has the same allele in both ladder strains) present in the *Salmonella* serotype Enteritidis internal ladder as they appear in the electropherogram:

SE9 (B)	181 - 184	181 – 184
SE1 (B)	190 - 193	211 - 213
SE3 (Y):	199 - 203	211 - 215
SE5 (G):	201 - 203	218 - 221
SE2 (Y):	317.5 - 324	363 - 364
SE8 (G):	346 - 350	433 - 436
SE6 (B):	446 - 447	479 - 482

NOTE: fragment size ranges for the internal ladders are based on multiple independent runs at CDC and PulseNet Participating Laboratories

Appendix PNL23-5

Steps for exporting and importing plate set ups on the ABI 3130

Note: the procedure described below will require the sequencer computer to have Microsoft Office. If the sequencer computer does not have Microsoft Office, the exported file needs to be saved on a flash drive so that steps 8 through 15 can be performed on a computer with Microsoft Office.

1. Under "GA Instruments", click on the + to expand the "ga3130" subfolders on the left-hand menu
2. Highlight "Plate Manager" and on the right side of the window click "Find All" to list all of the available plates
3. Find the most recent full plate. Click on the desired plate and then click the "Export..." button at the bottom of the screen
   3.1. **NOTE**: If you do not have a full plate, create a new one by filling in each sample ID space (A01 through H12) with "test" and saving it with the plate name "MLVA_Template". Be sure to add the results group "MLVA1" and instrument protocol "FragTest" for each sample. It is necessary to have all required wells filled in when exporting so that they will be available in your exported file
4. In the export window, create a new folder on the desktop by clicking the "Desktop" icon on the left side of the window. Find the "Create New Folder" button on the top right side of the window and name the new folder "MLVA Plate Setup"
5. Save the file to this folder by using your new plate name (e.g.– CDC101020) and clicking "Save"
6. A window will pop up letting you know that the plate has been successfully exported. Click "OK"
7. Minimize the 3130 Viewer and open the "MLVA Plate Setup" folder on the desktop
8. Right-click your plate name, select "Open With" and in the submenu select Microsoft Office Excel.
9. Sample IDs can be typed or copied and pasted from a separate Excel file into the spaces next to the correct wells under the "Sample Name" heading. The first sample ID *must* be directly under the "Sample Name" heading
   9.1. **NOTE**: Do not change/delete any of the column headings. The fields must be in the same format when importing as they were when exported. Additionally, the software will not import IDs with special characters (e.g. - ! , / ,) , etc) or spaces. You can use underscores and dashes
10. The "Container Name" should be changed to match the plate/file name, and initials should be placed under the "Owner" and "Operator" headings
    10.1. **NOTE**: If the container name is not changed it cannot be imported because it will be recognized as a plate that is already in the system
11. Once all of the IDs are inserted, make sure that for each sample ID and all controls the priority is "100", the results group name "MLVA1" is under the "Results Group 1" heading, and the running method "FragTest" is under the "Instrument Protocol 1" heading
12. For all *unused* wells, everything under the headings "Well", "Priority", "Results Group 1", and "Instrument Protocol 1" must be deleted. This can be done by clicking the first cell to be deleted under these columns and dragging until the entire section is highlighted. Right-click in the highlighted area and from the drop-down menu select "Clear Contents"
13. Under the "File" drop-down menu select "Save"

14. A warning window will appear asking if the workbook should be saved in the "Text (tab-delimited)" format. Click "Yes"
15. Exit Microsoft Excel, clicking "No" when prompted to save changes
16. Go back to the ABI 3130 plate manager window and click the "Import..." button at the bottom of the screen
17. In the import window, navigate to the recently created plate under Desktop/MLVA Plate Setup, select the appropriate .txt file and click "Open"
18. When the file has been imported, a "Progress" window will appear stating that the plate was "successfully imported". Click "OK"
    18.1. **NOTE**: For repeated use, plate templates can be created by exporting a plate to the "MLVA Plate Setup" folder. Save the file as a tab-delimited text file with 'test' as the sample ID and with a new plate name (e.g. "MLVA_Template"). After you have filled out the information for a new run on the template, save it in step 13 with the standardized run name (name (e.g. CDC101020).

1. **PURPOSE:** To describe standardized protocols for molecular subtyping of botulinum toxin producing clostridia by Pulsed-field Gel Electrophoresis (PFGE).

2. **SCOPE:** To provide the PulseNet participants with the same procedures for performing PFGE of botulinum toxin producing clostridia thus ensuring interlaboratory comparability of the generated results.

3. **DEFINITIONS/TERMS:**

   3.1 PFGE: Pulsed-field Gel Electrophoresis
   3.2 DNA: Deoxyribonucleic acid
   3.3 CDC: Centers for Disease Control and Prevention
   3.4 CLRW: Clinical Laboratory Reagent Water

4. **RESPONSIBILITIES/PROCEDURE:**

BIOSAFETY WARNING:

All samples received must be considered infectious. Botulinum toxin producing clostridia and/or botulinum toxin may be present in a variety of food products, clinical materials (serum, feces) and environmental samples (soil, surface water). Exposure to botulinum toxin is the primary laboratory hazard. The toxin may be absorbed after ingestion or following contact with the broken skin, eyes, or mucous membranes, including the respiratory tract. Accidental parenteral inoculation may also represent a significant exposure to toxin. Broth cultures grown under optimal conditions for toxin production may contain 2,000,000 mouse LD_{50} per ml of toxin.

Recommended Precautions: Biosafety Level 2 practices, containment equipment and facilities are recommended for all activities with materials known to or that may potentially contain botulinum toxin. Personal protective equipment (PPE) i.e., gloves, lab coat, safety glasses and/or face shield should be worn at all times when handling anything that has come into contact with the organism or toxin including pipette tips and plastic transfer pipettes used to transfer liquids. All liquids require disinfection using a freshly prepared 10% bleach solution. Solutions of sodium hypochlorite (0.1%) or sodium hydroxide (0.1N) readily inactivate the toxin and are recommended for decontaminating work surfaces and spills of cultures or toxin.

Please read all instructions carefully before starting protocol. All plasticware, glassware, pipets, spatulas, etc. that come in contact with the cell suspensions or plugs should be disinfected with 10% bleach for at least 1 hour before they are washed and reused.

SELECT AGENTS REQUIREMENTS:

All PFGE materials, including restricted plugs, may contain viable bacteria until the start of electrophoresis. Botulinum toxin producing clostridia are Select Agents (SA), and according to the APHIS/CDC SA Regulations all parts of the following procedure until electrophoresis must be performed by SA approved personnel and within SA approved space. In addition, long term stored plugs are subject to SA inventory requirements, as defined by APHIS/CDC SA Regulations. Please refer to www.selectagents.gov for additional information.

PREPARATION OF PFGE PLUGS FROM TEST CULTURES

Day 1

1. Streak each test culture for colony isolation onto Anaerobic Blood Agar Plate – CDC formulation (ANA-BAP).

2. Incubate the plates in an anaerobic chamber overnight at 35°C ± 2°C.

Day 2

1. Label small tubes (12-mm x 75-mm Falcon tubes or equivalent) with culture numbers.

2. Prepare **Cell Suspension Buffer** (**100 mM Tris:100 mM EDTA, pH 8.0**) as follows:

   200 ml of 1 M Tris-HCl, pH 8.0
   400 ml of 0.5 M EDTA, pH 8.0
   Dilute to 2000 ml with sterile Ultrapure water (Clinical Laboratory Reagent Water (CLRW))

Note: Acceptable options for CLRW are type I or Milli-Q water.

3. Transfer ≈1.5 ml of **Cell Suspension Buffer** (CSB) to the labeled tubes. Use a sterile polyester-fiber or cotton swab that has been moistened with sterile CSB to remove some of the growth from the ANA-BAP plate; suspend cells in CSB by spinning the swab gently so cells will be evenly dispersed and formation of aerosols is minimized.

4. Adjust concentration of cell suspensions to a milky turbidity of ~1 McFarland (0.18-0.20 on Dade Microscan Turbidity Meter) by diluting with sterile CSB or by adding additional cells.

5. Pipette 1000 µl of the cell suspensions into sterile microcentrifuge tubes and spin for 5 minutes at 5,000 rpm.

6. Remove the supernatants, resuspend cells in 1000 µl of the cell suspension buffer and spin for 5 minutes at 5,000 rpm. Decant supernatants in waste container containing bleach.

7. Remove the supernatants and resuspend cells in 500 µl of the cell suspension buffer. Decant supernatants in waste container containing bleach.

8. Use these washed cells to inoculate two EYA plates per sample for confluent growth (250 µl per plate), using the Kirby –Bauer technique.

9. Incubate the plates for 18-72 hours in an anaerobic chamber at 35°C ± 2°C.

Note: Remove plates from incubation as soon as there is sufficient growth for testing. 18 hours is typically optimal.

Day 3

1. Turn on shaker water bath or incubator (55°C ± 2°C) and stationary water baths (55°C ± 2°C and 37°C ± 2°C).

2. Add bottles of sterile Ultrapure water (CLRW) and TE buffer to the 55°C water bath to warm for the washing procedural steps.

Note: An acceptable option for CLRW is type I or Milli-Q water.

3. Prepare **2X Cell Lysis Buffer** (**12mM Tris, 2M NaCl, 200 mM EDTA, 1% Brij 58, 0.4% Deoxycholate, 5% Sarcosyl)** as follows:

   1.2 ml of 1 M Tris-HCl, pH 8.0
   40 ml of 5 M NaCl
   40 ml of 0.5 M EDTA, pH 8.0
   1 g Brij 58
   0.4 g Deoxycholate
   5 g Sarkosyl
   Dilute to 100 ml with sterile Ultrapure water (CLRW)

Note: An acceptable option for CLRW is type I or Milli-Q water.

4. Prepare Lysozyme (Sigma L7651 or equivalent) stock solution (20 mg/mL in TE) as follows:
   a. Weigh out 100 mg Lysozyme (keep the container of Lysozyme on ice)
   b. Add 5 mL TE buffer, swirl to mix
   c. Aliquot 250 uL amounts into small eppendorf tubes and freeze for future use.

5. Prepare Mutanolysin (Sigma M9901 or equivalent) stock solution (5 U/µL in TE) as follows:
   a. Add 1 mL TE buffer to vial of lyophilized Mutanolysin, swirl to mix
   b. Aliquot 50 µL amounts into small eppendorf tubes and freeze for future use.

6. Take out tubes of Lysozyme (20 mg/ml) and Mutanolysin (5U/µl) needed from the -20ºC freezer and pre-warm **2X Cell Lysis Buffer** to 55 ºC ± 2°C.

7. Prepare 1.2% SeaKem Gold agarose in **TE Buffer** (**10 mM Tris:1 mM EDTA, pH 8.0**) for PFGE plugs as follows:

   a. Weigh 0.12 g (or 0.24 g) SeaKem Gold (SKG) agarose into 250 ml screw-cap flask
   b. Add 10.0 ml (or 20.0 ml) TE Buffer; swirl gently to disperse agarose.
   c. Loosen or remove cap and cover loosely with clear film, and microwave for 30-sec; mix gently and repeat for 10-sec intervals until agarose is completely dissolved.
   d. Recap flask and place in 55°C ± 2°C water bath and equilibrate the agarose in the water bath for 15 minutes or until ready to use.

SAFETY WARNING: Use heat-resistant gloves when handling hot flasks after microwaving.

Note: SeaKem Gold agarose works well for making PFGE plugs because it provides added strength to the plugs minimizing breakage of plugs during the lysis and washing steps. The time and temperature needed to completely dissolve the agarose is dependent on the specifications of the microwave used, and will have to be determined empirically in each laboratory.

8. Label small tubes (12-mm x 75-mm Falcon tubes or equivalent) with culture numbers.
9. Prepare **PIV Buffer** as follows:

   5 ml of 1 M Tris-HCl, pH 8.0
   100 ml of 5 M NaCl
   Dilute to 500 ml with sterile Ultrapure water (CLRW)

Note: An acceptable option for CLRW is type I or Milli-Q water.

10. Transfer ≈1.5 ml of **PIV Buffer** to the labeled tubes. Use a sterile polyester-fiber or cotton swab that has been moistened with sterile PIV to remove some of the growth from the agar plate; suspend cells in PIV by spinning swab gently so cells will be evenly dispersed and formation of aerosols is minimized.

Note: If a large number of samples are being prepared, it is recommended that they are prepared in batches of ~10 samples at a time.

11. Adjust concentrations of cell suspensions to 0.68-0.72 as measured on the Dade Microscan Turbidity Meter by diluting with sterile PIV Buffer or by adding additional cells. These cell suspension concentrations give satisfactory results at CDC; each laboratory may need to establish the optimal concentration needed for satisfactory results.

Note: Cell suspensions need to be at room temperature when concentration is checked.

12. Pipette 1000 µl of the cell suspensions into microcentrifuge tubes and spin for 5 minutes at 5,000rpm.

13. Calculate the volume of 2X cell lysis buffer and Proteinase K required per sample as follows:

Note: Stock concentrations of Proteinase K may vary by lot and will need to be calculated for each shipment. Adjust total volume to compensate by changing amount of 2X cell lysis buffer used. The final concentration of Proteinase K for plugs is 0.665 mg/ml.

Example Calculation:
(stock conc. of Proteinase K) (X) = (final conc. desired) (final volume)

(stock conc. of Proteinase K) (X) = (0.665 mg/ml) (1 ml)

$$\frac{(0.665 \text{ mg/ml}) \times (1 \text{ ml})}{\text{(stock conc. of Proteinase K)}} = \text{volume of Proteinase K needed (ml)}$$

Total volume of 2X cell lysis buffer + Proteinase K = 316 µl

316 µl – volume of Proteinase K = volume of 2X cell lysis buffer needed (µl)

14. Remove the supernatants and resuspend cells in the calculated volume of 2X cell lysis buffer that has been pre-warmed to 55ºC ± 2°C. Then add 80 µl of lysozyme (20 mg/ml) and incubate in the waterbath for 20 minutes at 55°C ± 2°C. Decant supernatants in waste container containing bleach.

15. After removing the samples from the waterbath, add 4 µl of mutanolysin (5U/µl) and the calculated volume of proteinase K, and incubate another 10 minutes at 37ºC ± 2°C.

CASTING PLUGS

Label wells of PFGE plug molds with culture number. When reusable plug molds are used, put strip of tape on lower part of reusable plug mold before labeling wells.

Note: Unused plug agarose can be kept at room temperature and reused 1-2 times. Microwave on low-medium power for 10 -15 sec and mix; repeat for 5 -10 sec intervals until agarose is completely melted. This agarose melts rapidly!

1. Add 400 µl (0.4 ml) melted 1.2% SeaKem Gold agarose to the cell suspensions; mix by gently pipetting mixture up and down a few times. Maintain temperature of melted agarose by keeping flask in beaker of warm water (55-60ºC).

2. Immediately, dispense part of mixture into appropriate well(s) of reusable plug mold. Do not allow bubbles to form. Two plugs of each sample can be made from these amounts of cell suspension and agarose. Allow plugs to solidify at room temperature for 10-15 min.

Note: If plugs are being prepared from a large number of samples, it is recommended that they are prepared in batches of ~10 samples at a time.

LYSIS OF CELLS IN AGAROSE PLUGS

Note: Two plugs of the same strain can be lysed in the same 50-ml tube.

1. Label 50-ml polypropylene screw-cap tubes with culture numbers.

2. Prepare **ES Buffer** as follows:
   495 ml of 0.5 M EDTA, pH 8.0
   5 ml of 10 % Sarcosyl

3. Add 5 ml of ES Buffer to each labeled 50 ml tube.

4. Calculate the volume of **Proteinase K** needed per sample as follows:

Note: Stock concentrations of Proteinase K may vary by lot and will need to be calculated for each shipment. The final concentration of Proteinase K per tube is 0.14 mg/ml.

Calculation:
(stock conc. of Proteinase K) (X) = (final conc. desired) (final volume)

(stock conc. of Proteinase K) (X) = (0.14 mg/ml) (5 ml)

$$\frac{(0.14 \text{ mg/ml}) \times (5 \text{ ml})}{(\text{stock conc. of Proteinase K})} = \text{volume of Proteinase K needed (ml)}$$

Add the calculated volume of Proteinase K to each labeled 50 ml tube containing ES Buffer.

5. Trim excess agarose from top of plugs with scalpel, razor blade or similar instrument. Open reusable plug mold and transfer plugs from mold with a 6-mm wide spatula to appropriately labeled tubes containing ES Buffer + Proteinase K. Be sure plugs are under buffer and not on side of tube.

Note: Ensure that the green screen caps are in place on conical tubes containing the plugs to prevent the loss or damage of the plug

6. Place both sections of the plug mold, spatulas, and scalpel in 10% bleach. **Soak them for 1 hour before washing them.**

7. Place tubes in a rack and incubate in the 55°C ±2°C shaker water bath or incubator for a minimum of 2 h (4 h is optimal) with constant agitation (40-70 rpm). Be sure water level is above level of lysis buffer in tubes if using a water bath.

WASHING OF AGAROSE PLUGS AFTER CELL LYSIS

Note: Most laboratories will find that their plugs are sufficiently stable to perform the following washing steps at 54-55ºC. However, if you notice that your plugs are nicked along the edges or breaking it will be necessary for your laboratory to lower the water bath or incubator to 50ºC for the following washing steps.

1. Remove tubes from water bath and carefully pour off ES Buffer into an appropriate discard container; plugs can be held in tubes with the green screen caps.

Note: It is important to remove all of the liquid during this and subsequent wash steps by touching edge of tube or screened cap on an absorbent paper towel.

2. Add 20 ml sterile Ultrapure water (CLRW) that has been preheated to 55ºC ± 2°C to each tube and shake the tubes in a 55°C ± 2°C water bath for 15 min at 70 RPM. Decant used distilled water into a waste container containing bleach.

Note: An acceptable option for CLRW is type I or Milli-Q water.

3. Pour off water from the plugs and repeat wash step with 20 ml of preheated water (Step 2) one more time.

4. Pour off water, add a minimum of 20 ml preheated (55ºC ± 2°C) sterile TE Buffer, and shake the tubes in the 55ºC ± 2°C water bath for 15 min at 70 RPM. Decant used TE into a waste container containing bleach.

5. Pour off TE and repeat wash step with preheated TE five more times.

6. Decant last wash and add 15 ml sterile TE. Continue with step 1 in "Restriction Digestion" section or store plugs in TE Buffer at 4ºC until needed. Plugs can be transferred to smaller tubes containing 1 ml of TE for storage.

Day 4

RESTRICTION DIGESTION OF DNA IN AGAROSE PLUGS

Note: A small slice of the plug or the entire plug (made in disposable plug molds) can be digested with the restriction enzyme. Restriction digestion of a small slice of the plug is recommended because less enzyme is required and other slices of the plug can be subjected to restriction analysis with other enzymes. Restriction analysis with a secondary enzyme is important in situations where the PFGE patterns obtained with the primary enzyme from two or more isolates are indistinguishable.

1. Label 1.5 ml microcentrifuge tubes with sample numbers; label 3 (10-well gel) or 4 (15-well gel) tubes for *Salmonella* ser. Braenderup H9812 standards.

2. Prepare 1X restriction buffer by diluting the appropriate 10X restriction buffer (provided with each restriction enzyme by the vendor 1:10 with sterile Ultrapure water (CLRW) according to the following tables:

Note: An acceptable option for CLRW is type I or Milli-Q water.

***Sma*I digestion buffer**

Reagent	µl/Plug Slice
Sterile Ultrapure Water	180 µl
Restriction Buffer 4	20 µl
Total Volume	200 µl

***Xba*I digestion buffer**

Reagent	µl/Plug Slice
Sterile Ultrapure Water	180 µl
Restriction Buffer H	20 µl
Total Volume	200 µl

***Xho*I digestion buffer**

Reagent	µl/Plug Slice
Sterile Ultrapure Water	178 µl
Restriction Buffer 4	20 µl
100X BSA	2 µl
Total Volume	200 µl

Note: Use buffer H (Roche) for the standard plugs and buffer 4 (New England Biolabs) for the sample plugs. Buffer 4 requires the addition of BSA when the plugs are going to be digested with *XhoI*.

3. Add 200 µl diluted restriction buffer (1X) to the corresponding (samples or standards) 1.5 ml microcentrifuge tubes.

4. Carefully remove plug from TE with spatula and place in a sterile disposable Petri dish or on a large glass slide.

5. Cut a 2.0 to 2.5 mm wide slice from test sample plugs with a single edge razor blade (or scalpel, coverslip, etc.) and transfer to tube containing appropriate diluted restriction buffer. Be sure plug slice is under buffer. Replace rest of plug into the original tube that contains TE buffer. Store at 4ºC.

Note: The shape and size of the plug slice that is cut will depend on the size of the comb teeth that are used for casting the gel. PulseNet recommends that the combs with larger teeth (10 mm wide teeth) be used to cast the gels because computer analysis of the gel lanes is more accurate and less tedious than analysis of gel lanes cast with combs with the smaller teeth (5.5-mm). The number of slices that can be cut from the plugs will depend on the skill and experience of the operator, integrity of the plug, and whether the slices are cut vertically or horizontally (plugs made in disposable molds).

6. Cut three or four 2.0 to 2.5 mm wide slices from plug of the *S.* ser. Braenderup H9812 standard and transfer to tubes with diluted restriction buffer H. Be sure plug slices are under buffer. Replace rest of plug in original tube that contains TE buffer. Store at 4ºC.

7. Incubate sample and standard plug slices in 37ºC water bath (for plugs that will be digested with *Xba*I or *Xho*I) or 25ºC water bath (for plugs that will be digested with *Sma*I) for 10 minutes.

8. Prepare a restriction enzyme master mix with the appropriate restriction enzyme per sample according to the following tables. Prepare enough for each plug slice plus one additional aliquot.

***Sma*I (20U/µl stock concentration)**

Reagent	µl/Plug Slice
Sterile Ultrapure Water	177.5 µl
10X Restriction Buffer 4	20 µl
Enzyme (50 U/sample)	2.5 µl
Total Volume	200 µl
Incubate	25°C, 4 hours

***Xba*I (20U/µl stock concentration)**

Reagent	µl/Plug Slice
Sterile Ultrapure Water	175 µl
10X Restriction Buffer H	20 µl
Enzyme (100 U/sample)	5 µl
Total Volume	200 µl
Incubate	37°C, 2 hours

***Xho*I (20U/µl stock concentration)**

Reagent	µl/Plug Slice
Sterile Ultrapure Water	173 µl
10X Restriction Buffer 4	20 µl
100X BSA	2 µl
Enzyme (100 U/sample)	5 µl
Total Volume	200 µl
Incubate	37°C, 3 hours

Note: Digest *Salmonella* standard plugs with Roche *Xba*I and sample plugs with New England Biolabs *Sma*I or *Xho*I. These restriction enzymes give satisfactory results at CDC. Restriction enzymes provided by other vendors have not been evaluated. Be certain that the restriction buffer that is used is recommended by the vendor for the corresponding restriction enzyme and calculate the volume of enzyme needed to achieve the same final concentrations as indicated in the tables. Keep vials of restriction enzymes on ice or in insulated storage box (-20˚C) at all times.

a. **Addition of Bovine Serum Albumin (BSA):** Several restriction enzyme vendors specifically recommend the addition of 1X BSA to enzyme restriction mixtures. However, BSA can be added to all enzyme restriction mixtures and may assist in reducing the incidence of incomplete restriction. If BSA is added to the enzyme reaction mixture, the volume of BSA added should be deducted from the volume of water to maintain the total volume of 200 µl per slice.

9. After incubation, remove buffer from plug slice using a pipet fitted with 200-250 µl tip all the way to bottom of tube and aspirate buffer. Be careful not to damage the plug slice with pipet tip and that plug slice is not discarded with pipet tip.

10. Add 200µl of the appropriate restriction enzyme cocktail to each of the tubes, making sure the plug is completely submerged in the solution. Close the tubes and mix by gently tapping.

11. Incubate the plug slices with restriction enzymes at each of their respective temperatures and times.

LOADING PFGE PLUG SLICES AND POURING AGAROSE GEL

1. **Confirm that water bath is equilibrated to 55°C ±2°C.**

2. Make volume of 0.5X Tris-Borate EDTA Buffer (TBE) that is needed for both the gel and electrophoresis running buffer according to one of the following tables.

5X TBE:

Reagent	Volume in milliliters (ml)					
5X TBE	200	210	220	230	240	250
Reagent Grade Water[1]	1800	1890	1980	2070	2160	2250
Total Volume of 0.5X TBE	2000	2100	2200	2300	2400	2500

10X TBE:

Reagent	Volume in milliliters (ml)					
10X TBE	100	105	110	115	120	125
Reagent Grade Water	1900	1995	2090	2185	2280	2375
Total Volume of 0.5X TBE	2000	2100	2200	2300	2400	2500

2. Make 1% SeaKem Gold (SKG) agarose in 0.5X TBE as follows:
   a. Weigh appropriate amount of SKG into 500 ml screw-cap flask.
   b. Add appropriate amount of 0.5X TBE; swirl gently to disperse agarose.
   c. Loosen or remove cap and cover loosely with clear film, and microwave for 60-sec; mix gently and repeat for 15-sec intervals until agarose is completely dissolved.
   d. Recap flask and return to 55°C ±2°C water bath and equilibrate the agarose in the water bath for 15 minutes or until ready to use.

   Mix 1.0 g agarose with 100 ml 0.5X TBE for 14-cm-wide gel form (10 wells)
   Mix 1.5 g agarose with 150 ml 0.5X TBE for 21-cm-wide gel form (15 wells)

SAFETY WARNING: Use heat-resistant gloves when handling hot flasks after microwaving.

4. Place the gel mold on a leveling table and adjust until perfectly leveled. Place the comb holder so the front part (side with small metal screws) and teeth face the bottom of gel frame and the comb teeth touch the gel platform.

5. Remove restricted plug slices from the water baths. Remove enzyme/buffer mixture and add 200 µl 0.5X TBE. Incubate at room temperature for 5 min.

6. Remove plug slices from tubes; put comb on bench top and load plug slices on the bottom of the comb teeth as follows:

   a. Load *S.* ser. Braenderup H9812 standards on teeth (lanes) 1, 5, 10 (10-well gel) or on teeth 1, 5, 10, 15 (15-well gel).
   b. Load samples on remaining teeth.

7. Remove excess buffer with tissue. Allow plug slices to air dry on the comb for a minimum of 15 minutes.

[1] De-ionized water (does not need to be sterilized).

8. Position comb in gel mold and confirm that the plugs slices are correctly aligned on the bottom of the comb teeth, that the lower edge of the plug slice is flush against the black platform, and there are no bubbles.

9. Carefully pour the agarose (cooled to 55°C ± 2°C) into the gel mold and allow the gel to solidify for a minimum of 15 minutes at room temperature.

10. Put black gel frame in electrophoresis chamber and add 2.2 L freshly prepared 0.5X TBE. Close cover of unit. (The amount of buffer needed depends on whether residual buffer was left in tubing or if unit was flushed with water after the last gel was run.)

11. Turn on cooling module (14ºC), power supply, and pump (set at ≈70 to achieve a flow rate of 1 liter/minute).

12. After the gel has solidified, add 5-10 ml of 0.5X TBE to the comb and gently remove from the gel.

13. Unscrew and remove end gates from gel mold; remove excess agarose from sides and bottom of casting platform with a tissue. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber.

14. Add 860 µl of Thiourea (10mg/ml) to 0.5X TBE in the electrophoresis chamber. Close cover of chamber.

SAFETY WARNING: Thiourea is a toxic chemical. Weigh thiourea in a chemical fume hood; use gloves, eye protection, and disposable spatula when handling this chemical. Clean up any spills, and wipe down balance and surrounding area with a moistened towel. Discard gloves, spatula, weighing paper, etc. as hazardous waste, according to the guidelines of your institution. Re-cap bottle tightly after use.

ELECTROPHORESIS CONDITIONS

1. Select the following conditions on the CHEF MAPPER:

a. Select the following conditions on the CHEF MAPPER
Auto Algorithm
30 kb - low MW
600 kb - high MW
Initial switch time: 0.5 s
Final switch time: 40 s
Change run time to 18 - 20 h (See note below)
(Default values: Initial switch time = 2.16 s; Final switch time = 54.17 s)

b. Select following conditions on **CHEF-DR III**
Initial switch time: 2.2 s
Final switch time: 54.2 s
Voltage: 6 V
Included Angle: 120°
Run time: 18-20 h (See note below)

Note: The electrophoresis running times recommended above are based on the equipment and reagents used at the CDC. Run times may be different in your laboratory and will have to be optimized for your gels so that the lowest band in the *S.* ser. Braenderup H9812 standard migrates 1.0 - 1.5 cm

from the bottom of the gel. Make note of the initial milliamp (mAmp) reading on the instrument. The initial mAmps should be between 110-170 mAmps. A reading outside of this range may indicate that the 0.5X TBE buffer was prepared improperly and the buffer should be remade.

Day 5

STAINING AND DOCUMENTATION OF PFGE AGAROSE GEL

1. When electrophoresis run is over, turn off equipment; remove and stain gel with ethidium bromide. Dilute 50 µl of ethidium bromide stock solution (10 mg/ml) with 500 ml of reagent grade water (this volume is for a staining box that is approximately 14-cm x 24-cm; a larger container may require a larger amount of staining solution). Stain gel for 20 minutes in covered container with gentile agitation.

Note: Use extreme caution when handling Ethidium Bromide, as it is carcinogenic and a mutagenic chemical. Stock solutions of 10 mg/ml Ethidium Bromide (EtBr) in water are available from several commercial companies (Amresco X328; Bio-Rad, 161-0433; Sigma, E-1510). CDC does not recommend disposing of EtBr down the drain. Aqueous solutions containing EtBr can be filtered through charcoal or degraded using activated carbon destaining or "tea" bags from Amresco (E732-25 Destaining Bags) or other companies, which effectively and safely remove EtBr from solutions and gels. Once the EtBr is removed, the treated aqueous solutions can be discarded down the drain. If you have further questions about EtBr please refer to the **Material Safety Data Sheets** (**MSDS**) provided by the vendor or manufacturer.

2. Destain gel in approximately 500 ml reagent grade water for 60 min; changing water every 20 minutes. Capture image using a Gel Doc 1000, 2000, EQ, or XR, or equivalent documentation system. If too much background is observed destain for an additional 30-60 min.

   a. Alternative Method for PFGE Gel Staining (GelStar): Dilute 40 ul of GelStar (10,000X stock solution) into 400 ml of 1X TBE. Stain gel for 60 minutes in covered container with gentile agitation.

   Note: Destaining is not necessary when staining the gel with GelStar. After staining, proceed to capturing the image with a gel documentation system. Stock solutions of GelStar are available from Lonza, 50535. **Use the same precautions when handling and disposing of GelStar as indicated above for EtBr.**

3. Capture image using a Gel Doc 1000, 2000, EQ, or XR, or equivalent documentation system.

4. Follow directions given with the imaging equipment to save gel image as an ***.img** or ***.1sc** file; convert this file to ***.tif** file for analysis with the BioNumerics software program. The gel image should fill the entire window of the imaging equipment (computer) screen (without cutting off wells or lower bands). Ensure that the image is in focus and that there is little to no staturation (over-exposure) in the bands. Additional instructions are provided in PNL07 of the PulseNet QA/QC manual.

5. Drain buffer from electrophoresis chamber and discard. Rinse chamber with 2-4 L reagent grade water or, if unit is not going to be used for several days, flush lines with water by letting pump run for 5-10 min before draining water from chamber and hoses. Remove and clean loose pieces of agarose in the electrophoresis chamber.

6. If the lowest band in the H9812 standard does not migrate within 1 -1.5 cm of the bottom of the gel, the run time will need to be determined empirically for the conditions in each laboratory.

Please note the following if PFGE results do not have to be available within 24-28 hours:

1. Plugs can be lysed for longer periods of time (3-16 hours).

2. The washing steps with TE to remove the lysis buffer from the PFGE plugs can be done for longer periods of time (30-45 min) and at lower temperatures (37°C or room temperature). They can be started on Day 1 and finished on Day 2 after overnight refrigeration of the plugs in TE.

3. The restriction digestion can be done for longer periods of time (3-16 hours).

Use of trade names and commercial sources is for identification purposes only and does not imply endorsement by CDC or the U.S. Department of Health and Human Services.

ALTERNATIVE PROCEDURE FOR PFGE OF CLOSTRIDIUM BOTULINUM

Some strains may not be typeable by the method described above. The following procedure can be used as an alternative method to perform PFGE on botulinum toxin producing clostridia.

PREPARATION OF PFGE PLUGS FROM TEST CULTURES

Day 1

1. Streak each test culture for colony isolation onto Egg Yolk Agar Plate.

2. Incubate the plates at 35± 2°C under anaerobic conditions until isolated colonies are present (18-48 hours).

Day 2

1. Inoculate a single colony from each plate into 10ml of Trypticase Peptone Glucose Yeast Extract (TPGY) medium.

2. Incubate the TPGY tubes at 35± 2°C under anaerobic conditions until growth is evident in the TPGY media.

Day 3

1. Prepare 2X Cell Lysis Buffer (12mM Tris, 2M NaCl, 200 mM EDTA, 1% Brij 58, 0.4%, Deoxycholate, 5% Sarcosyl) as follows:

   1.2 ml of 1 M Tris-HCl, pH 8.0
   40 ml of 5 M NaCl
   40 ml of 0.5 M EDTA, pH 8.0
   1 g Brij 58
   0.4 g Deoxycholate
   5 g Sarkosyl
   Dilute to 100 ml with sterile Ultrapure water (CLRW)

Note: Acceptable options for CLRW is type I or Milli-Q water.

2. Prepare PIV Buffer as follows:

   5 ml of 1 M Tris-HCl, pH 8.0

100 ml of 5 M NaCl
Dilute to 500 ml with sterile Ultrapure water (CLRW)

Note: Acceptable options for CLRW is type I or Milli-Q water.

3. Prepare Lysozyme (Sigma L7651 or equivalent) stock solution (20 mg/mL in TE) as follows:
   a. Weigh out 100 mg Lysozyme (keep the container of Lysozyme on ice)
   b. Add 5 mL TE buffer, swirl to mix
   c. Aliquot 250 uL amounts into small eppendorf tubes and freeze for future use.

4. Prepare Mutanolysin (Sigma M9901 or equivalent) stock solution (5 U/µL in TE) as follows:
   a. Add 1 mL TE buffer to vial of lyophilized Mutanolysin, swirl to mix
   b. Aliquot 50 µL amounts into small eppendorf tubes and freeze for future use.

5. Prepare 1.8% SKG Gold Agarose in TE Buffer (10 mM Tris: 1 mM EDTA, pH 8.0) for PFGE plugs as follows:

   a. Weigh 0.18 g (or 0.36 g) of SeaKem Gold (SKG) agarose into 250 ml screw-cap flask
   b. Add 10.0 ml (or 20.0 ml) TE Buffer; swirl gently to disperse agarose.
   c. Loosen or remove cap and cover loosely with clear film, and microwave for 30-sec; mix gently and repeat for 10-sec intervals until agarose is completely dissolved.
   d. Recap flask and place in 55± 2°C water bath and equilibrate the agarose in the water bath for 15 minutes or until ready to use.

SAFETY WARNING: Use heat-resistant gloves when handling hot flasks after microwaving.

Note: SeaKem Gold agarose works well for making PFGE plugs because it provides added strength to the plugs minimizing breakage of plugs during the lysis and washing steps. The time and temperature needed to completely dissolve the agarose is dependent on the specifications of the microwave used, and will have to be determined empirically in each laboratory.

6. Take out tubes of Lysozyme (20 mg/ml) and Mutanolysin (5U/µl) needed from the -20ºC freezer.

7. Place the 2X Cell Lysis Buffer in a 37± 2°C water bath and the PIV buffer on ice.

8. Remove the TPGY cultures from the anaerobic incubator and pipet the entire 10 ml into labeled Nalgene 50 ml centrifuge tubes and keep on ice.

9. Centrifuge at 1500 × *g* for 15 minutes at 4°C.

10. Prepare a master mix containing diluted formaldehyde according to the following table. Prepare enough for each sample plus one additional aliquot.

Reagent	µl/Plug Slice
PIV buffer	3.6 ml
Formaldehyde (35-40%)	400 µl

Note: This step should be done using a chemical fume hood.

11. Remove the Nalgene tubes containing the bacterial pellets from the centrifuge and decant the supernatant into a waste container containing bleach.

12. Gently resuspend each bacterial pellet with 4 ml of PIV + formaldehyde and place on ice for 1 hour with gentle shaking every 15 minutes.

Note: Steps 11 and 12 should be performed in a Class 2 biological safety cabinet.

13. Centrifuge at 1500 × *g* for 15 minutes at 4°C.

14. Decant the supernatants into a waste container in a chemical fume hood.

15. Resuspend each bacterial pellet in 4 ml of cold PIV buffer.

16. Repeat step 13 - 15 two more times.

17. Centrifuge at 1500 × g for 15 minutes at 4°C.

18. Decant the supernatants into a waste container in a chemical fume hood.

19. Prepare a master mix containing 2X Cell Lysis Buffer (pre-warmed to 37°C), RNase A, mutanolysin, and lysozyme according to the following table. Prepare enough for each sample plus one additional aliquot.

Reagent	µl/Plug Slice
2X Cell Lysis Buffer	500 µl
RNase (100mg/ml)	0.2 µl
Mutanolsyin	4 µl
Lysozyme	50 µl

20. Resuspend each bacterial pellet in 500 µl of this master mix and transfer to labeled microcentrifuge tubes.

21. Add 500 µl of melted agarose to each cell suspension, mix gently a few times, and immediately transfer to PFGE plug molds. Agarose suspension is enough to fill two plug molds.

Note: Prewarm the cell suspensions at 55± 2°C for ≥30 seconds prior to adding the agarose.

22. Allow the plugs to solidify in the plug molds at 4± 2°C for at least 15 minutes.

23. Prepare a master mix containing 1X Cell Lysis Buffer (prewarmed to 37°C), RNase A, mutanolysin, and lysozyme according to the following table. Prepare enough for each sample plus one additional aliquot.

Reagent	µl/Plug Slice
2X Cell Lysis Buffer	2 ml
Distilled Water	2 ml
RNase (100mg/ml)	0.8 µl
Mutanolsyin	16 µl
Lysozyme	200 µl

24. Pipet 4 ml of this master mix into separate, labeled 50 ml conical tubes for each sample. Fit a green screen cap onto each 50 ml conical tube and prewarm at 37± 2°C.

25. Remove the plug molds from the refrigerator and using a spatula remove the sample plugs and place into the corresponding 50 ml conical tubes containing the prewarmed lysis solutions.

26. Place the conical tubes containing the plugs into the shaking water bath at 37± 2°C and incubate overnight shaking at approximately 60 rpm overnight.

27. Place both sections of the plug mold, spatulas, and scalpel in 10% bleach. Soak them for 1 hour before washing them.

Day 4

1. Prepare ES buffer by mixing 500 ml of 0.5M EDTA and 50 g of Sarkosyl.

2. Place one bottle of distilled water and two bottles of TE buffer in a water bath at 50± 2°C. One glass bottle containing distilled water and one glass bottle of TE should be equipped with dispensing apparatuses.

3. Decant the lysis solution from each conical tube containing PFGE plugs into a waste container containing bleach.

4. Add 4 ml of pre-warmed TE into each 50 ml conical tube containing plugs and decant the TE into a waste container containing bleach.

5. Calculate the volume of Proteinase K required to yield 0.1 mg/ml final concentration as follows:

   (stock conc. of Proteinase K) (X) = (final conc. desired) (final volume)

   (stock conc. of Proteinase K) (X) = (0.1 mg/ml) (4 ml)

   $$\frac{(0.1\ \text{mg/ml}) \times (4\ \text{ml})}{\text{(stock conc. of Proteinase K)}} = \textbf{volume of Proteinase K needed (ml)/sample}$$

6. Add 4 ml ES buffer and the calculated volume of Proteinase K into each conical tube. Incubate the conical tubes containing the plugs at 50± 2°C shaking at approximately 60 rpm for 3 hours.

7. Repeat steps 3, 4, and 6 one more time.

8. Decant the lysis solution from each conical tube containing PFGE plugs into a waste container containing bleach.

9. Add 10 ml of pre-warmed distilled water into each 50 ml conical tube containing plugs and incubate at 50± 2°C shaking at approximately 60 rpm for 10 minutes. Decant used distilled water into a waste container containing bleach.

10. Repeat step 9 one more time.

11. Add 20 ml of pre-warmed TE buffer into each 50 ml conical tube containing plugs and incubate at 50± 2°C shaking at approximately 60 rpm for 10 minutes. Decant used TE buffer into a waste container containing bleach..

12. Repeat step 11 four more times.

13. Store the PFGE plugs in the 50 ml conical tubes containing ≥10 ml TE at 4± 2°C until ready for digestion. Continue with step 1 in "Restriction Digestion" section or store plugs in TE Buffer at 4ºC until needed. Plugs can be transferred to smaller tubes containing 1 ml of TE for storage.

Day 5

RESTRICTION DIGESTION OF DNA IN AGAROSE PLUGS

Note: A small slice of the plug or the entire plug (made in disposable plug molds) can be digested with the restriction enzyme. Restriction digestion of a small slice of the plug is recommended because less enzyme is required and other slices of the plug can be subjected to restriction analysis with other enzymes. Restriction analysis with a secondary enzyme is important in situations where the PFGE patterns obtained with the primary enzyme from two or more isolates are indistinguishable.

1. Label 1.5 ml microcentrifuge tubes with sample numbers.

2. Prepare 1X restriction buffer by diluting the appropriate 10X restriction buffer (provided with each restriction enzyme by the vendor) 1:10 with distilled water according to the following tables:

***Sma*I digestion buffer**

Reagent	µl/Plug Slice
Sterile Distilled Water	180 µl
Restriction Buffer 4	20 µl
Total Volume	200 µl

***Xho*I digestion buffer**

Reagent	µl/Plug Slice
Sterile Distilled Water	178 µl
Restriction Buffer 4	20 µl
100X BSA	2 µl
Total Volume	200 µl

3. Add 200 µl diluted restriction buffer (1X) to the corresponding 1.5 ml microcentrifuge tubes.

4. Carefully remove plug from TE with spatula and cut a 2.0 to 2.5 mm wide slice from test sample plugs with a single edge razor blade.

Note: The shape and size of the plug slice that is cut will depend on the size of the comb teeth that are used for casting the gel. PulseNet recommends that the combs with larger teeth (10 mm wide teeth) be used to cast the gels because computer analysis of the gel lanes is more accurate and less tedious than analysis of gel lanes cast with combs with the smaller teeth (5.5-mm). The number of slices that can be cut from the plugs will depend on the skill and experience of the operator, integrity of the plug, and whether the slices are cut vertically or horizontally (plugs made in disposable molds).

5. Transfer the plug slices into microcentrifuge tube containing appropriate diluted restriction buffer. Be sure plug slice is under buffer. Replace rest of plug into the original tube that contains TE buffer.

6. Incubate 1.5 ml microcentrifuge tubes containing plugs with restriction buffer for *SmaI* digestions at 25± 2°C and restriction buffer for *XhoI* digestions at 37± 2°C for 1 hour.

7. Prepare restriction enzyme master mixes with the appropriate restriction enzyme per sample according to the following tables. Prepare enough for each plug slice plus one additional aliquot.

***Sma*I (20U/µl stock concentration)**

Reagent	µl/Plug Slice
Sterile Distilled Water	175 µl
10X Restriction Buffer 4	20 µl
Enzyme (100 U/sample)	5 µl
Total Volume	200 µl

***Xho*I (20U/µl stock concentration)**

Reagent	µl/Plug Slice
Sterile Distilled Water	173 µl
10X Restriction Buffer 4	20 µl
100X BSA	2 µl
Enzyme (100 U/sample)	5 µl
Total Volume	200 µl

8. After incubation, remove buffer from plug slice using a pipet fitted with 200-250 µl tip all the way to bottom of tube and aspirate buffer. Be careful not to damage the plug slice with pipet tip and that plug slice is not discarded with pipet tip.

9. Add 200µl of the appropriate restriction enzyme cocktail to each of the tubes, making sure the plug is completely submerged in the solution. Close the tubes and mix by gently tapping.

10. Incubate 1.5 ml microcentrifuge tubes containing plugs with restriction buffer *SmaI* at 25± 2°C and with restriction buffer *XhoI* at 37± 2°C overnight.

11. Soak the used green screen caps in 10% bleach for at least one hour and rinse with sterile water prior to storage.

Day 6

1. Label 1.5 ml microcentrifuge tubes for *Salmonella* ser. Braenderup H9812 standards. One standard plug is needed per every 3-4 samples.

2. Prepare 1X restriction buffer by diluting the appropriate 10X restriction buffer (provided with each restriction enzyme by the vendor) 1:10 with distilled water according to the following tables:

***Xba*I digestion buffer**

Reagent	µl/Plug Slice
Sterile Ultrapure Water	180 µl
Restriction Buffer H	20 µl
Total Volume	200 µl

3. Add 200 µl diluted restriction buffer (1X) to the corresponding 1.5 ml microcentrifuge tubes.

4. Carefully remove standard plugs from cryovials containing TE and cut 2.0 to 2.5 mm wide slices with a single edge razor blade.

5. Transfer the plug slices to tubes containing the diluted restriction buffer. Be sure each plug slice is under the buffer. Replace the rest of the plug into the cryovial and store at $4^{o}C \pm 2^{o}C$.

6. Incubate 1.5 ml microcentrifuge tubes containing plugs with restriction buffer for *XbaI* digestions at $37^{o}C \pm 2^{o}C$ for 10 minutes.

7. Prepare a restriction enzyme master mix with the appropriate restriction enzyme per sample according to the following table. Prepare enough for each plug slice plus one additional aliquot.

***Xba*I (20U/µl stock concentration)**

Reagent	µl/Plug Slice
Sterile Distilled Water	175 µl
10X Restriction Buffer H	20 µl
Enzyme (100 U/sample)	5 µl
Total Volume	200 µl

8. After incubation, remove buffer from plug slice using a pipet fitted with 200-250 µl tip all the way to bottom of tube and aspirate buffer. Be careful not to damage the plug slice with pipet tip and that plug slice is not discarded with pipet tip.

9. Add 200µl of the *XbaI* restriction enzyme cocktail to each of the tubes, making sure the plug is completely submerged in the solution. Close the tubes and mix by gently tapping.

10. Incubate 1.5 ml microcentrifuge tubes containing plugs with *XbaI* at $37^{o}C \pm 2^{o}C$ for 2 hours.

LOADING PFGE PLUG SLICES AND POURING AGAROSE GEL

11. Make 0.5X Tris-Borate EDTA Buffer (TBE) that is needed for both the gel and electrophoresis running buffer by adding 125 ml of 10X TBE into 2,375 ml distilled water.

Note: Steps 12-24 are repeated for each gel that will be run.

12. Prepare 1% agarose by measuring 1.5 g of SKG Gold Agarose into a 250 ml bottle. Add 150 ml of 0.5X TBE buffer to the bottle and microwave on high power until completely melted. Transfer the bottle containing 1% agarose into the water bath set at 55± 2°C.

13. Place the gel mold on a leveling table and adjust until perfectly leveled. Place the comb holder so the front part (side with small metal screws) and teeth face the bottom of gel frame and the comb teeth are slightly above the surface of the gel mold.

14. Remove all of the restricted plug slices (digested with *SmaI*, *XhoI*, or *XbaI*) from the water baths. Remove enzyme/buffer mixture and add 200 µl 0.5X TBE. Incubate at room temperature for 5 min.

15. Remove plug slices from tubes; put comb on bench top and load plug slices on the bottom of the comb teeth.

16. Position comb in gel mold and confirm that the plugs slices are correctly aligned on the bottom of the comb teeth and that the lower edge of the plug slice is flush against the black platform, and there are no bubbles.

17. Carefully pour the agarose (cooled to 55°C ± 2°C) into the gel mold and allow the gel to solidify for a minimum of 15 minutes at room temperature.

18. Put black gel frame in electrophoresis chamber and add 2.2 L freshly prepared 0.5X TBE. Close cover of unit. (The amount of buffer needed depends on whether residual buffer was left in tubing or if unit was flushed with water after the last gel was run.)

19. Turn on cooling module (14ºC), power supply, and pump (set at ≈70 to achieve a flow rate of 1 liter/minute).

20. After the gel has solidified, add 5-10 ml of 0.5X TBE to the comb and gently remove from the gel.

21. Unscrew and remove end gates from gel mold; remove excess agarose from sides and bottom of casting platform with a tissue. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber.

22. Add 860 µl of Thiourea (10mg/ml) to 0.5X TBE in the electrophoresis chamber. Close cover of chamber.

SAFETY WARNING: Thiourea is a toxic chemical. Weigh thiourea in a chemical fume hood; use gloves, eye protection, and disposable spatula when handling this chemical. Clean up any spills, and wipe down balance and surrounding area with a moistened towel. Discard gloves, spatula, weighing paper, etc. as hazardous waste, according to the guidelines of your institution. Re-cap bottle tightly after use.

ELECTROPHORESIS CONDITIONS

1. Select the following conditions on the CHEF MAPPER:

   a. Select the following conditions on the CHEF MAPPER
   Auto Algorithm
   30 kb - low MW
   600 kb - high MW
   Initial switch time: 0.5 s
   Final switch time: 40 s
   Change run time to 18 - 20 h (See note below)
   (Default values: Initial switch time = 2.16 s; Final switch time = 54.17 s)

   b. Select following conditions on **CHEF-DR III**
   Initial switch time: 2.2 s
   Final switch time: 54.2 s
   Voltage: 6 V
   Included Angle: 120°
   Run time: 18-20 h (See note below)

Day 7

STAINING AND DOCUMENTATION OF PFGE AGAROSE GEL

1. When electrophoresis run is over, turn off equipment; remove and stain gel with ethidium bromide. Dilute 50 µl of ethidium bromide stock solution (10 mg/ml) with 500 ml of reagent grade water (this volume is for a staining box that is approximately 14-cm x 24-cm; a larger container may require a larger amount of staining solution). Stain gel for 20 minutes in covered container with gentile agitation.

Note: Use extreme caution when handling Ethidium Bromide, as it is carcinogenic and a mutagenic chemical. Stock solutions of 10 mg/ml Ethidium Bromide (EtBr) in water are available from several commercial companies (Amresco X328; Bio-Rad, 161-0433; Sigma, E-1510). CDC does not recommend disposing of EtBr down the drain. Aqueous solutions containing EtBr can be filtered through charcoal or degraded using activated carbon destaining or "tea" bags from Amresco (E732-25 Destaining Bags) or other companies, which effectively and safely remove EtBr from solutions and gels. Once the EtBr is removed, the treated aqueous solutions can be discarded down the drain. If you have further questions about EtBr please refer to the Material Safety Data Sheets (MSDS) provided by the vendor or manufacturer.

2. Destain gel in approximately 500 ml reagent grade water for 60 min; changing water every 20 minutes. Capture image using a Gel Doc 1000, 2000, EQ, or XR, or equivalent documentation system. If too much background is observed destain for an additional 30-60 min.

a. Alternative Method for PFGE Gel Staining (GelStar): Dilute 40 ul of GelStar (10,000X stock solution) into 400 ml of 1X TBE. Stain gel for 60 minutes in covered container with gentile agitation. Capture image using a Gel Doc 1000, 2000, EQ, or XR, or equivalent documentation system.

Note: Destaining is not necessary when staining the gel with GelStar. After staining, proceed to capturing the image with a gel documentation system. Stock solutions of GelStar are available from Lonza, 50535. Use the same precautions when handling and disposing of GelStar as indicated above for EtBr.

3. Follow directions given with the imaging equipment to save gel image as an *.img or *.1sc file; convert this file to *.tif file for analysis with the BioNumerics software program. The gel image should fill the entire window of the imaging equipment (computer) screen (without cutting off wells or lower bands). Ensure that the image is in focus and that there is little to no staturation (over-exposure) in the bands. Additional instructions are provided in PNL07 of the PulseNet QA/QC manual.

4. Drain buffer from electrophoresis chamber and discard. Rinse chamber with 2-4 L reagent grade water or, if unit is not going to be used for several days, flush lines with water by letting pump run for 5-10 min before draining water from chamber and hoses. Remove and clean loose pieces of agarose in the electrophoresis chamber.

5. If the lowest band in the H9812 standard does not migrate within 1 -1.5 cm of the bottom of the gel, the run time will need to be determined empirically for the conditions in each laboratory.

Use of trade names and commercial sources is for identification purposes only and does not imply endorsement by CDC or the U.S. Department of Health and Human Services.

NOTE: CLIA LABORATORY PROCEDURE MANUAL REQUIREMENTS

Efforts have been made to assure that the procedures described in this protocol have been written in accordance with the 1988 Clinical Laboratory Improvement Amendments (CLIA) requirements for a procedure manual (42 CFR 493.1211). However, due to the format required for training, the procedures will require some modifications and additions to customize them for your particular laboratory operation.

Any questions regarding the CLIA requirements for a procedure manual, quality control, quality assurance, etc., should be directed to the agency or accreditation organization responsible for performing your laboratory's CLIA inspection. In addition, some states and accreditation organizations may have more stringent requirements that will need to be addressed.

5. FLOW CHART:

6. BIBLIOGRAPHY:

7. CONTACTS:

7.1 Carolina Lúquez, PhD
Botulism Outbreak Investigations Unit
National Botulism Laboratory Team
Centers for Disease Control and Prevention
E-mail: CLuquez@cdc.gov
Phone: (404) 639-0896

8. AMENDMENTS:

8.1 1/25/12 the line **"**or 50 ml of a 10% Sarkosyl Solution" was removed from the Cell Lysis Buffer Preparation section under Day 3 step 3. This leaves only the 5g Sarkosyl in order to make the final concentration 5% Sarkosyl and the final volume as 100 ml.

8.2 **10/2012** An alternative procedure was added beginning on page 13 of this document.

8.3 **5/2/2013** *Xho*I digestion buffer amounts were corrected under Day 4 step 2 (page 7).

1. PURPOSE: to describe the standardized laboratory protocol for molecular subtyping of Shiga toxin-producing *Escherichia coli* O157 (STEC O157) and *Salmonella enterica* serotypes Typhimurium and Enteritidis.

2. SCOPE: to provide the PulseNet participants with a single protocol for performing MLVA of STEC O157 and *Salmonella* serotypes Typhimurium and Enteritidis, thus ensuring inter-laboratory comparability of the generated results.

3. DEFINITIONS:

3.1 MLVA: Multiple-locus variable-number tandem repeat analysis
3.2 VNTR: Variable-number tandem repeat
3.3 DNA: Deoxyribonucleic acid
3.4 DNase: Deoxyribonuclenase
3.5 PCR: Polymerase chain reaction
3.6 HPLC: High purity liquid chromatography
3.7 dNTP: Deoxyribonucleotide triphosphate
3.8 CDC: Centers for Disease Control and Prevention
3.9 SOP: Standard Operating Procedure

4. RESPONSIBILITIES/PROCEDURE

4.1. Biosafety warning: STEC O157 and *Salmonella* serotypes Typhimurium and Enteritidis with an infectious dose as low as 100 cells is a human pathogen capable of causing serious disease. Always use a minimum of Biosafety level 2 practices and extreme caution when transferring and handling strains of these serotypes. Work in a biological safety cabinet when handling large amounts of cells. Disinfect or dispose of all plastic ware and glassware that come in contact with the cultures in a safe manner.

4.2. Reagents, supplies and equipment needed for DNA template preparation

4.2.1 Trypticase soy agar with 5 % sheep blood (TSA-SB) or comparable media
4.2.2 1 µl inoculation loops
4.2.3 0.5 ml microcentrifuge tubes
4.2.4 DNase-free, molecular biology -grade water
4.2.5 Vortex
4.2.6 Boiling water bath or thermocycler/thermal block accommodating 0.5 ml tubes
4.2.7 Tabletop centrifuge for high rpm (up to 13,000-14,000 rpm) spinning
4.2.8 Pipets (200 µl) for aliquoting 100 µl of DNase-free, molecular biology-grade water
4.2.9 Filtered Sterile Pipet tips

4.3. Reagents, supplies and equipment needed for PCR

4.3.1 DNA templates from isolates (keep at -20^{o}C or -80^{o}C freezer for long term)
4.3.2 PCR primers (see appendix PNL28-1)
4.3.2.1 Fluorescent-labeled forward primers
4.3.2.1.1 HPLC-purified
4.3.2.2 Unlabeled reverse primers

4.3.2.2.1 Regular gel filtration purification

4.3.2.3 Biosearch Technologies (Novato, CA; www.biosearchtech.com; 1-800-436-6631) sells primers labeled with the three dyes needed for the protocol

4.3.2.4 Divide the concentrated stocks (100 µM) in portions and store at -80ºC freezer

4.3.2.4.1 One vial should contain enough to prepare 25-50 µl of working solution. Avoid repetitive freeze-thaw cycles of concentrated primer stocks.

4.3.2.5 The 1.0, 2.5, 5.0, 12.5 and 25.0 µM working solutions can be stored at either -20^{o}C or -80ºC freezer

4.3.2.6 Prepare new working solutions every month or if a significant drop in the fluorescence level is observed (for instructions refer to PNQ06_MLVA ABI certification, appendix PNQ06-5)

4.3.3 96-well polypropylene PCR plates (Fisher, Cat. No. 07-200-613) or Microamp PCR tubes without caps (Life Technologies, Cat. No. N8010533)

4.3.4 8-well strip caps for the polypropylene plate (Fisher, Cat. No. 07-200-639) or MicroAmp strip caps for the individual tubes (Life Technologies, Cat. No. N8010535)

4.3.5 DNase-free, molecular biology -grade water

4.3.6 1.5 ml Eppendorf microcentrifuge tubes

4.3.7 PCR Nucleotide Mix (ready-to-use dNTP mix containing all four nucleotides; Roche, Cat. No. 11 814 362 001)

4.3.8 Platinum Taq Polymerase with 50 mM $MgCl_2$ and 10X buffer (Life Technologies, Cat. No. 10966-034)

4.3.9 PCR Cooling block (VWR International, Cat. No. 62111-762)

4.3.10 DNA Engine (Biorad), GeneAmp (Life Technologies) or similar thermocycler with a heated lid option and 96-well block format

4.3.11 Parafilm M, 4" width (VWR, Cat. No. 52858-032)

4.3.12 Complete set (1000 µl, 200 µl, 100 µl, 20 µl, 10 µl and 2 µl) of single channel pipettors for mastermix set-up ("clean set")

4.3.13 1-10 µl single channel pipettor for adding DNA templates

4.3.14 Filtered tips for pipettors

4.3.15 Microfuge for low (up to 6,000 rpm) rpm spinning

4.4. Reagents, supplies and equipment needed for Genetic Analyzer 3500

4.4.1 DNase-free, molecular biology -grade water

4.4.2 PCR Cooling block (VWR International, Cat. No. 62111-762)

4.4.3 10 µl, 100 µl, and 1000 µl single channel pipettors

4.4.4 10 µl and 200 µl multichannel pipettors

4.4.5 Filtered pipette tips

4.4.6 Sterile solution basins

4.4.7 1.5 ml Eppendorf microcentrifuge tubes

4.4.8 96-well polypropylene (non-PCR) V-bottom plate (for dilutions; Fisher Scientific, Cat. No. 07-200-698)

4.4.9 MicroAmp Optical 96-well reaction plates (Life Technologies, Cat. No. 4306737)

4.4.10 96-well plate base (Life Technologies, Cat. No. 4317237)

4.4.11 Rubber septa for 96-well reaction plates (Life Technologies, Cat. No. 4315933)
4.4.12 96-well plate retainer (Life Technologies, Cat. No. 4317241)
4.4.13 Hi-Di Formamide (Life Technologies, 25 ml Cat. No. 4311320)
4.4.14 GeneFlo 625 DNA size standard – ROX, 800 µl (Chimerx, Cat. No. 3125-02)
4.4.15 GeneScan Fragment Install Standard DS-33 (Life Technologies, Cat. No. 4376911)
4.4.16 DS-33 Matrix Standard Kit (Dye Set G5) (Life Technologies, Cat. No. 4345833)
4.4.17 Multi-Capillary DS-30 (Dye Set D) Matrix Standard Kit (Life Technologies, Cat. No. 4345827)
4.4.17.1 Needed to establish the system dye color spectra for the instrument. Required when analyzing fragments labeled with FAM, HEX, NED, and ROX.
4.4.17.2 **NOTE:** DS-33 Matrix Standard (Dye set G5, required for analyzing fragments labeled with FAM, VIC, NED, PET, and LIZ) and the GeneScan Fragment Installation Standard are typically installed as default as part of the instrument installation process. If any of the three standards is missing from the instrument, you are not going to be able to start the run.
4.4.17.3 In order to install the DS-30 Matrix, follow the instructions of the kit insert and the "Getting Started Guide", chapter "Performing a Spectral Calibration"
4.4.18 3500xL Genetic Analyzer 24-Capillary Array 50 cm (Life Technologies, Cat. No. 4404689) or the 3500 Genetic Analyzer 8-Capillary Array (Life Technologies, Cat. No. 4404685)
4.4.19 Genetic Analyzer Cathode Buffer Container (4 pack), 1X (Life Technologies, Cat. No. 4408256)
4.4.20 Genetic Analyzer Anode Buffer Container (4 pack), 1X (Life Technologies, Cat. No. 4393927)
4.4.21 3500 Conditioning Reagent (Life Technologies, Cat. No. 4393718)
4.4.22 3500 POP7 Performance Optimized Polymer, (384 samples) (Life Technologies, Cat. No. 4393708)
4.4.23 Parafilm M, 4" width (VWR, Cat. No. 52858-032)
4.4.24 Centrifuge with a microtiter plate rotor
4.4.25 Heating block or thermal cycler accommodating a 96-well plate for denaturation
4.4.26 Microfuge for low (up to 6,000 rpm) rpm spinning

4.5. DNA template preparation

4.5.1 **Day 0:**

4.5.1.1 Streak an isolated colony from test cultures to TSA-SB plate (or comparable media). Incubate cultures at 37ºC for 14-18 hrs.

4.5.2 **Day 1:**

4.5.2.1 For each isolate to be typed, aliquot 100 µl of sterile, molecular biology-grade water into 0.5 ml microfuge tubes. Use a sterile, disposable 1 µl loop to pick 2-3 colonies (about half of a loopful); rotate the loop in the microfuge tube to release the bacteria into the water. Cap and vortex for 10-15 seconds to disperse any clumps.

4.5.2.2 Place the tubes in a 99-100ºC water bath or heat block for 10-15 minutes. Cool briefly on ice or in fridge and centrifuge for 10 minutes at 10,000 rpm. Place on ice or in fridge while preparing PCR reactions. These DNA templates can be stored at -20ºC or -80ºC for several years.

4.6. PCR procedure

4.6.1 **Day 1:**

4.6.1.1 Fill out, save with the run name, and an organism specific (copy and paste the appropriate sizing tables for the controls) MLVA Fragment Analysis ABI Worksheet (see appendix PNL28-2) with appropriately labeled samples (a maximum of 46 isolates/plate; 44 unknowns + two positive controls and a negative control; two wells are reserved for the internal ladder).

4.6.1.1.1 For each isolate, two wells must be labeled as follows: "BNkeyR1" where "BNkey" represents the isolate-specific state laboratory identification number (be sure to use the exact same isolate ID that is used in the PFGE gels uploaded to the national database) and "R1" represents one of the two specific multiplex PCR reactions (R1, R2).

4.6.1.2 Fill out, and print a PCR mastermix calculation worksheet (see appendices PNL28-3a, PNL28-3b and PNL28-3c) by typing the number of isolates to be tested (plus 2-3 extra) in the PCR mastermix calculators labeled R1 and R2. This number is highlighted in RED and is next to "number of samples to be analyzed". The mastermixes for reactions 1 and 2 (R1, R2) for one sample are as follows:

STEC O157:H7 (appendix PNL28-3a)

R1	Volume (µl)	Final conc.
PCR water	5.30	
PCR buffer (10x)	1.00	1x
$MgCl_2$ (50 mM)	0.40	2.00 mM
dNTPs (10 mM)	0.20	0.20 mM
VNTR-3F (25 µM)	0.27	0.67 µM
VNTR-3R (25 µM)	0.27	0.67 µM
VNTR-34F (5 µM)	0.24	0.12 µM
VNTR-34R (5 µM)	0.24	0.12 µM
VNTR-9F (5 µM)	0.24	0.12 µM
VNTR-9R (5 µM)	0.24	0.12 µM
VNTR-25F (2.5 µM)	0.20	0.05 µM
VNTR-25R (2.5 µM)	0.20	0.05 µM
Taq (5 U/µl)	0.20	1 U
	= 9.00	

R2	Volume (µl)	Final conc.
PCR water	5.92	

PCR buffer (10x)	1.00	1x
$MgCl_2$ (50 mM)	0.40	2.00 mM
dNTPs (10 mM)	0.20	0.20 mM
VNTR-17F (5 µM)	0.30	0.15 µM
VNTR-17R (5 µM)	0.30	0.15 µM
VNTR-19F (1 µM)	0.16	0.016 µM
VNTR-19R (1 µM)	0.16	0.016 µM
VNTR-36F (1 µM)	0.11	0.011 µM
VNTR-36R (1 µM)	0.11	0.011 µM
VNTR-37F (2.5 µM)	0.07	0.017µM
VNTR-37R (2.5 µM)	0.07	0.017µM
Taq (5 U/µl)	0.20	1 U
	= 9.00	

<u>*Salmonella* serotype Typhimurium (appendix PNL28-3b)</u>

<u>R1</u>	Volume (µl)	Final conc.
PCR water	3.81	
PCR buffer (10x)	1.00	1x
$MgCl_2$ (50 mM)	0.45	2.25 mM
dNTPs (10 mM)	0.20	0.20 mM
ST3-F (5 µM)	0.10	0.05 µM
ST3-R (5 µM)	0.10	0.05 µM
ST5-F (25 µM)	0.60	1.50 µM
ST5-R (25 µM)	0.60	1.50 µM
ST7-F (5 µM)	0.80	0.40 µM
ST7-R (5 µM)	0.80	0.40 µM
ST10-F (2.5 µM)	0.12	0.03 µM
ST10-R (2.5 µM)	0.12	0.03 µM
Taq (5 U/µl)	0.30	1.50 U
	= 9.00	

<u>R2</u>	Volume (µl)	Final conc.
PCR water	4.38	
PCR buffer (10x)	1.00	1x
$MgCl_2$ (50 mM)	0.32	1.60 mM
dNTPs (10 mM)	0.20	0.20 mM
ST2-F (25 µM)	0.36	0.90 µM
ST2-R (25 µM)	0.36	0.90 µM
ST6-F4 (5 µM)	0.56	0.28 µM
ST6-R2 (5 µM)	0.56	0.28 µM
ST8-F3 (5 µM)	0.48	0.24 µM

ST8-R2 (5 µM)	0.48	0.24 µM
Taq (5 U/µl)	0.30	1.50 U
	= 9.00	

<u>*Salmonella* serotype Enteritidis (appendix PNL28-3c)</u>

<u>R1</u>	Volume (µl)	Final conc.
PCR water	4.91	
PCR buffer (10x)	1.00	1x
$MgCl_2$ (50 mM)	0.40	2.00 mM
dNTPs (10 mM)	0.20	0.20 mM
SE1-F (2.5 µM)	0.20	0.05 µM
SE1-R (2.5 µM)	0.20	0.05 µM
SE2-F (12.5 µM)	0.32	0.40 µM
SE2-R (12.5 µM)	0.32	0.40 µM
SE8-F (2.5 µM)	0.28	0.07 µM
SE8-R (2.5 µM)	0.28	0.07 µM
SE6-F (12.5 µM)	0.34	0.43 µM
SE6-R (12.5 µM)	0.34	0.43 µM
Taq (5 U/µl)	0.20	1.00 U
	= 9.00	

<u>R2</u>	Volume (µl)	Final conc.
PCR water	5.52	
PCR buffer (10x)	1.00	1x
$MgCl_2$ (50 mM)	0.40	2.00 mM
dNTPs (10 mM)	0.20	0.20 mM
SE5-F (2.5 µM)	0.20	0.05 µM
SE5-R (2.5 µM)	0.20	0.05 µM
SE3-F (12.5 µM)	0.40	0.50 µM
SE3-R (12.5 µM)	0.40	0.50 µM
SE9-F (2.5 µM)	0.08	0.02 µM
SE9-R (2.5 µM)	0.08	0.02 µM
Taq (5 U/µl)	0.20	1.00 U
	= 9.00	

4.6.1.2.1. **NOTE:** these primer concentrations serve as a starting point. Since laboratory-specific factors, such as the age of the primer stocks, calibration status of the thermocyclers and pipettes, etc., affect amplification efficiency, each laboratory will have to re-optimize the primer concentrations for optimal detection of all targets. However, any other parameters stated in the SOP should not be changed.

4.6.1.3 Thaw all reagents and supplies needed for PCR reactions and place on ice; keep primers light protected as much as possible

4.6.1.3.1 **NOTE:** PCR mastermixes should be set up in a clean hood that is dedicated just for this purpose and where no cultures or DNA are handled.

4.6.1.4 Prepare the two separate PCR mastermixes in 1.5 ml Eppendorf tubes following the instructions in the PCR mastermix calculation worksheet (see appendices PNL28-3a, PNL28-3b and PNL28-3c). Keep the mastermix on ice while preparing. Add the mastermix components in the following order: water, 10x PCR buffer, Mg_2Cl, dNTPs, primers, and then finally Taq polymerase. Mix the reaction mixture by vortexing briefly.

4.6.1.4.1 **NOTE**: All components except Taq polymerase should be vortexed thoroughly before adding to the mastermix. Taq may be briefly centrifuged with low rpm, if necessary, to pull the enzyme down to the bottom of the tube.

4.6.1.5 Place a 96-well PCR plate or required number of PCR tubes in a PCR cooling block.

4.6.1.6 Dispense 9.0 µl of each mastermix into the appropriate rows of the 96-well polypropylene plate / PCR tubes as noted in the MLVA Fragment Analysis ABI Worksheet (see appendix PNL28-2).

4.6.1.7 Add 1 µl of PCR water to each of the two different wells representing the negative controls of the two reactions.

4.6.1.8 Add 1.0 µl of DNA template to each of the two different wells representing the two PCR reactions for each isolate to be tested.

4.6.1.9 Add the positive controls (it is recommended to run the positive control as a duplicate).

4.6.1.9.1 Use STEC O157 strain EDL933 (ATCC 43895) as a positive control. The internal ladder to be used will be comprised of pooled PCR products of the isolates EC04PN0139 and EC04PN0570 (see appendix PNL28-4 for instructions for ladder preparation).

4.6.1.9.2 Use *S. enterica* serotype Typhimurium strain LT2 (ATCC 29946) as a positive control. The internal ladder to be used will be comprised of pooled PCR products of the isolates CDC_2009K0825 and CDC_2009K0826 (see appendix PNL28-4 for instructions for ladder preparation).

4.6.1.9.3 Use *S. enterica* serotype Enteritidis strain K1891 (ATCC25928) as a positive control. The internal ladder to be used will be comprised of pooled PCR products of the isolates H9560 and 2010K0017 (see appendix PNL28-4 for instructions for ladder preparation).

4.6.1.10 Cover all wells / tubes with 8-well strip caps and firmly clamp down to avoid any evaporation during PCR amplification.

4.6.1.11 Recommendation: briefly spin down the plate / tubes to remove any air bubbles.

4.6.1.12 Program and save the following two PCR cycling conditions:

STEC O157:H7 and *Salmonella* serotype Enteritidis

"O157-SEMLVA"

* 95ºC for 5 min	Step 1
* 94ºC for 20 sec	Step 2
* 65ºC for 20 sec	Step 3
* 72ºC for 20 sec	Step 4
* Go to step 2, 34x	Step 5
* 72ºC for 5 min	Step 6
* Indefinite hold at 4ºC	Step 7

Salmonella serotype Typhimurium

"STMLVA"

* 95ºC for 5 min	Step 1
* 94ºC for 20 sec	Step 2
* 63ºC for 20 sec	Step 3
* 72ºC for 20 sec	Step 4
* Go to step 2, 34x	Step 5
* 72ºC for 5 min	Step 6
* Indefinite hold at 4ºC	Step 7

4.6.1.12.1 **NOTE:** Make sure to use the heated lid option on the PCR block and tube (calculated) temperature control.

4.6.1.13 When the PCR is complete store the amplification products light-protected at 4ºC until ready to run on the sequencer. If the fragment analysis is not performed the same day, the plate should be stored at -20ºC or -80ºC. The PCR products are stable for approximately one month, when stored frozen.

4.7 Initial setup of Genetic Analyzer 3500 instrument before the first run:

NOTE: step 4.7 only needs to be performed before the very first run. In future runs continue directly to step 4.8.

4.7.1 Click on the "3500" icon to open the 3500 Data Collection Software. The software will open a window and the main screen will display the "Dashboard". If the connections are functioning properly, the status light on the sequencer will be green, the "State" of the instrument will be "Idle", and all of the consumables should have the number of days/samples remaining for each item.

4.7.1.1 **NOTE:** Prior to the first run, but after capillary array installation, the GeneScan Fragment Install Standard DS-33, DS-33 and DS-30 matrix standards must be installed. In order to run the DS-30 dye matrix standard, the "dye set D" parameters must be set up in the Library under the submenu "Dye Set". To do this, click "Create" in the toolbar at the top of the window. In the new window, name the new dye set "D" and from the "dye" drop-down menu select "Any

Dye". Uncheck the boxes (not shaded in gray) for the Purple and Orange dyes, and click "Save" at the bottom of the window.

4.7.2 Setting up the Size Standard

4.7.2.1 Click on the "Library" icon at the top right of the screen.

4.7.2.2 At the left-hand side of the screen select the "Size Standards" menu option.

4.7.2.3 At the top of this screen select "Create" in the toolbar.

4.7.2.4 A new window will appear and in the box next to "Size Standard" enter in "GeneFlo625".

4.7.2.5 From the "Dye Color" drop-down menu select "Red".

4.7.2.6 In the "Enter new Size Standard definition" box enter the fragment sizes for each peak (refer to the GeneFlo 625 product insert for the peak sizes). Separate each size using a comma.

4.7.2.7 When finished, click the "Add Size(s)" button, then click "Save" and "Close"

4.7.3 Setting up Run Parameters:

4.7.3.1 Once the fragment sizes have been assigned the Assay, File Name Conventions, and Results Group will need to be set up.

4.7.3.1.1 <u>Assay</u>

4.7.3.1.1.1 At the left-hand side of the screen select the "Assays" menu option and click "Create" in the toolbar at the top of the screen.

4.7.3.1.1.2 For the "Assay Name" enter "Fragtest" and from the "Application Type" drop-down menu select "Fragment". Under the "Protocols" heading make sure "No" is selected for assigning multiple instrument protocols.

4.7.3.1.1.3 Next to "Instrument Protocol" select the "Create New" button. A new window will appear.

4.7.3.1.1.3.1 From the "Dye Set" drop-down menu select "D".

4.7.3.1.1.3.2 For the "Run Module" select "FragmentAnalysis50_POP7xl".

4.7.3.1.1.3.3 An alert window will pop up asking if you would wish to assign a protocol name. Click "Yes" and next to the "Protocol Name" replace the '_1' with '_2' (i.e. - FragmentAnalysis50_POP7xl_2). Use the default values for all other parameters. This will create a new protocol with the specified criterion.

4.7.3.1.1.3.3.1 **NOTE:** These are the instrument default running conditions for Fragment Analysis with a 50 cm capillary array and POP7 polymer. You can check the running conditions by clicking the "Library" button at the top right of the dashboard and then selecting the "Assays" menu option on the left-hand side of the screen. The default conditions are:

* Oven_Temperature: 60^{o}C
* PreRun_Voltage: 15.0 kVolts
* Pre-Run-Time: 180 sec.
* Injection_Voltage: 1.6 kVolts
* Injection_Time: 15 sec.

* Data_Delay_Time: 1 sec.
* Run_Voltage: 19.5 kVolts
* Run time: 1330 sec.

4.7.3.1.1.3.4 When finished, click "Save to Library" and then click "Yes" in the pop-up window that asks to apply the changes to the assay. Click "OK" to confirm, and then close this window.

4.7.3.1.1.4 Next to the "Sizecalling Protocol" option select the "Create New" button.

4.7.3.1.1.4.1 In the new window enter "MLVAsizecalling" as the "Protocol Name".

4.7.3.1.1.4.2 From the "Size Standard" drop-down menu select "GeneFlo625"

4.7.3.1.1.4.3 Input the following analysis settings:

* Analysis: Full Range
* Sizing: Partial Sizing
    * Start Size: 50
    * Stop Size 625
* Size Calling Method: Local Southern Method
* Primer Peak: Present
* Minimum Peak Height:
    * Blue: 600
    * Green: 600
    * Yellow: 600
    * Red: 20
    * Uncheck Purple and Orange
* Smoothing: none
* Baseline window: 51 pts
* Min. Peak Half Width: 2 pts
* Peak Window Size: 21 pts
* Polynomial Degree: 2
* Slope Threshold Peak Start: 0.0
* Slope Threshold Peak End: 0.0

4.7.3.1.1.4.4 Click on "Save to Library" and "Yes" to apply to the assay. Select "OK" to confirm and close this window when finished.

4.7.3.1.1.5 Once all protocols have been established for the new assay, click "Save" and close the window to return to the "Library" main screen.

4.7.3.1.2 <u>File Name Conventions</u>

4.7.3.1.2.1 Access the "File Name Conventions" menu on the left-hand side of the screen. In the toolbar at the top of the screen select "Create".

4.7.3.1.2.2 In the window that appears enter "MLVA" next to the "Name" option.

4.7.3.1.2.3 Use the default settings and click "Save" and close the window.

4.7.3.1.3 <u>Results Groups</u>

4.7.3.1.3.1 Access the "Results Groups" menu on the left-hand side of the screen. In the toolbar at the top of the screen select "Create".

4.7.3.1.3.2 In the window that appears, enter "MLVA1" as the name for this results group.

4.7.3.1.3.2.1 When the results group reaches its upper size limit set up a new results group with a different name (MLVA2, MLVA3…).

4.7.3.1.3.3 Under the "Delimiters" section heading click the "Add" button to insert a dash in the "Selected Attributes" box on the right-hand side of the window.

4.7.3.1.3.4 From the "Available Attributes" list, scroll down to the "Plate Name" option. Select it and click the "Add" button in between the two boxes to add it to the "Selected Attributes" list.

4.7.3.1.3.5 Uncheck the "Include an Instrument Run Name folder" and "Include an Injection folder" options, keeping all other default parameters.

4.7.3.1.3.6 Save the results group to the library and close this window.

4.8 Genetic Analyzer 3500 instrument preparation before each run

4.8.1. **Day 1**

4.8.1.1 Make sure the service console is open and the status light on the sequencer is green. Make sure a capillary array is installed in the instrument. For installation, follow the instructions of the "Install Capillary Array" wizard. You can find the wizards by clicking on the "Maintenance" option in the toolbar at the top right of the screen and then clicking on the "Maintenance Wizards" menu option in the left-hand panel. Spectral and spatial calibrations will need to be run for each array installation. Before setting up a run, check to make sure that the consumables are not expired.

4.8.1.1.1 **NOTE:** The RFID tags on the packaging of the consumables automatically log in the hours spent on the instrument. The run will not start with expired reagents.

4.8.1.2 Go back to the Dashboard screen by clicking the "Dashboard" button at the top left of the screen

4.8.1.3 Select the "Create New Plate" icon at the top of the screen. Name the run following the standardized PulseNet naming system: use the unique identifier code that was assigned to your laboratory by PulseNet for the first two to four letters of the file name. The next two spaces will indicate the year and the next four spaces will indicate the month and the date the run was performed. For example GA070426 is a run made at the GA Public Health Laboratory on April 26, 2007. If several runs are performed the same day, separate the file names by using sequential numbers, for example GA070426-1, GA070426-2.

4.8.1.4 From the "Plate Type" drop-down menu, select "Fragment".

4.8.1.5 Type in the Owner name or initials.

4.8.1.6 Use the default settings (50cm array, POP-7 polymer) for the remaining options and click on the "Assign Plate Contents" button at the bottom of the screen.

4.8.1.7 The plate view screen will appear

4.8.1.8 Here you can type in the sample IDs or import a sample plate file from a template (see Appendix PNL28-5 for instructions on importing plate .txt files).

4.8.1.9 To assign the Assay to the plate, proceed as follows:

4.8.1.9.1 Click on "Add from Library" in the "Assays" box at the bottom of the screen.

4.8.1.9.2 In the new window select "Fragtest" from the list of assays.

4.8.1.9.3 Add this assay by clicking on the "Add to Plate" button, and then close the window.

4.8.1.10 Adding the File Name Conventions (MLVA) and Results Groups (MLVA1) can be done in the same manner, by selecting the "Add from Library" link in the respective boxes.

4.8.1.11 Once the assay, file name convention and results group have been added to the plate, select the entire sample ID set by highlighting the individual cells or by clicking on column/row headings.

4.8.1.12 Check the box next to each of the run parameters: Assay, File Name Convention, and Results Group.

4.8.1.13 When each of the run parameters have been successfully assigned a dot will appear in the center of the cells in which the parameters were applied.

4.8.1.13.1 **NOTE1:** If a plate layout has been imported from a .txt file the Assay, File Name Convention and Results Group should automatically be applied to the plate and check-marked for all samples.

4.8.1.13.2 **NOTE2:** What identifiers are shown in the sample ID cells can be changed by selecting or deselecting options in the "Show In Wells" drop-down menu above the sample plate layout. Assays, file name conventions, and results groups can be color coded by double-clicking the black dot to the left of the file name or during initial setup.

4.8.1.14 Save the plate by clicking the "Save Plate" button in the toolbar at the top of the screen and selecting "Save" from the drop-down menu.

4.8.1.15 Go back to the dashboard screen by clicking the "Dashboard" button in the top left corner of the screen and continue with fragment analysis preparation.

4.8.1.16 If needed, install the POP7 polymer in the instrument.

4.8.1.16.1 Click on the "Maintenance" button in the toolbar at the top right of the window, and then select the "Maintenance Wizards" menu option on the left-hand side.

4.8.1.16.2 Follow the instructions of the "Replenish Polymer" wizard.

4.8.1.17 If needed, also install new anode and cathode buffer containers.

4.8.1.17.1 **NOTE1:** If an old polymer pouch (been on the instrument > 7 days) is switched to a new one, follow the instructions of the "Wash Pump and Channels" wizard instead. This process takes 30 minutes to complete and requires a conditioning pouch.

4.8.1.17.2 **NOTE2:** To update the system after new reagents have been installed, click the "Refresh" button under the "Consumables Information" heading on the Dashboard.

4.9. Fragment analysis sample preparation

4.9.1 **Day 1**

4.9.1.1 **NOTE:** The fragment analysis method is not organism specific therefore; STEC O157 and *Salmonella* serotypes Typhiumurium and Entertidis may be run on a single fragment analysis plate.

4.9.1.2 Thaw the Hi-Di Formamide, the GeneFlo 625 DNA size standard and the internal ladder (see appendix PNL28-4) and place on ice.

4.9.1.2.1 **NOTE:** aliquot Hi-Di Formamide (500 µl / tube) and the size standard (50 µl / tube) in order to avoid frequent freeze-thaw cycles.

4.9.1.3 Prepare a 96-well V-bottom plate for diluting the PCR reactions. Using a 200 µl multichannel pipettor and a solution basin, dispense 19 µl of molecular-grade water in the required number of wells.

4.9.1.4 Remove the plate / tubes with the PCR reactions from the thermocycler. Briefly spin down the plate / tubes, if necessary. Use a 10 µl multichannel pipettor to transfer 1 µl of each PCR reaction directly across to the corresponding set of wells in the dilution plate. In order to avoid cross-contamination, remove the strip cap from just one column at a time and recap the column before opening the next one.

4.9.1.5 For the internal ladder, combine R1 and R2 PCR products from the four PCR reactions of both internal ladder isolates to end up with a total of 40 µl. Mix well by pipetting up and down a few times and add 3 µl of internal ladder in two wells.

4.9.1.6 Using a 200 µl multichannel pipettor, mix the dilutions by pipetting up and down a few times. Cover the plate with parafilm and put in the fridge or on ice.

4.9.1.7 Prepare a fragment analysis master mix containing DNA size standard and Hi-Di Formamide for the samples following the calculations indicated in the table below. The fragment analysis mastermix calculations can also be performed using the autocalculate box at the bottom of the MLVA Fragment Analysis ABI Worksheet (see appendix PNL28-2). Vortex briefly and place on ice.

Reagents	Frag. anal. mastermix
Hi-Di Formamide	8 µl x (# samples +3)=
GeneFlo 625 bp size standard	1 µl x (# samples +3)=

4.9.1.8 Place a MicroAmp Optical 96-well sample plate in a cold block. Aliquot 9 µl of the prepared fragment analysis mastermix to the required number of wells. Cover the plate loosely with Parafilm.

4.9.1.9 Using the 10 µl multichannel pipettor, add 1 µl of 1:20 diluted PCR reactions to the appropriate columns in the sample plate. Keep sliding the Parafilm sheet from column to column to keep track of the sample order.

4.9.1.10 Denaturate templates by heating the reaction plate uncovered in a thermocycler at 95°C for 3 min.

4.9.1.11 While the templates denaturate, turn the oven of the 3500 on using the "Start Pre-Heat" button located on the middle, right-hand side of the dashboard. The default set point should be 60°C.

4.9.1.12 Briefly spin down the sample plate to remove any air bubbles.

4.9.1.13 Seal the plate with the rubber septa. Place the sample plate in a plate base. Snap the plate retainer onto the plate and the plate base.

4.9.1.14 Place the plate assembly and the buffer into the Genetic Analyzer 3500.

4.9.1.14.1 Push the tray button at the front of the Genetic Analyzer 3500 to bring the autosampler to the forward position. Open the instrument door.

4.9.1.14.2 Place the plate assembly on the autosampler in position A or B with the notched corner of the plate base facing you.

4.9.1.14.3 Close the instrument door and wait for the green light to illuminate.

4.9.1.14.4 Open the previously created plate setup by clicking the blue "Edit Existing Plate" button at the top of the dashboard screen.

4.9.1.14.5 Select the plate to be run and click "Open".

4.9.1.14.6 Link the plate by clicking "Link Plate for Run" at the bottom of the screen

4.9.1.14.7 A "Load Plate" pop-up window will verify that the plate has been successfully loaded. Click "OK".

4.9.1.15 The "Start Run" button at the bottom of the screen will become functional indicating that the run can be started. Make sure the plate is linked to the correct position (A or B), then click the "Start Run" button. Click "OK" on the alert window. The run will start.

4.10. Viewing and exporting data from the Genetic Analyzer 3500

4.10.1 **Day 2**

4.10.1.1 **NOTE**: Steps 4.10.1.3 and 4.10.1.4 only need to be performed before the very first analysis.

4.10.1.2 Double-click on the shortcut icon for GeneMapper v4.1 or higher and enter the appropriate password to access the software. The main menu window will open.

4.10.1.3 Set up the size standard:

4.10.1.3.1 From the "Tools" drop-down menu, select "GeneMapper Manager". A "GeneMapper Manager" window will open.

4.10.1.3.2 Select the "Size Standards" tab.

4.10.1.3.3 Click on "New", leave the default option "Basic or Advanced" selected and click "OK". The "Size Standard Editor" window will appear.

4.10.1.3.4 Name the new size standard "GeneFlo625".

4.10.1.3.5 Leave the default option "Red" as "Size Standard Dye".

4.10.1.3.6 Enter sizes for each peak in the table (refer to the GeneFlo 625 product insert for the peak sizes).

4.10.1.3.7 When finished, click "OK".

4.10.1.4 Set up analysis method:

4.10.1.4.1 From the "Tools" drop-down menu, select "GeneMapper Manager". A "GeneMapper Manager" window will open.

4.10.1.4.2 Select the "Analysis Methods" tab.

4.10.1.4.3 Click on "New", leave the default option "Microsatellite" selected and click "OK". The "Analysis Methods Editor" window will appear.

4.10.1.4.4 Name the new method "PNMLVA".and click "OK".

4.10.1.4.5 Highlight the new method name "PNMLVA" and click "Open".

4.10.1.4.6 Select the "Peak Detector" tab and change "Peak Detection Algorithm" to "Advanced" from the drop-down menu.

4.10.1.4.7 Input the following analysis settings and click "OK" when finished:

*Analysis: Full Range
* Sizing: Partial Sizing
*Start Size: 50
* Stop Size 625
* Smoothing: none
* Baseline window: 51 pts
* Size Calling Method: Local Southern Method
* Peak Amplitude Thresholds:
***** B: 600
***** G: 600
***** Y: 600
***** R: 20
***** P: 50
***** O: 50
* Min. Peak Half Width: 2 pts
* Polynomial Degree: 2
* Peak Window Size: 21 pts
* Slope Threshold:
* Peak Start: 0.0
* Peak End: 0.0
* Leave the "Enable Normalization" option unchecked

4.10.1.4.8 Click "Done" to close the GeneMapper Manager window and return to the main screen.

4.10.1.5 From the "File" drop-down menu select "Add Samples to Project".

4.10.1.6 Find the folder containing the data file to be analyzed: My Computer → AB SW & DATA (D:) → Applied Biosystems → 3500 → Data → MLVA1-<PlateName>.

4.10.1.7 Highlight the desired file(s) and click on "Add to List". File(s) will appear in the window on the right. Click "Add" below the file list to return to the original screen.

4.10.1.8 Samples in the selected file(s) will be listed in a new window and the "Analyze" (play) button appears in green color in the toolbar indicating that the files are ready to be analyzed.

4.10.1.9 Select the size standard GeneFlo625 for the first sample, highlight the size standard column by selecting the heading, and select "Fill Down" from the "Edit" drop-down menu.

4.10.1.10 Select the analysis method PNMLVA for the first sample, highlight the analysis method column, and select "Fill Down" from the "Edit" drop-down menu.

4.10.1.11 Click on the "Play" icon.

4.10.1.12 Name the project with the run name (for example, "GA070426") and click "OK".

4.10.1.13 A successful analysis is indicated by green squares. Yellow triangles indicate problematic components (i.e. missing size standard peaks). Red circles indicate results fell below acceptable quality values. Samples with yellow or red circles in the SQ column should be selected for re-analysis.

4.10.1.13.1 To resolve failed analyses due to sub-optimal molecular marker peak profile (i.e. miscalling of peaks), select a row(s) with a yellow triangle or a red circle in the SQ column and click on the "Size Match Editor" icon on the toolbar. The "Size Match Editor" view will appear.

4.10.1.13.2 Place the cursor near the X-axis to activate the magnifying lens, and then pull up (mouse left-click and hold) to zoom in a specific area to facilitate editing

4.10.1.13.3 Left-click at the base of a peak to select. Right-click and select "Add", "Delete", or "Change".

4.10.1.13.4 Select the correct molecular weight for the selected peak from the drop-down menu. Repeat this process for all other miscalled peaks. Click "OK" when finished.

4.10.1.13.5 After the size standard has been adjusted, click the "Play" button to re-analyze the data. After a successful analysis, the samples will have green squares under the SQ column. If the size standard cannot be adjusted, the reaction is considered a fragment analysis failure and must be re-run

4.10.1.14 Check the fragment result data (the fluorescent peaks) for each well by highlighting the well ID and by clicking on the "Display Plots" icon on the toolbar.

4.10.1.14.1 Make sure that all VNTRs amplified in the positive control and that the fragment sizes are within the range specified in the appendix PNL28-2 and record the fragment sizes on the MLVA Fragment Analysis ABI Worksheet.

4.10.1.14.2 The size calling for the internal ladder should also be within the range specified in the appendix PNL28-2 (or PNL28-4).

4.10.1.14.3 Write down any failed reactions in the MLVA Fragment Analysis ABI Worksheet. Make a note of non-specific bands and primer-dimers.

4.10.1.15 Export the peak file:

4.10.1.15.1 **NOTE1:** the following column headings should appear in the exported table in the following order from left to right: "Dye/Sample Peak", "Sample File Name", "Marker", "Allele", "Size", "Height", "Area in Point", "Data Point". You can modify the format of the table by selecting the "Table Setting Editor" from the "Tools" drop-down menu. Select the "Genotypes" tab and make sure that *only* the boxes for the following are checked: "Sample File Name", "Marker", "Dye", "Allele", "Size", "Height", "Area in Point", and "Data Point".

4.10.1.15.2 **NOTE 2:** You can import the peak file also from the Data Collection Foundation software, but the file format is not correct; the "Allele" and "Marker" columns are missing and there are additional columns in the file. This file cannot be imported into BioNumerics without first manually modifying (adding and deleting appropriate columns) the file.

4.10.1.15.3 Highlight the samples for which you want to export peak data.

4.10.1.15.4 Click on the "Display Plots" icon on the toolbar.

4.10.1.15.5 Click on the "Sizing Table" icon on the toolbar and a table will appear below the electropherograms.

4.10.1.15.6 From the "File" drop-down menu, select "Export Table".

4.10.1.15.7 Select the location (for example a flash drive) where you want to export the data.

4.10.1.15.8 Name the export file with the run name (for example GA070426) and make sure the file type is tab-delimited text (.txt) file.

4.10.1.16 The remaining gel can stay in the instrument if it is going to be used within 7 days.

4.10.1.16.1 **NOTE:** To extend life of polymer, remove after run, place in refrigerator, and replace with an old polymer (on instrument longer than 7 days) until next use. Polymer should not be on instrument for more than a total of 7 days.

5. FLOW CHART:

6. REFERENCES:

6.1 Hyytiä-Trees, E., Smole, S. C., Fields, P. I.., Swaminathan, B., and Ribot, E. M. (2006) Second generation subtyping: a proposed PulseNet protocol for multiple-locus variable-number tandem repeat analysis (MLVA) of Shiga toxin-producing *Escherichia coli O157 (STEC O*157). Foodborne Pathog. Dis. 3, 118-131.

6.2 Hyytia-Trees, E., Lafon, P., Vauterin, P., and Ribot, E. (2010) Multi-laboratory validation study of standardized multiple-locus VNTR analysis (MLVA) protocol for Shiga toxin-producing

E*scherichia coli* O157 (STEC O157): a novel approach to normalize fragment size data between capillary electrophoresis platforms. Foodborne Path. Dis. 7, 129-136.

7. CONTACTS:

7.1 Eija Trees, D.V.M., Ph.D.
PulseNet Next Generation Subtyping Methods Unit, CDC
(404) 639-3672
EHyytia-Trees@cdc.gov

7.2 Patti Lafon
PulseNet Next Generation Subtyping Methods Unit, CDC
(404) 639-2828
PLafon@cdc.gov

7.3 Ashley Sabol
PulseNet Next Generation Subtyping Methods Unit, CDC
(404) 639-2947
ASabol@cdc.gov

8. AMENDMENTS:

5/6/2013: former appendix PNL28-4 (BioNumerics specifications for the *E. coli* O157 VNTR loci) was moved to SOP PND16 (PulseNet standard operating procedure for analysis of MLVA data of Shiga toxin-producing *Escherichia coli* in BioNumerics – Applied Biosystems Genetic Analyzer 3130/3500 data). Former appendices PNL28-5 and PNL28-6 were renamed PNL28-4 and PNL28-5, respectively.
2/26/2014: the three laboratory SOPs for STECO157 (PNL28), and *Salmonella* serotypes Typhimurium (PNL29) and Enteritidis (PNL30) using the ABI 3500 platform were combined into a single SOP (PNL28).

Appendix PNL28-1

MLVA PCR Primer sequences for STEC O157:H7 and *Salmonella* serotypes Typhimurium and Enteritidis

Locus	Dye[1]	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
STEC O157:H7			
VNTR-3	CalRed590	GG CGG TAA GGA CAA CGG GGT GTT TGA ATT G	GAA CAA CCT AAA ACC CGC CTC GCC ATC G
VNTR-34	FAM	GA CAA GGT TCT GGC GTG TTA CCA ACG G	GTT ACA ACT CAC CTG CGA ATT TTT TAA GTC CC
VNTR-9	FAM	GC GCT GGT TTA GCC ATC GCC TTC TTC C	GTG TCA GGT GAG CTA CAG CCC GCT TAC GCT C
VNTR-25	HEX	GC CGG AGG AGG GTG ATG AGC GGT TAT ATT TAG TG	GCG CTG AAA AGA CAT TCT CTG TTT GGT TTA CAC GAC
VNTR-17	CalRed590	GC AGT TGC TCG GTT TTA ACA TTG CAG TGA TGA	GGA AAT GGT TTA CAT GAG TTT GAC GAT GGC GAT C
VNTR-19	FAM	GC AGT GAT CAT TAT TAG CAC CGC TTT CTG GAT GTT C	GGG GCA GGG AAT AAG GCC ACC TGT TAA GC
VNTR-36	FAM	GG CGT CCT TCA TCG GCC TGT CCG TTA AAC	GCC GCT GAA AGC CCA CAC CAT GC
VNTR-37	HEX	GC CGC CCC TTA CAT TAC GCG GAC ATT C	GCA GGA GAA CAA CAA AAC AGA CAG TAA TCA GAG CAG C
***Salmonella* Typhimurium**			
ST3	HEX	GT TCT TCT GCA ACG CAG GCA	GAT GGC ATG ACG CTG CAA CG
ST5	FAM	TT TTC GCT CAA CAA ACT T	ACA GCA CCA GAA GCA AT
ST7	CalRed590	CG ATT GAC GAT ATC TAT GAC TT	GTT TTT CAC GTT TGC CTT TC
ST10	HEX	CG GGC GCG GCT GGA GTA TTT G	GAA GGG GCC GGG CAG AGA CAG C
ST2	FAM	CA ACG CCT GTT CAG CAA C	ATC AAC AGC GGG TGG AT
ST6	CalRed590	AG CAG TGG CTG GCG GGA AAC C	GCA GCC GGA CAG GGG ATA AGC C
ST8	HEX	GC AGG TGT GGC TAT TGG CGT TGA AA	GAT GGT GAC GCC GTT GCT GAA GG
***Salmonella* Enteritidis**			
SE-1	FAM	TGT GGG ACT GCT TCA ACC TTT GGG C	CCA GCC ATC CAT ACC AAG ACC AAC ACT CTA TGA
SE-2	CallRed590	GTG CTT CCT CAG GTT GCT TTT AGC CTT GTT CG	GGG GAA TGG ACG GAG GCG ATA GAC G
SE-8	HEX	GGT AGC TTG CCG CAT AGC AGC AGA AGT	GGC GGC AAG CGA GCG AAT CC
SE-6	FAM	CTG GTC GCA GGT GTG GC	GGT GAC GCC GTT GCT GAA GGT AAT AAC AGA GTC
SE-5	HEX	GGC TGG CGG GAA ACC ACC ATC	GCC GAA CAG CAG GAT CTG TCC ATT AGT CAC TG
SE-3	CallRed590	CGG GAT AAG TGC CAC ATA ACA CAG TCG CTA AGC	CGC CAG TGT TAA AGG AAT GAA TGA ACC TGC TGA TG
SE-9	FAM	CCA CCT CTT TAC GGA TAC TGT CCA CCA GC	GGC GTT ACT GGC GGC GTT CG

[1] Only the 5' of the forward primer is fluorescently labeled

Note: this appendix has been posted on the SharePoint site as an Excel file so that it can be saved locally for data entry and autocalculation

Appendix PNL28-2

PCR run date:
PCR instrument:
ABI run date/Initials:
ABI instrument:

MLVA Fragment Analysis ABI Worksheet

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

	1	[put in # of samples only]
Hi-Di	32	
625 bp	4.00	

Hi-Di lot no. **Exp. Date**
625 bp lot no. **Exp. Date**
Polymer lot no. **Exp. Date**
Buffer lot no. **Exp Date**

1. For each isolate, fill in an appropriate BioNumerics key number for PCR reactions R1 and R2 (for example: for an isolate CDC_K1720 the BN key numbers are CDC_K1720R1 and CDC_K1720R2
2. Fill in the exact number of fragment analysis reactions (2 per isolate + controls) in the red box (extra already included in calculation). Mix the Hi-Di Formamide and 625 bp size standard in an appropriate tube. Add 9 µl of mixture to each well.
3. Using a multichannel pipettor, add 1 µl of 1:20 diluted PCR reaction to wells containing the Formamide / 625 bp mixture.
4. Denaturate the plate at 95°C for 3 min.
5. Briefly spin down the plate.
6. Place the plate in the plate base; cover wells with a rubber septa; finish the plate assembly by snapping on the plate retainer.

Organism **– Positive Control**			
Multiplex Reaction fragment sizes		Multiplex Reaction fragment sizes	
VNTR (size range)		**VNTR (size range)**	
VNTR (size range)		**VNTR (size range)**	
VNTR (size range)		**VNTR (size range)**	
VNTR (size range)			

Organism **– Internal Ladder fragment sizes**				
	Expected Size Ranges		Fragment Sizes	
VNTR				
VNTR				
VNTR				
VNTR				
VNTR				
VNTR				
VNTR				

Appendix PNL28-2 continued

Expected fragment sizes for positive controls and internal ladders

E. coli O157 - Positive Control (EDL933)			
R1 fragment sizes		R2 fragment sizes	
VNTR_25 (138 - 140)		VNTR_17 (159 - 161)	
VNTR_34 (278 - 280)		VNTR_36 (157 - 158)	
VNTR_3 (379 - 382)		VNTR_37 (187 - 189)	
VNTR_9 (530 - 534)		VNTR_19 (308 - 310)	

E. coli O157 - Internal Ladder fragment sizes				
	Expected Size Ranges		Fragment Sizes	
VNTR_25	127 - 128	138 - 140		
VNTR_17	153 - 155	177 - 179		
VNTR_36	170 - 172			
VNTR_37	192 - 194	198 - 200		
VNTR_34	222 - 224	260 - 262		
VNTR_19	296 - 298	320 - 322		
VNTR_3	397 - 401	433 - 435		
VNTR_9	518 - 522	565 - 569		

Salmonella Enteritidis - Positive Control (K1891)			
R1 fragment sizes		R2 fragment sizes	
SE1 (190 - 193)		SE9 (181 - 184)	
SE2 (335 - 339)		SE5 (201 - 203)	
SE8 (433 - 436)		SE3 (211 - 215)	
SE6 (479 - 482)			

Salmonella Enteritidis - Internal Ladder fragment sizes				
	Expected Size Ranges		Fragment Sizes	
SE9	181 - 184			
SE1	190 - 193	211 - 213		
SE3	199 - 203	211 - 215		
SE5	201 - 203	218 - 221		
SE2	317.5 - 324	363 - 364		
SE8	346 - 350	433 - 436		
SE6	446 - 447	479 - 482		

Salmonella Typhimurium - Positive Control (LT2)			
R1 fragment sizes		R2 fragment sizes	
ST7 (157 - 158)		ST6 (271 - 273)	
ST3 (188 - 192)		ST2 (370 - 373)	
ST5 (219 - 220)		ST8 (556 - 558)	
ST10 (376 - 378)			

Salmonella Typhimurium - Internal Ladder fragment sizes				
	Expected Size Ranges		Fragment Sizes	
ST7	137 - 139	147 - 151		
ST3	177 - 180	188 - 192		
ST5	184 - 185	231 - 233		
ST6	253 - 255	283 - 285		
ST2	370 - 373	399 - 401		
ST10	383 - 384	413 - 415		
ST8	582 - 584	589- 591		

Note: this appendix has been posted on the SharePoint site as an Excel file so that it can be saved locally for data entry and autocalculation

Appendix PNL28-3a

PCR Mastermix Calculations

Type in the number of reactions (+2 extra), currently a red one next to "Number to analyze" for autocalculations

Date:

Technician:

Thermocycler:

E. coli multiplex PCR **R1**

1 Number of samples to be analyzed

10 ul Total Reaction Volume

		Start		Final		Add	
	PCR Buffer	10	X	1	X	**1.00**	ul
	MgCl2	50	mM	2	mM	**0.40**	ul
	dNTPs	10	mM	0.2	mM	**0.20**	ul
black	VNTR-3F	25	uM	0.67	uM	**0.27**	ul
	VNTR-3R	25	uM	0.67	uM	**0.27**	ul
blue	VNTR-34F	5	uM	0.12	uM	**0.24**	ul
	VNTR-34R	5	uM	0.12	uM	**0.24**	ul
blue	VNTR-9F	5	uM	0.12	uM	**0.24**	ul
	VNTR-9R	5	uM	0.12	uM	**0.24**	ul
green	VNTR-25F	2.5	uM	0.05	uM	**0.20**	ul
	VNTR-25R	2.5	uM	0.05	uM	**0.20**	ul
	Platinum taq	5	U/ul	1	U	**0.20**	ul
	PCR Water					**5.30**	ul

	Lane 1	Lane 2	Lane 3	Lane 4
A				
B				
C				
D				
E				
F				
G				
H				

	Date	**PCR Reagents**	**Lot#**	**Exp.**
VNTR-3F		10X Buffer		
VNTR-3R		MgCl2		
VNTR-34F		dNTPs		
VNTR-34R		Platinum Taq		
VNTR-9F				
VNTR-9R				
VNTR-25F				
VNTR-25R				

E. coli multiplex PCR **R2**

1 Number of samples to be analyzed

10 ul Total Reaction Volume

		Start		Final		Add	
	PCR Buffer	10	X	1	X	**1.00**	ul
	MgCl2	50	mM	2	mM	**0.40**	ul
	dNTPs	10	mM	0.2	mM	**0.20**	ul
black	VNTR-17F	5	uM	0.15	uM	**0.30**	ul
	VNTR-17R	5	uM	0.15	uM	**0.30**	ul
blue	VNTR-19F	1	uM	0.016	uM	**0.16**	ul
	VNTR-19R	1	uM	0.016	uM	**0.16**	ul
blue	VNTR-36F	1	uM	0.011	uM	**0.11**	ul
	VNTR-36R	1	uM	0.011	uM	**0.11**	ul
green	VNTR-37F	2.5	uM	0.017	uM	**0.07**	ul
	VNTR-37R	2.5	uM	0.017	uM	**0.07**	ul
	Platinum taq	5	U/ul	1	U	**0.20**	ul
	PCR Water					**5.92**	ul

	Lane 4	Lane 5	Lane 6	Lane 7
A				
B				
C				
D				
E				
F				
G				
H				

VNTR-17F	
VNTR-17R	
VNTR-19F	
VNTR-19R	
VNTR-36F	
VNTR-36R	
VNTR-37F	
VNTR-37R	
VNTR-37F	
VNTR-37R	

Note: this appendix has been posted on the SharePoint site as an Excel file so that it can be saved locally for data entry and autocalculation

PCR Mastermix calculations **Appendix PNL28-3b**

Type in the number of reactions (+ 2 extra), currently a red zero, next to "Number to analyze" for autocalculations.

Date:
Technician:
Thermocycler

S. Typhimurium multiplex PCR **R1**

1 Number of samples to be analyzed
10 ul Total Reaction Volume

		Start		Final		Add	
	PCR Buffer	10	X	1	X	1.00	ul
	MgCl2	50	mM	2.25	mM	0.45	ul
	dNTPs	10	mM	0.2	mM	0.20	ul
green	ST3-F	5	uM	0.05	uM	0.10	ul
	ST3-R	5	uM	0.05	uM	0.10	ul
blue	ST5-F	25	uM	1.5	uM	0.60	ul
	ST5-R	25	uM	1.5	uM	0.60	ul
yellow	ST7-F	5	uM	0.4	uM	0.80	ul
	ST7-R	5	uM	0.4	uM	0.80	ul
green	ST10-F	2.5	uM	0.03	uM	0.12	ul
	ST10-R	2.5	uM	0.03	uM	0.12	ul
	Platinum taq	5	U/ul	1.5	U	0.30	ul
	PCR Water					3.81	ul

Increased ST5 from 1.2 to 1.5
Decreased ST3 from .07 to .05

S. Typhimurium multiplex PCR **R2**

1 Number of samples to be analyzed
10 ul Total Reaction Volume

		Start		Final		Add	
	PCR Buffer	10	X	1	X	1.00	ul
	MgCl2	50	mM	1.6	mM	0.32	ul
	dNTPs	10	mM	0.2	mM	0.20	ul
blue	ST2-F	25	uM	0.9	uM	0.36	ul
	ST2-R	25	uM	0.9	uM	0.36	ul
yellow	ST6-F4	5	uM	0.28	uM	0.56	ul
	ST6-R2	5	uM	0.28	uM	0.56	ul
green	ST8-F3	5	uM	0.24	uM	0.48	ul
	ST8-R2	5	uM	0.24	uM	0.48	ul
	Platinum taq	5	U/ul	1.5	U	0.30	ul
	PCR Water					4.38	ul

	Row 1	Row 2	Row 3	Row 4	Row 5	Row 6
A						
B						
C						
D						
E						
F						
G						
H						

Primer	Dateprepared
ST3-F	
ST3-R	
ST5-F	
ST5-R	
ST7-F	
ST7-R	
ST10-F	
ST10-R	

PCR Reagents	Lot	Exp.
10X Buffer		
MgCl2		
dNTPs		
Platinum Taq		

	Row 7	Row 8	Row 9	Row 10	Row 11	Row 12
A						
B						
C						
D						
E						
F						
G						
H						

Primers	Dateprepared
ST2-F	
ST2-R	
ST6-F4	
ST6-R2	
ST8-F3	
ST8-R2	

Note: this appendix has been posted on the SharePoint site as an Excel file so that it can be saved locally for data entry and autocalculation

PCR Mastermix calculations **Appendix PNL28-3c**

Type in the number of reactions (+ 2 extra), currently a red zero, next to "Number to analyze" for autocalculations.

PCR run by:
Date:
Thermocycler:

S. Enteritidis multiplex PCR **R1**

1 Number of samples to be analyzed
10 ul Total Reaction Volume

		Start		Final		Add		
	PCR Buffer	10	X	1	X	1.00	ul	B
	MgCl2	50	mM	2	mM	0.40	ul	C
	dNTPs	10	mM	0.2	mM	0.20	ul	D
Blue	SE1F	2.5	uM	0.05	uM	0.20	ul	E
	SE1R	2.5	uM	0.05	uM	0.20	ul	F
Black	SE2F	12.5	uM	0.4	uM	0.32	ul	G
	SE2R	12.5	uM	0.4	uM	0.32	ul	H
Green	SE8F	2.5	uM	0.07	uM	0.28	ul	
	SE8R	2.5	uM	0.07	uM	0.28	ul	
Blue	SE6F	12.5	uM	0.43	uM	0.34	ul	
	SE6R	12.5	uM	0.43	uM	0.34	ul	
	Platinum taq	5	U/ul	1	U	0.20	ul	
	PCR Water					4.91	ul	

Row 1	Row 2	Row 3	Row 4	Row 5	Row 6

Primer	Date prepared	PCR Reagents		
SE1F			Lot#	Exp.
SE1R		**10X Buffer**		
SE2F		**MgCl2**		
SE2R		**dNTPs**		
SE8F		**Platinum Taq**		
SE8R				
SE6F				
SE6R				

S. Enteritidis multiplex PCR **R2**

1 Number of samples to be analyzed
10 ul Total Reaction Volume

		Start		Final		Add		
	PCR Buffer	10	X	1	X	1.00	ul	C
	MgCl2	50	mM	2	mM	0.40	ul	D
	dNTPs	10	mM	0.2	mM	0.20	ul	E
Green	SE5F	2.5	uM	0.05	uM	0.20	ul	F
	SE5R	2.5	uM	0.05	uM	0.20	ul	G
Black	SE3F	12.5	uM	0.5	uM	0.40	ul	H
	SE3R	12.5	uM	0.5	uM	0.40	ul	
Blue	SE9F	2.5	uM	0.02	uM	0.08	ul	
	SE9R	2.5	uM	0.02	uM	0.08	ul	
	Platinum taq	5	U/ul	1	U	0.20	ul	
	PCR Water					5.84	ul	

Row 1	Row 2	Row 3	Row 4	Row 5	Row 6

Primers	Date prepared
SE5F	
SE5R	
SE3F	
SE3R	
SE9F	
SE9R	

Appendix PNL28-4

Instructions to prepare the internal ladders

1. Prepare DNA templates from isolates as described in the protocol step 4.5. Store the templates at -20°C or -80°C freezer.
   1.1 For STEC O157:H7, use the strains EC04PN0139 and EC04PN0570
   1.2 For *Salmonella* serotype Typhimurium, use strains 2009K0825 and 2009K0826
   1.3 For *Salmonella* serotype Enteritidis, use strains H9560 and 2010K0017
2. Use the DNA templates to set up and run the PCR reactions R1 and R2 as described in the protocol step 4.6.
3. After PCR amplification, pool the R1 and R2 reactions for the two strains into one single PCR tube to end up with a final volume of 40 µl. Mix by pipetting up and down a few times.
4. A new lot of internal ladder must be tested against the old ladder lot by running them in the same fragment analysis run.
5. Store the ladder in -20°C or -80°C freezer. It should remain stable at least 5-6 freeze-thaw cycles for a period of one month.

Expected fragment sizes (bp) of the fifteen fragments (locus VNTR_36 is a null allele in EC04PN0139) present in the STEC O157:H7 internal ladder as listed in the peak file:

VNTR-36 (B):	170 - 172	NA
VNTR-34 (B):	222 - 224	260 - 262
VNTR-19 (B):	296 - 298	320 - 322
VNTR-9 (B):	518 - 522	565 - 569
VNTR-25 (G):	127 - 128	138 - 140
VNTR-37 (G):	192 - 194	198 - 200
VNTR-17 (Y):	153 - 155	177 - 179
VNTR-3 (Y):	397 - 401	433 - 435

Expected fragment sizes (bp) of the fifteen fragments (locus VNTR_36 is a null allele in EC04PN0139) present in the STEC O157:H7 internal ladder as they appear in the electropherogram:

VNTR-25 (G):	127 - 128	138 - 140
VNTR-17 (Y):	153 - 155	177 - 179
VNTR-36 (B):	170 - 172	NA
VNTR-37 (G):	192 - 194	198 - 200
VNTR-34 (B):	222 - 224	260 - 262
VNTR-19 (B):	296 - 298	320 - 322
VNTR-3 (Y):	397 - 401	433 - 435
VNTR-9 (B):	518 - 522	565 - 569

Expected fragment sizes (bp) of the fourteen fragments present in the *Salmonella* serotype Typhimurium internal ladder as listed in the peak file:

ST5 (B):	184 - 185	231 - 233
ST2 (B):	370 - 373	399 - 401
ST3 (G):	177 - 180	188 - 192
ST10 (G):	383 - 384	413 - 415
ST8 (G):	582 - 584	589 - 591
ST7 (Y):	137 - 139	147 - 151
ST6 (Y):	253 - 255	283 - 285

Expected fragment sizes (bp) of the fourteen fragment present in the *Salmonella* serotype Typhimurium internal ladder as they appear in the electropherogram:

ST7 (Y):	137 - 139	147 - 151
ST3 (G):	177 - 180	188 - 192
ST5 (B):	184 - 185	231 - 233
ST6 (Y):	253 - 255	283 - 285
ST2 (B):	370 - 373	399 - 401
ST10 (G):	383 - 384	413 - 415
ST8 (G):	582 - 584	589 - 591

Expected fragment sizes (bp) of the thirteen fragments (SE9 has the same allele in both ladder strains) present in the *Salmonella* serotype Enteritidis internal ladder as listed in the peak file:

SE9 (B)	181 - 184	181 - 184
SE6 (B):	446 - 447	479 - 482
SE1 (B)	190 - 193	211 - 213
SE5 (G):	201 - 203	218 - 221
SE8 (G):	346 - 350	433 - 436
SE3 (Y):	199 – 203	211 - 215
SE2 (Y):	317.5 - 324	363 - 364

Expected fragment sizes (bp) of the fifteen fragments (SE9 has the same allele in both ladder strains) present in the *Salmonella* serotype Enteritidis internal ladder as they appear in the electropherogram:

SE9 (B)	181 - 184	181 - 184
SE1 (B)	190 - 193	211 - 213
SE3 (Y):	199 - 203	211 - 215
SE5 (G):	201 - 203	218 - 221
SE2 (Y):	317.5 - 324	363 - 364
SE8 (G):	346 - 350	433 - 436
SE6 (B):	446 - 447	479 - 482

NOTE: fragment size ranges for the internal ladders are based on multiple independent runs at CDC and PulseNet Participating Laboratories

Appendix PNL28-5

Steps for Exporting and Importing Plate Set ups on the ABI 3500

Note: the procedure described below will require the sequencer computer to have Microsoft Office. If the sequencer computer does not have Microsoft Office, the exported file needs to be saved on a flash drive so that steps 10 through 16 can be performed on a computer with Microsoft Office.

1. On the Dashboard screen click on the "Edit Existing Plate" button.
2. Select "Fragment" from the "Filter" drop-down menu.
3. Find the most recent full plate. Click on the ID to highlight the desired plate and then click the "Open" button at the bottom of the window.
   3.1. **NOTE**: If you do not have a full plate, create a new one by filling in each sample ID space (A01 through H12) with "test" and saving it with the plate name "MLVA_Template". Be sure to add the results group "MLVA1", file name convention "MLVA", and Assay "Fragtest" to each sample, making sure each one is check-marked. It is necessary to have all required wells filled in when exporting so that they will be available in your exported file.
4. In the plate layout window, click the "Export…" button in the toolbar at the top of the screen.
5. In the export window, create a new folder on the desktop by clicking the "Desktop" icon on the left side of the window. Find the 'New Folder' button in the toolbar at the top of the window and name the new folder "MLVA Plate Setup".
6. Save the file to this folder by using your new plate name (e.g. - CDC110726) and clicking "Save".
7. A window will pop up letting you know that the plate has been successfully exported. Click "OK".
8. Click the "Close Plate" button in the toolbar at the top of the plate layout screen, and click "Yes" to close without saving changes.
9. Minimize the 3500 program window and open the "MLVA Plate Setup" folder on the desktop.
10. Right-click your plate name, select "Open With" and in the submenu select Microsoft Office Excel.
11. Sample IDs can be typed or copy and pasted from a separate Excel file into the spaces next to the correct wells under the "Sample Name" heading.
    11.1. **NOTE**: Do not change/delete any of the column headings. The fields must be in the same format when importing as they were when exported. Additionally, the software will not import the file if the sample IDs contain special characters (e.g. - ! , / ,) , etc). You can use underscores and dashes.
12. The "Plate Name" should be changed to match the plate/file name, and initials should be placed under the 'Owner Name' heading. The defaults for "Application Type", "Capillary Length (cm)", "Polymer", and "Number of Wells" should be used.
13. Once all of the IDs are inserted, make sure that for each sample ID and all controls, the assay is "Fragtest", the results group name is "MLVA1", the file name convention is "MLVA", and "Sample" is listed under "Sample Type".
14. Under the "File" drop-down menu select "Save".
15. A warning window will appear asking if the workbook should be saved in the "Text (tab-delimited)" format. Click "Yes".
16. Exit Microsoft Excel, clicking "No" when prompted to save changes.
    16.1. **NOTE:** The removal of additional spaces from lines in the .txt file after modification in Excel may be necessary before importing into the "3500" program. To do this, open the file in "Notepad" making

sure the window is opened in full screen. In lines 1, 2, and 4 remove any additional spaces after the last character in the line or from the left side of the page. Once all spaces have been removed from those lines, save the file by clicking "Save" in the "File" drop-down menu and close the window.

17. Go to the ABI 3500 plate layout window by clicking "Create New Plate" on the Dashboard.
18. Enter in the name of the plate and from the "Plate Type" drop-down menu select "Fragment".
19. Click on the "Assign Plate Contents" button at the bottom of the screen.
20. In the toolbar at the top of the "Plate View" screen, click the "Import..." button.
21. In the import window, navigate to the recently created plate under Desktop/MLVA Plate Setup. Select the appropriate .txt file and click "Open".
    21.1. At this point an error message may appear asking if you would like to proceed. If you encounter this message, click the "Proceed" button at the bottom of the window.
22. When the file has been imported, an alert window will appear stating that the plate "has been successfully imported". Click "OK".
23. In the toolbar at the top of the screen click on "Save Plate" and from the drop-down menu select "Save As". Enter the plate name matching the name of the .txt file, and click "OK".
    23.1. **NOTE:** When importing a plate layout from a .txt file the Assay, File Name Convention and Results Group should automatically be applied to the plate and check-marked for all samples. If the .txt file does not contain the correct assay file name (e.g. – the name is misspelled, etc.), the assay will not be imported and the file name convention and results group will be imported but not check-marked.
    23.2. **NOTE2:** For repeated use, plate templates can be created by exporting a plate to the "MLVA Plate Setup" folder. Save the file as a tab-delimited text file with "test" as the sample ID and with a new plate name (e.g. - "MLVA_Template"). After you have filled out the information for a new run on the template, save it in step 14 with the standardized run name (name (e.g. - CDC101020).

1. **PURPOSE:** To describe the One-Day (24-26 h) Standardized Laboratory Protocol for Molecular Subtyping of *Cronobacter* species by Pulsed-field Gel Electrophoresis (PFGE).

2. **SCOPE:** To provide the PulseNet participants with a standardized procedure for performing PFGE of *Cronobacter* spp., thus ensuring inter-laboratory comparability of the generated results.

3. **DEFINITIONS/TERMS:**
   3.1 PFGE: Pulsed-field Gel Electrophoresis
   3.2 DNA: Deoxyribonucleic acid
   3.3 CDC: Centers for Disease Control and Prevention
   3.4 CLRW: Clinical Laboratory Reagent Water

4. **RESPONSIBILITIES/PROCEDURE:**

BIOSAFETY WARNING: *Cronobacter* species are human pathogens and can cause serious disease. Always use Biosafety Level 2 practices (at a minimum) and extreme caution when transferring and handling strains of this genus. Disinfect or dispose of all plasticware and glassware that come in contact with the cultures in a safe manner.

Please read all instructions carefully before starting protocol. Treat all plasticware, glassware, pipets, spatulas, etc. that come in contact with the cell suspensions or plugs as contaminated materials and dispose of, or disinfect according to the guidelines of your institution. Disinfect reusable plug molds before they are washed; the disposable plug molds, including the tape and the tab that is used to push the plugs out of the wells, are also contaminated and should be disinfected 1% Lysol/Amphyll or 90% ethanol for at least 30 minutes if they will be washed and reused.

Day 0

Streak an isolated colony from test cultures onto Trypticase Soy Agar with 5% defibrinated sheep blood (TSA-SB) plates (or comparable non-selective media) for confluent growth. It is recommended that a storage vial of each culture be created. To do this stab small screw cap tubes of TSA, HIA, or similar medium with the same inoculating loop used to streak the plate. This will ensure that the same colony can be retested if necessary. Incubate cultures at 37ºC for 14-18 h.

Day 1

1. **Turn on shaker water bath or incubator (54-55ºC), stationary water baths (55-60ºC) and spectrophotometer** (or equivalent instrument such as the Dade Microscan Turbidity meter or bioMérieux Vitek colorimeter).

2. Prepare **TE Buffer** (**10 mM Tris:1 mM EDTA, pH 8.0**)[1] as follows:
   10 ml of 1 M Tris, pH 8.0
   2 ml of 0.5 M EDTA, pH 8.0
   Dilute to 1000 ml with sterile Ultrapure Clinical Laboratory Reagent Water (CLRW)

Note: The TE Buffer is used to make the plug agarose and also to wash lysed PFGE plugs.

[1]Additional information is found on pages 11 and 12 of this document.

3. Prepare 1% SeaKem Gold agarose in **TE Buffer** (**10 mM Tris:1 mM EDTA, pH 8.0**) for PFGE plugs as follows:
   a. Weigh 0.50 g (or 0.25 g) SeaKem Gold (SKG) agarose into 250 ml screw-cap flask.
   b. Add 50.0 ml (or 25.0 ml) TE Buffer; swirl gently to disperse agarose.
   c. Loosen or remove cap, cover loosely with clear film, and microwave for 30 sec; mix gently and repeat for 10 sec intervals until agarose is completely dissolved.
   d. Recap flask and return to 55-60ºC water bath and equilibrate the agarose in the water bath for 15 minutes or until ready to use.

SAFETY WARNING: Use heat-resistant gloves when handling hot flasks after microwaving.

Note: SeaKem Gold agarose works well for making PFGE plugs because it provides added strength to the plugs minimizing breakage of plugs during the lysis and washing steps. The time and temperature needed to completely dissolve the agarose is dependent on the specifications of the microwave used, and will have to be determined empirically in each laboratory.

4. Label small transparent tubes (12 mm x 75 mm Falcon 2054 tubes or equivalent) with culture numbers.

5. Prepare **Cell Suspension Buffer** (**100 mM Tris:100 mM EDTA, pH 8.0**) as follows:
   10 ml of 1 M Tris, pH 8.0
   20 ml of 0.5 M EDTA, pH 8.0
   Dilute to 100 ml with sterile Ultrapure water (CLRW)

6. Transfer ≈2 ml of **Cell Suspension Buffer** (CSB) to small labeled tubes. Use a sterile polyester-fiber or cotton swab that has been moistened with sterile CSB to remove some of the growth from agar plate; suspend cells in CSB by spinning swab gently so cells will be evenly dispersed and formation of aerosols is minimized.

Note: The minimum volume of the cell suspension needed will depend on size of the cuvettes or tubes used to measure the cell concentration and are dependent on the manufacturer's specifications for the spectrophotometer, turbidity meter, or colorimeter. Keep suspensions on ice if you have more than 6 cultures to process or refrigerate cell suspensions if you cannot adjust their concentration immediately.

7. Adjust concentration of cell suspensions to one of values given below by diluting with sterile CSB or by adding additional cells.
   a. Spectrophotometer: 610 nm wavelength, absorbance (Optical Density) of 1.00 (range of 0.8-1.0)
   b. Dade Microscan Turbidity Meter: **0.40 - 0.45** (measured in Falcon 2054 tubes)
      0.58 - 0.63 (measured in Falcon 2057 tubes)
   c. bioMérieux Vitek colorimeter: **≈17-18%** transmittance (measured in Falcon 2054 tubes)

Note: The values in Steps 7a, 7b and 7c give satisfactory results at CDC; each laboratory may need to establish the optimal concentration needed for satisfactory results.

CASTING PLUGS

Label wells of PFGE plug molds with culture number. When reusable plug molds are used, put strip of tape on lower part of reusable plug mold before labeling wells.

Note: Unused plug agarose can be kept at room temperature and reused 1-2 times. Microwave on low-medium power for 10 -15 sec and mix; repeat for 5-10 sec intervals until agarose is completely melted. This agarose melts rapidly!

Note: Proteinase K solutions (20 mg/ml) are available commercially. Alternatively, a stock solution of Proteinase K can be prepared from the powder in sterile Ultrapure water (CLRW). For best results, aliquot 300-500 µl into small tubes and store in a freezer (-20ºC) until ready to use. Just before use, thaw appropriate number of vials needed for the samples; keep Proteinase K solutions on ice. If the Proteinase K stock solution was prepared from powder, discard any thawed solution at the end of the work day. Store commercially prepared Proteinase K solutions according to directions provided by the supplier.

1. Transfer 400 µl (0.4 ml) adjusted cell suspensions to labeled, sterile 1.5 ml microcentrifuge tubes.

2. Add 20 µl of Proteinase K (20 mg/ml stock) to each tube and mix gently with pipet tip. (200 µl are needed for 10 cell suspensions.)

3. Add 400 µl melted 1% SeaKem Gold agarose to 400 µl cell suspension; mix by gently pipetting mixture up and down a few times. **Over-pipeting can cause DNA shearing**. Maintain temperature of melted agarose by keeping flask in beaker of warm water (55-60ºC).

4. Immediately, dispense part of mixture into appropriate well(s) of reusable plug mold. Do not allow bubbles to form. Two plugs of each sample can be made from these amounts of cell suspension and agarose and are useful if repeat testing is required. Allow plugs to solidify at room temperature for 10-15 min. They can also be placed in the refrigerator (4ºC) for 5 minutes

Note: If disposable plug molds are used for making plugs with 1% SeaKem Gold agarose, use 200 µl cell suspension, 10 µl of Proteinase K (20 mg/ml stock) and 200 µl of agarose; up to 4 plugs can be made from these amounts of cell suspension and agarose.

Note: The generation of cell suspension and the subsequent casting of the plugs should be performed as rapidly as possible in order to minimize premature cell lysis. If large numbers of samples are being prepared, it is recommended that they be processed in batches of ~10 samples at a time. Once the first batch of isolates are in the cell lysis incubation, then start preparing the cells suspensions the next group samples, and so on. All batches can be lysed and washed together, since additional lysis time will not affect the initial batches.

LYSIS OF CELLS IN AGAROSE PLUGS

Note: Two plugs (reusable molds) or up to four plugs (disposable molds) of the same strain can be lysed in the same 50ml tube.

1. Label 50 ml polypropylene screw-cap or 50 ml Oak Ridge tubes with culture numbers.

2. Prepare **Cell Lysis Buffer** (**50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl**) as follows:
   25 ml of 1 M Tris, pH 8.0
   50 ml of 0.5 M EDTA, pH 8.0
   50 ml of 10 % Sarcosyl (N-Lauroylsarcosine, Sodium salt)[2]
   Dilute to 500 ml with sterile Ultrapure water (CLRW)

[2]The N-Lauroylsarcosine, Sodium salt can be added directly to the other ingredients and allowed to dissolve. See page 12 of this document.

3. Calculate the total volume of **Cell Lysis**/**Proteinase K Buffer** needed as follows:
   a. 5 ml **Cell Lysis Buffer** (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl) is needed per tube (e. g., 5 ml x 10 tubes = 50 ml).
   b. 25 µl **Proteinase K** stock solution (20 mg/ml) is needed per tube of the cell lysis buffer (e. g., 25 µl x 10 tubes = 250 µl).
   c. Prepare the master mix by measuring the correct volume of Cell Lysis Buffer and Proteinase K into appropriate size test tube or flask and mix well.

Note: The final concentration of Proteinase K in the lysis buffer is **0.1 mg/ml** and is different from the concentration that was added to the cell suspension (0.5 mg/ml).

4. Add 5 ml of Proteinase K/Cell Lysis Buffer to each labeled 50 ml tube.

5. Trim excess agarose from top of plugs with scalpel, razor blade or similar instrument. Open reusable plug mold and transfer plugs from mold with a 6-mm wide spatula to appropriately labeled tube. If disposable plug molds are used, remove white tape from bottom of mold and push out plug(s) into appropriately labeled tube. **Be sure plugs are under buffer and not on side of tube**.

Note: The excess agarose, plug mold, spatula, etc. are contaminated. Discard or disinfect appropriately.

6. **Remove tape from reusable mold.** Place both sections of the plug mold, spatulas, and scalpel in 90% ethanol, 1% Lysol/Amphyll other suitable disinfectant. **Soak them for 15 minutes before washing them.** Discard disposable plug molds or disinfect them in or 90% ethanol for 30-60 minutes if they will be washed and reused.

7. Place tubes in rack and incubate in a 54-55ºC shaker water bath or incubator for 1.5 - 2 h with **constant and vigorous agitation** (150-175 rpm). If lysing in water bath, be sure water level is **above** level of lysis buffer in tubes.

8. Pre-heat enough sterile Ultrapure water (CLRW) to 54-55ºC so that plugs can be washed two times with 10-15 ml water (200-250 ml for 10 tubes).

WASHING OF AGAROSE PLUGS AFTER CELL LYSIS

Note: Most laboratories will find that their plugs are sufficiently stable to perform the following washing steps at 54-55ºC. However, if you notice that your plugs are nicked along the edges or breaking it will be necessary for your laboratory to lower the water bath or incubator to 50ºC for the following washing steps.

1. Remove tubes from water bath or incubator, and carefully pour off lysis buffer into an appropriate discard container; plugs can be held in tubes with a screened cap or spatula.

Note: It is important to remove all of the liquid during this and subsequent wash steps by touching edge of tube or screened cap on an absorbent paper towel.

2. Add at 10-15 ml sterile Ultrapure water (CLRW) that has been pre-heated to 54-55ºC to each tube and shake the tubes in a 54-55ºC water bath or incubator for 10-15 min.

3. Pour off water from the plugs and repeat wash step with pre-heated water (Step 2) one more time.

a. Pre-heat enough sterile **TE Buffer** (10 mM Tris:1 mM EDTA, pH 8.0) in a 54-55ºC water bath so that plugs can be washed four times with 10-15 ml TE (400-600 ml for 10 tubes) after beginning last water wash.

4. Pour off water, add 10-15 ml pre-heated (54-55ºC) sterile TE Buffer, and shake the tubes in 54-55ºC water bath or incubator for 10-15 min.

5. Pour off TE and repeat wash step with pre-heated TE three more times.

6. Decant last wash and add 5-10 ml sterile TE. Continue with step 1 in "Restriction Digestion" section or store plugs in TE Buffer at 4ºC until needed. Plugs can be transferred to smaller tubes for long term storage.

Note: If restriction digestion is to be done the same day, complete Steps 1-3 of next section (Restriction Digestion) during last TE wash step for optimal use of time.

RESTRICTION DIGESTION OF DNA IN AGAROSE PLUGS

Note: A small slice of the plug (not the entire plug) should be digested with the primary restriction enzyme because less enzyme is required and other slices of the plug can be subjected to restriction analysis with secondary or tertiary enzymes, according to the table below. The use of a secondary (or tertiary) enzyme is useful in situations where the PFGE patterns obtained with the primary enzyme from two or more isolates are indistinguishable.

Organism	Primary Enzyme (Concentration)	Secondary Enzyme (Concentration)
Cronobacter	*Xba*I (50 U/sample)	*Spe*I (30 U/sample)

1. Label 1.5 ml microcentrifuge tubes with culture numbers; label 3 (10-well gel) or 4 (15-well gel) tubes for *Salmonella* ser. Braenderup H9812 standards.
   a. **Pre-Restriction Incubation Step (highly recommended)**: Prepare a master mix by diluting the appropriate 10X restriction buffer (Roche Applied Science or equivalent) 1:10 with sterile Ultrapure water (CLRW) according to the following table:

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices
Sterile Clinical Laboratory Reagent Water (CLRW)	180 µl	1800 µl	2700 µl
10X Restriction Buffer	20 µl	200 µl	300 µl
Total Volume	200 µl	2000 µl	3000 µl

   b. Add 200 µl diluted restriction buffer (1X) to labeled 1.5 ml microcentrifuge tubes.
   c. Carefully remove plug from TE with spatula and place in a sterile disposable Petri dish or on large glass slide.
   d. Cut a 2.0 to 2.5 mm wide slice from each test samples and the appropriate number of *S.* ser. Braenderup H9812 standards with a single edge razor blade (or scalpel, cover slip, etc.) and transfer to tube containing diluted

restriction buffer. **Be sure plug slice is under buffer.** Replace rest of plug into the original tube that contains 5 ml TE buffer and store at 4ºC.

Note: PulseNet recommends that the combs with larger teeth (10 mm wide teeth) be used to cast the gels because computer analysis of the gel lanes is more accurate and less tedious than analysis of gel lanes cast with combs with the smaller teeth (5.5 mm). Using combs with smaller teeth is not advised. The number of slices that can be cut from the plugs will depend on the skill and experience of the operator, integrity of the plug, and whether the slices are cut vertically or horizontally (plugs made in disposable molds).

e. Incubate sample and control plug slices in a 37ºC water bath for 5-10 min or at room temp for 10-15 min.
f. After incubation, remove buffer from plug slice using a pipet fitted with 200-250 µl tip all the way to bottom of tube and aspirate buffer. Be careful not to damage the plug slice with pipet tip and that plug slice is not discarded with pipet tip.

2. Prepare the restriction enzyme master mix according to the following table[3]. May mix in the same tube that was used for the diluted restriction buffer.

Note: Enzymes may be purchased in several different stock concentrations. The calculations outlined here are based on using an enzyme at a concentration of 10 U/µl. If the enzyme used is of a different concentration, make necessary adjustments to the volume of enzyme and water to achieve a final concentration of 50 U/ sample.

Note: Keep vial of restriction enzyme on ice or in insulated storage box (-20˚C) at all times.

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices
Sterile Clinical Laboratory Reagent Water (CLRW)	174 µl	1740 µl	2610 µl
10X Restriction Buffer	20 µl	200 µl	300 µl
BSA (20mg/ml)	1 µl	10 µl	15 µl
Enzyme (10 U/µl)	5 µl	50 µl	75 µl
Total Volume	200 µl	2000 µl	3000 µl

Note: Addition of Bovine Serum Albumin (BSA; highly recommended): Several restriction enzyme vendors specifically recommend the addition of 1X BSA to enzyme restriction mixtures while others do not. PulseNet Central recommends adding BSA to all enzyme restriction mixtures to minimize the incidence of incomplete restriction.

3. Add 200 µl restriction enzyme master mix to each tube. Close tube and mix by tapping gently; **be sure plug slices are under enzyme mixture**.

4. Incubate sample and control plug slices in 37˚C water bath for 1.5-2 h.

5. If plug slices will be loaded into the wells (Option B, page 8), continue with Steps 1-4 of the next section (**CASTING AGAROSE GEL**) approximately 1 h before restriction digest reaction is finished so the gel can solidify for at least 30 minutes before loading the restricted PFGE plugs.

CASTING AGAROSE GEL

A. Loading Restricted Plug Slices on the Comb:

1. Confirm that water bath is equilibrated to 55-60ºC.

2. Make volume of 0.5X Tris-Borate EDTA Buffer (TBE) that is needed for both the gel and electrophoresis running buffer according to one of the following tables.

5X TBE:

Reagent	Volume in milliliters (ml)	
5X TBE	200	220
Clinical Laboratory Reagent Water (CLRW)	1800	1980
Total Volume of 0.5X TBE	2000	2200

10X TBE:

Reagent	Volume in milliliters (ml)	
10X TBE	100	110
Clinical Laboratory Reagent Water (CLRW)	1900	2090
Total Volume of 0.5X TBE	2000	2200

3. Make 1% SeaKem Gold (SKG) Agarose in 0.5X TBE as follows:
   a. Weigh appropriate amount of SKG into 500 ml screw-cap flask.
   b. Add appropriate amount of 0.5X TBE; swirl gently to disperse agarose.
      i. Mix 1.0 g agarose with 100 ml 0.5X TBE for 14 cm wide gel form (10 wells)
      ii. Mix 1.5 g agarose with 150 ml 0.5X TBE for 21 cm wide gel form (15 wells)
   c. Loosen or remove cap and cover loosely with clear film, and microwave for 60 sec; mix gently and repeat for 15 sec intervals until agarose is completely dissolved.
   d. Recap flask and return to 55-60ºC water bath and equilibrate the agarose in the water bath for 15 minutes or until ready to use.

SAFETY WARNING: Use heat-resistant gloves when handling hot flasks after microwaving.

Note: Agarose LF™ (Amresco, X174) is the only acceptable alternative to SeaKem Gold, at this time. The time and temperature needed to completely dissolve the agarose is dependent on the specifications of the microwave used and will have to be determined empirically in each laboratory. Similarly, the optimal running time for each agarose will have to be determined empirically in each laboratory.

4. A small volume (2-5 ml) of melted and cooled (55-60ºC) 1% SKG agarose may be wanted to seal wells after plugs are loaded. Prepare as described above. Unused SKG agarose can be kept at room temperature, melted, and reused several times.

Note: Place the gel form on a leveling table and adjust until perfectly leveled. Place the comb holder so the front part (side with small metal screws) and teeth face the bottom of gel frame and the comb teeth touch the gel platform.

5. Remove restricted plug slices from 37ºC water bath. Remove enzyme/buffer mixture and add 200 µl 0.5X TBE. Incubate at room temperature for 5 min.

6. Remove plug slices from tubes; put comb on bench top and load plug slices on the bottom of the comb teeth as follows:
   a. Load *S.* ser. Braenderup H9812 standards on teeth (lanes) 1, 5, 10 (10 well gel) or on teeth 1, 5, 10, 15 (15 well gel).
   b. Load samples on remaining teeth and note locations.

7. Remove excess buffer with tissue or kimwipe. Allow plug slices to air dry on the comb for 5-10 minutes or seal them to the comb with 1% SKG agarose (55-60ºC).

8. Position comb in leveled gel form and confirm that the plugs slices are correctly aligned on the bottom of the comb teeth, and that the lower edge of the plug slice is flush against the black platform.

9. Carefully pour the agarose (cooled to 55-60ºC) into the gel form and remove any bubbles or debris.

10. Put black gel frame in electrophoresis chamber. Add 2-2.2 L freshly prepared 0.5X TBE. Close cover of unit. The amount of buffer needed depends on whether residual buffer was left in tubing or if unit was flushed with water after the last gel was run.

11. Turn on power supply, pump calibrated to a flow rate of 1 liter/minute (setting of ≈70) and cooling module (14ºC) approximately 30 minutes before gel is to be run.

12. Remove comb after gel solidifies, about 30-45 minutes.

13. Fill in wells of gel with melted and cooled (55-60ºC) 1% SKG Agarose (optional). Unscrew and remove end gates from gel form; remove excess agarose from sides and bottom of casting platform with a tissue or kimwipe. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber. Close cover of chamber.

B. Loading Restricted Plug Slices into the Wells:

1. Follow steps 1-4 in Section A on pages 7-8 (**Loading Restricted Plug Slices on the Comb**).

Note: Place the gel form on a leveling table and adjust until perfectly leveled before pouring gel. Position the comb holder so that front part (side with small metal screws) and teeth face the bottom of gel and the **bottom edge of the comb is 2 mm above the surface of the gel platform**.

2. Cool melted SKG agarose in 55-60ºC water bath for 15-20 min; carefully pour agarose into gel form (casting stand) fitted with comb. Be sure there are no bubbles.

3. Put black gel frame in electrophoresis chamber. Add 2-2.2 L freshly prepared 0.5X TBE. Close cover of unit. (The amount of buffer depends on whether residual buffer was left in tubing, or if unit was flushed with water after the last gel was run).

4. Turn on power supply, pump calibrated to a flow rate of 1 liter/minute (setting at ~70), and cooling module (14ºC) approximately 30 minutes before gel is to be run.

5. Remove restricted plug slices from 37ºC water bath. Remove enzyme/buffer mixture and add 200 µl 0.5X TBE. Incubate at room temperature for 5 minutes.

6. Remove comb after gel solidifies, about 30 – 45 minutes.

7. Remove restricted plug slices from tubes with tapered end of spatula and load into appropriate wells. Gently push plugs to bottom and front of wells with wide end of spatula. Manipulate position with spatula and be sure that are no bubbles.
   a. Load *S.* ser. Braenderup H9812 standards in wells (lanes) 1, 5, 10 (10 well gel) or in wells 1, 5, 10, 15 (15 well gel).
   b. Load samples in remaining wells.

Note: Loading the plug slices can be tedious; each person has to develop his/her own technique for consistently placing the plug slices in the wells so the lanes will be straight and the bands sharp.

8. Fill in wells of gel with melted 1% SKG Agarose (equilibrated to 55-60ºC). Allow to harden for 3-5 min. Unscrew and remove end gates from gel form; remove excess agarose from sides and bottom of casting platform with a tissue or kimwipe. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber. Close cover of chamber.

ELECTROPHORESIS CONDITIONS

1. Select following conditions for ***Cronobacter*** strains restricted with *Xba*I or *SpeI*:
   a. Select following conditions on **CHEF Mapper**
      Auto Algorithm
      25 kb - low MW
      286 kb - high MW
      Select default values except where noted by pressing "enter."
      Change run time to 18 - 19 h (See note below)
      (Default values: Initial switch time = 1.8 s; Final switch time = 25 s)

   b. Select following conditions on **CHEF-DR III**
      Initial switch time: 1.8 s
      Final switch time: 25 s
      Voltage: 6 V
      Included Angle: 120°
      Run time: 18-19 h (See note below)

   c. Select following conditions on **CHEF-DR II**
      Initial A time: 1.8 s
      Final A time: 25 s
      Start ratio: 1.0 (if applicable)
      Voltage: 200 V
      Run time: 19-20 h (See note below)

Note: The electrophoresis running times recommended above are based on the equipment and reagents used at the CDC. **Run times may be different in your laboratory and will have to be optimized for your gels so that the lowest band in the *S.* ser. Braenderup H9812 standard migrates 1.0-1.5 cm from the bottom of the gel.**

Note: Make note of the initial milliamp (mAmp) reading on the instrument. The initial mAmps should be between 110-150 mAmps. A reading outside of this range may indicate that the 0.5X TBE buffer was prepared improperly and the buffer should be remade.

Day 2

STAINING AND DOCUMENTATION OF PFGE AGAROSE GEL

Note: The following staining procedure describes the use of ethidium bromide to stain PFGE gels. Alternate DNA stains may be used. Please see the "Alternate DNA Stains-Results and Recommendations" posting within the PulseNet Documents forum on the SharePoint site for additional information.

1. When electrophoresis run is over, turn off equipment; remove and stain gel with ethidium bromide by diluting 40 µl of ethidium bromide stock solution (10 mg/ml) with 400 ml of Ultrapure water (CLRW). This volume is for a staining box that is approximately 14 cm x 24 cm; a larger container may require a larger amount of staining solution. Stain gel for 20-30 min in covered container.

Note: Ethidium bromide is toxic and a mutagen. Stock solutions of 10 mg/ml Ethidium Bromide (EtBr) in water are available from several commercial companies (Amresco X328; Bio-Rad, 161-0433; Sigma, E-1510). The diluted solution can be kept in dark bottle and reused 6-8 times before discarding according to your institution's guidelines for hazardous waste. CDC does not recommend disposing of EtBr down the drain. Aqueous solutions containing EtBr can be filtered through charcoal or degraded using activated carbon destaining or "tea" bags from Amresco (E732-25 Destaining Bags) or other companies, which effectively and safely remove EtBr from solutions and gels. Once the EtBr is removed, the treated aqueous solutions can be discarded down the drain. If you have further questions about EtBr please refer to the **Material Safety Data Sheets** (**MSDS**) provided by the vendor or manufacturer.

Note: Currently, the only acceptable alternative stain options are GelRed™ (Biotium, 31010), SYBR® Safe (Invitrogen, S-33102) and SYBR® Gold (Invitrogen, S-11494). Labs are strongly encouraged to follow manufacturer's instructions and test stains in their labs before adopting them for routine use. If one of the alternative stains is used, the destaining steps should be omitted.

2. Destain gel in approximately 500 ml CLRW for 60 - 90 min, changing water every 20 minutes. Capture image using a Gel Doc 1000, 2000, EQ, XR, or equivalent documentation system. If too much background is observed destain for an additional 30-60 min.

3. Follow directions given with the imaging equipment to save gel image as a ***.1sc** file; convert this file to ***.tif** file for analysis with the BioNumerics software program. The gel image should fill the entire window of the imaging equipment (computer) screen (without cutting off wells or lower bands). Ensure that the image is in focus and that there is little to no saturation (over-exposure) in the bands (signified by red pixilation in the QuantityOne or ImageLab software). Additional instructions are provided in PNL07 of the PulseNet QA/QC manual.

4. Drain buffer from electrophoresis chamber and discard. Rinse chamber with 2 L Ultrapure water (CLRW) or, if unit is not going to be used for several days, flush lines with water by letting pump run for 5-10 min **before** draining water from chamber and tubing.

5. If the lowest band in the H9812 standard does not migrate within 1-1.5 cm of the bottom of the gel, the proper run time will need to be determined empirically for the conditions in each laboratory.

Note: The following options are available if PFGE results do not have to be available within 24-28 hours:

- Plugs can be lysed for longer periods of time (3-16 hours).
- The washing steps with TE to remove the lysis buffer from the PFGE plugs can be done for longer periods of time (30-45 min) and at lower temperatures (37°C or room temperature). They can be started on Day 1 and finished on Day 2 after overnight refrigeration of the plugs in TE.

Use of trade names and commercial sources is for identification purposes only and does not imply endorsement by CDC or the U.S. Department of Health and Human Services.

NOTE: CLIA LABORATORY PROCEDURE MANUAL REQUIREMENTS

Efforts have been made to assure that the procedures described in this protocol have been written in accordance with the 1988 Clinical Laboratory Improvement Amendments (CLIA) requirements for a procedure manual (42 CFR 493.1211). However, due to the format required for training, the procedures will require some modifications and additions to customize them for your particular laboratory operation.

Any questions regarding the CLIA requirements for a procedure manual, quality control, quality assurance, etc., should be directed to the agency or accreditation organization responsible for performing your laboratory's CLIA inspection. In addition, some states and accreditation organizations may have more stringent requirements that will need to be addressed.

Formulas of Selected Reagents used in PulseNet Standardized Laboratory Protocol for PFGE

Tris:EDTA Buffer, pH 8.0 (TE, 10 mM Tris:1 mM EDTA, pH 8.0)

10 ml of 1 M Tris, pH 8.0
2 ml of 0.5 M EDTA, pH 8.0
Dilute to 1000 ml with sterile Ultrapure water (CLRW)

Cell Lysis Buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosine + 0.1 mg/ml Proteinase K)

25 ml (50 ml) of 1 M Tris, pH 8.0
50 ml (100 ml) of 0.5 M EDTA, pH 8.0
50 ml (100 ml) 10% N-Lauroylsarcosine, Sodium salt (Sarcosyl)

OR

5 g (10 g) of N-Lauroylsarcosine, Sodium salt (Sarcosyl)[3]
Dilute to 500 ml (1000 ml) with Sterile Ultrapure water (CLRW)

Add **25 µl** Proteinase K stock solution (20 mg/ml) per 5 ml of cell lysis buffer **just before use** for a final concentration in the lysis buffer of 0.1 mg/ml Proteinase K.

[3]If Sarcosyl powder is added directly to the other components of this reagent, warm the solution to 50-60ºC for 30-60 minutes, or leave at room temperature for ≈2 hours to completely dissolve the Sarcosyl; adjust to the final volume with sterile Ultrapure water (CLRW).

Use the following calculations for *Spe*I (30 Units/plug slice):

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices
Sterile Clinical Laboratory Reagent Water (CLRW)	178.25 µl	1782.5 µl	2673.75 µl
10X Restriction Buffer	20 µl	200 µl	300 µl
BSA (20mg/ml)	1 µl	10 µl	15 µl
***Spe*I (40 U/µl)**	0.75 µl	7.5 µl	11.25 µl
Total Volume	200 µl	2000 µl	3000 µl

Note: Keep vial of restriction enzyme on ice or in insulated storage box (-20ºC) at all times.

5. FLOW CHART:

6. BIBLIOGRAPHY:

1. Brengi, et al. 2012. Development and validation of a PulseNet standardized protocol for subtyping Isolates of *Cronobacter* species. Foodborne Pathog Dis. 9:861 – 867.
2. Ribot, at al. 2006. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. Foodborne Pathog Dis. 3:59 – 67.

7. CONTACTS:

8. AMENDMENTS:

1. **PURPOSE:** To describe the minimal requirements for computer hardware and software needed for analysis of PulseNet PFGE gels.

2. **SCOPE:** This procedure applies to all PulseNet participating laboratories analyzing PFGE gels.

3. **DEFINITIONS/TERMS:**

   3.1 PFGE: Pulsed-field Gel Electrophoresis
   3.2 BioNumerics: Gel analysis software used by PulseNet, developed by Applied Maths, Belgium
   3.3 CDC: Centers for Disease Control and Prevention

4. **RESPONSIBILITIES/PROCEDURE:**

   4.1 Participating laboratories must use the following software and hardware
      4.1.1 BioNumerics Software v3.0, v3.5, v4.01, or v5.0
      4.1.2 CDC PulseNet masterscripts
      4.1.3 Personal computer equipped with an Intel Pentium CPU or better, 256 MB RAM or more, 65 K color graphics or better and Windows 98, Windows NT 4.0, or higher
      4.1.4 Keyboard, mouse, CD-ROM
      4.1.5 High resolution screen 1024x768 or higher; true color
      4.1.6 Large screen recommended (17" or more)

   4.2 For quotes and additional information in USA and Canada, contact Applied Maths, Inc.
   4.3 For quotes and additional information in Europe, contact Applied Maths BVBA.

Use of trade names and commercial sources is for identification only and does not imply endorsement by CDC or the U.S. Department of Health and Human Services.

5. **FLOW CHART:**

6. **BIBLIOGRAPHY:**

7. **CONTACTS:**

   7.1 Applied Maths, Inc.

   512 East 11th Street, Suite 207
   Austin, TX 78701
   Phone: 512-482-9700
   Fax: 512-482-9708
   E-mail: **info-US@applied-maths.com**
   www.applied-maths.com

   7.2 Applied Maths BVBA

   Keistraat120
   B-9830 Sint-Martens-Latem
   Belgium
   Phone: +32-9-2222-100
   Fax: +32-9-2222-102
   E-mail: **info@applied-maths.com**
   www.applied-maths.com

8. **AMENDMENTS:**

1. **PURPOSE :** To describe the guidelines for standardization of TIFF image analysis in order to accurately compare PFGE patterns between PulseNet participating laboratories.

2. **SCOPE:** This procedure applies to all analyses submitted to PulseNet, thereby allowing comparison of results with other PulseNet laboratories.

3. **DEFINITIONS/TERMS:**

   3.1 SOP: Standard Operating Procedure
   3.2 PFGE: Pulsed-field Gel Electrophoresis
   3.3 TIFF: Tagged Image File Format. A file of a gel image that can be analyzed in BioNumerics
   3.4 BioNumerics: Gel analysis software used by PulseNet, developed by Applied Maths, Belgium

4. **RESPONSIBILITIES:**

   4.1 After an electronic image of a gel is obtained using the procedure in SOP PNL07, the file **MUST** be analyzed using the PulseNet customized version of BioNumerics Analysis Software version 3.0 or higher.

   4.2 The SOPs PNG05, PNL01 – PNL06 and PND01 contain settings and instructions to allow users to create and compare analyzed PFGE fingerprint patterns sent to the PulseNet national databases. Masterscripts for setting up databases in *E. coli*, *Listeria*, *Salmonella*, *Shigella*, and *Campylobacter* are available from the PulseNet Task Force by sending an e-mail to pfge@cdc.gov. In order for results to be compatible with the PulseNet national databases, databases must be set up using the masterscripts provided by the PulseNet Task Force.

   4.3 TIFFs can either be copied to the appropriate images directory or imported directly into BioNumerics.

   4.4 Local labs can add information fields to their databases; however, they should add their lab ID either to the beginning or end of the information field name. This is recommended to ensure that the local labs are able to differentiate between CDC fields and their local lab fields.

   4.5 When creating a bundle file to be shared with other PulseNet participating labs, and/or on the WebBoard, the "PulseNet Bundle" tool must be used.

5. **PROCEDURE:**

   5.1 Installing BioNumerics Software
      5.1.1 Insert the BioNumerics CD into the CD-ROM drive and click on "Install BioNumerics."
      5.1.2 Note the directory the installation will be in and use the default setting to install a sample database (DemoBase).
      5.1.3 Click "Yes" to create a shortcut icon
      5.1.4 Click "OK" for the protection key installer
      5.1.5 Click "Next" to install the sentinel system driver
      5.1.6 Accept the terms and click "Next"
      5.1.7 Choose the default folder and then click "Next"
      5.1.8 Choose the default "Complete" setup type and then click "Next"
      5.1.9 Clink "Install" to begin the installation process
      5.1.10 Insert the license string, which is found in the BioNumerics package
      5.1.11 Click "Finish"

   5.2 Creating a new database

5.2.1 Double-click on the BioNumerics icon and click the "New" button.
5.2.2 When the new database window appears, enter a name for the new database and click "Next."
5.2.3 Choose which directory you would like to install the database into. Click "Next" to accept the default directory or change the directory path by using the browse key, and then click "Next."
5.2.4 When asked if you would like to create log files for the new database, select "Yes" and click the "Finish" button. The log files allow you to track any changes made to the database.

5.3 Running masterscripts
5.3.1 Insert the most recent PulseNet masterscripts CD (provided by PulseNet) into the CD-ROM drive.
5.3.2 Start the BioNumerics software, highlight the database name that you wish to customize for PulseNet and click the "Analyze" button.
5.3.3 In the upper toolbar click "Scripts" and select "Run script from file…"
5.3.4 Choose "Install.BNS" from the PulseNet Master Scripts CD.
5.3.5 Select the appropriate organism and enter your lab ID; then click "OK."
5.3.6 The program will ask to be restarted, click "OK" to finish the installation of Master Scripts.

5.4 Changing database settings
5.4.1 Highlight the database of interest and click "Settings."
5.4.2 By clicking on the tabs, various settings can be changed, including the background color and the order of the database fields.

5.5 Importing a TIFF into BioNumerics
5.5.1 Highlight the database of interest and click "Analyze."
5.5.2 Right-click under the "Files" panel in the main BioNumerics screen and click on "Add new experiment file…," or click on the folder shortcut button at the top of the Files menu.
5.5.3 "Browse," and choose the location of the TIFF, highlight it, and click "Open."
5.5.4 In BioNumerics v4.0, a confirmation window will ask, "Do you want to edit the image before adding it to the database?"
5.5.4.1 Choose "No" if you do not need to edit the image.
5.5.4.2 If you choose "Yes," you can rotate or crop the image, or invert colors.
5.5.5 There should now be a red "N" in front of the TIFF name (the "N" denotes a new TIFF that has not been analyzed).

5.6 Saving a TIFF into an organism-specific BioNumerics database from an e-mail or WebBoard
5.6.1 When you save a TIFF to analyze in BioNumerics, you will need to save it in a specific file location.
5.6.2 Save the TIFF in the "Images" folder in the BioNumerics directory for the specified organism. For example, to save a TIFF in the *E. coli* BioNumerics database, you would save it under: C:\Program Files\BioNumerics\data\Ecoli\Images.
5.6.3 The next time the specified database is open, the TIFF will appear under the "Files" panel with a red "N" in front of it.
5.6.4 **Note:** The TIFF will not appear if it is saved in the "Images" folder while BioNumerics is open. To see the newly imported TIFF, close, then reopen BioNumerics.

5.7 Analyzing a gel
5.7.1 Locate the TIFF you wish to analyze in the "Files" area and double-click on the file name, or highlight the TIFF name and click on the blue arrow shortcut button.
5.7.2 When the "Fingerprint File" window opens, click on the **"**Edit fingerprint data" button.
5.7.3 Select the appropriate fingerprint type (this will be the enzyme used for the majority of the gel) and click "OK."

5.7.4 The gel should be white with black bands. If the gel appears black with white bands, go to "Edit settings" and check the box for "Inverted values."

5.7.5 Once the Fingerprint data window opens, the selected TIFF image appears in a green box.

5.7.6 Using the green nodes, adjust the size of the green box so that the left and right sides of the green box are at the edge of the left and rightmost gel lanes. Adjust the top of the green box so that it is just below the wells of the gel. Position the bottom of the green box at the bottom of the gel or the bottom of the TIFF (if the bottom of the gel is not visible).

5.7.7 If the gel is curved, holding down the <Shift> key while adjusting the nodes will allow for the box to curve around the gel.

5.7.8 Next, indicate the lanes on the gel by clicking on the "Auto search lanes" button (magnifying glass) to perform a lane search.

5.7.9 The Search lanes window will appear and prompt you for an estimated number of lanes on the gel. This number doesn't have to be exact but it should be close to the number of lanes since this will help the program to designate the correct number of lanes. Enter the number of lanes and click "OK." Each lane that encompasses the DNA fingerprint is termed a "Gel Strip."

5.7.10 If the exact number of lanes is known, you may choose to add them by choosing "Lanes" and "Define group of lanes…" and entering the exact number of lanes on the gel.

5.7.11 When the lane auto search is complete, the "Gel Strip" may need to be adjusted to encompass the entire PFGE pattern. Blue nodes located inside of the "Gel Strips" allow these lane indicators to be moved and curved to match the lanes on the gel. To move the entire gel strip, click on any node and move the gel strip left or right. To adjust the curvature of a gel strip, hold down the <Shift> key and click on a node and drag it (hold down the mouse button while you move it) to curve the gel strip. How the gel strip is curved depends on the selected node.

5.7.12 Further adjustments to the fingerprint conversion can be made in the Fingerprint conversion settings window. Click on the "Edit Settings" button or choose "Edit" then "Edit Settings" from the top menu to open this window. From this window you can adjust the thickness of the image gel strips so that they fit the gel lanes. You can also adjust the number of nodes in the lane indicators to allow for more adjustment flexibility. When you are done with adjustments click "OK" to close the window and apply the changes.

5.7.13 Once the gel strips have been placed and adjusted to your satisfaction, adjustments to the appearance of the gel image can be made in the "Gel tone curve" window. To open the window, click on the "Edit" menu and click on "Edit tone curve." The "Gel tone curve" window has a set of image adjustment buttons and a pair of gel lane windows that show a "before" and "after" preview of any adjustments before they are applied. Furthermore, by clicking in the bottom preview window and dragging the mouse, the gel image can be moved around within the "after" window.

5.7.14 The "Linear" adjustment on the "Gel tone curve" window is a good starting point to adjust the color of a gel image. This adjustment selects for the "best fit" Optical Density (OD) range or densitometric curve values for optimizing the TIFF image. To use this function click on the "Linear" button. Other adjustments that can be used to improve a gel image are "Enhance weak bands" and "Enhance dark bands." These two functions are complementary, so using one and then the other will cancel any effect. Click on the "Enhance weak bands" button to apply this adjustment. The button can be clicked a number of times to increase the level of adjustment.

5.7.15 When satisfied with all tone curve adjustments click "OK" to accept the changes (or click "Cancel" to reject any changes), which will close the "Gel tone curve" window.

5.7.16 When you are satisfied with the gel strip positions and any image adjustments that have been made, click on the "Next step" button (right arrow) to proceed to Step 2 of Analysis.

5.7.17 The goal of Step 2 is to place the Curve strips so that they are located in the area of the lane that gives the most accurate representation of the gel bands. The densitometric curve for the selected lane is shown on the right side of the window. The curve peaks indicate where the gel lane is "darkest" or has the highest optical density. Click on the nodes in the Curve strips and drag the mouse to move the whole strip from left to right. By holding down the <Shift> key and clicking

on a node, the Curve strip can be curved. You should avoid placing the curve strip in areas with artifacts (specks, etc.) on the gel.

5.7.18 Once the Curve strips have been successfully placed, click on the "Next Step" button to go to Step 3 of Analysis, which is a step for Normalization where the gel lanes are normalized against the PulseNet global standard.

5.7.19 **Note:** During any step of analysis, you can save your work, undo and redo actions, and zoom in or out by clicking on the various buttons in the upper toolbar.

5.7.20 Step 3, "Normalization," allows laboratories to compare PFGE results and is the most important step of analysis. Click on a reference lane to select it (the number above the lane should turn yellow) and click on the "Use selected lane as reference lane" button (looks like a weight). This will mark the lane as a reference lane. Repeat this process for all of the reference lanes.

5.7.21 Match the bands in the reference lanes from the gel strips with the global standard band markers (flags with fragment sizes on them).

5.7.22 To mark a gel strip reference lane band, first select the desired lane by clicking on it. The gel strip number turns yellow when selected. Place the band selection cursor (small black arrow) on a band of a reference lane by clicking on it with the mouse and then clicking on its corresponding global standard lane band marker (the selected band marker will turn white). You can also drag the mouse within the gel strip (hold down the <Tab> key to take off "Snap to Peaks" and move pixel by pixel).

5.7.23 **Note:** Make sure to click the band first and then the global standard band marker to ensure the correct band marker is highlighted. Enlarging the image using the zoom buttons helps with placement of band markings.

5.7.24 Once both of the markers are correctly placed, press the <Enter> key to insert the normalization marker. If you make a mistake in placing the normalization marker, then you can press the <Delete> key to erase it. Repeat these steps to mark the rest of the bands in the reference lanes.

5.7.25 Another option is to mark only some of the bands for each reference lane. Click on the "Auto assign reference positions" button to have BioNumerics search and place the normalization markers. When the "Confirmation" window appears stating, "Do you want to preserve the existing bands?" click "Yes" to save the selected bands that have been marked. Double-check to make sure the correct bands in the reference lanes of the gel were matched up with the correct band markers.

5.7.26 After the bands for the reference lanes have been marked, and their placements are correct, then click on the "Show normalized view" button to show the gel strips as they will appear when normalized. If changes are made in the normalized view, click on the "Update normalization" button to update normalization.

5.7.27 To check normalization, click on "Normalization" and "Show Distortion Bars" (in version 3.5, this option is saved). The gel strip lanes will now show colored distortion bars to the right of the gel lanes. The colors in the distortion bars indicate the degree of adjustment that was made to each section of the gel lane. The colors run from light blue for a small adjustment to red and black for a large adjustment. An unusually high amount of orange, red or black in the distortion bar may indicate a normalization error, and should be examined. The colors that are seen should be consistent as they move horizontally across the gel lanes. In the case of an abnormal gel run, dark distortion bars may remain, even if normalization was performed correctly.

5.7.28 Once the normalized gel strips have been checked for any normalization errors, click on the "Next step" button to proceed to Step 4 to complete band marking.

5.7.29 Click on the "Auto search bands" button from the toolbar to have BioNumerics search the lanes for band positions. When the "Band search" window appears, accept the default search values and click **"**OK**"** to perform the band auto search. If the gel is atypical, adjust the minimum profiling percentage. When the "Confirmation" window appears with a question that asks, "There are already some bands defined on the gel. Do you want to keep the existing bands?" click "Yes" to preserve the existing bands marked, "No" for BioNumerics to auto search for all bands again, or "Cancel."

5.7.30 After bands are found by the auto search function, look at the whole gel and delete erroneous or unnecessary bands. Be sure to go through each lane individually and check band markings by looking at a printout of the gel image. This will ensure that extra bands, such as those near the top or bottom of a TIFF image, are deleted. If artifacts are present on a TIFF image or the TIFF image is dark, the auto search function may find many extra bands.

5.7.31 Refer to the document "PulseNet Gel Analysis Guidelines" (PND04) for more information on how to mark bands.

5.7.32 To manually insert a band mark, click on the position where you would like the band, and this will put the band placement cursor where the band mark will be placed. Press the <Enter> key to insert the band mark. To remove a band mark, highlight the band mark and press the <Delete> key. If you are uncertain about a band mark, then you can classify a band mark as uncertain (press <F5>), and this mark will not be included in comparisons (dendrograms, etc.) unless you choose to have them included. Do not place more than two uncertain bands per gel. If you need more, you should rerun the corresponding isolates or the entire gel. To access bands that may be marked outside of the standard, you can un-normalize the gel.

5.7.33 Once all of the lanes have been checked, click the "Save" button to save the normalized gel strips with band marks. Close the Fingerprint data window after saving. This completes the analysis.

5.8 Changing enzymes/Fingerprint type

5.8.1 It is now time to link lanes; but first, make sure the correct fingerprint type (e.g., PFGE-XbaI, PFGE-BlnI, etc.) is indicated. If the lanes are linked with incorrect fingerprint types, possible duplicate entries in the database can occur.

5.8.2 To change the fingerprint type, right-click on the desired lane and select "Change fingerprint type of lane," then select the appropriate enzyme.

5.9 Linking isolates to patterns/database entries

5.9.1 To link a lane, either right-click on the desired lane and select the "Link lane" option or highlight the lane and click on the pink arrow on the top toolbar.

5.9.2 The "Link lane" window will appear and is where a unique isolate number or identifier should be entered. This code will be known as a "Key" in BioNumerics. Click "OK" after entering the new key. If the key is not currently present in the database, then a "Confirmation" window appears to ask if you would like to create the new key. If the key is correct, then click "Yes." If the key is incorrect, click "Cancel" and reenter the key to make sure the key is correct.

5.9.3 If an isolate is a re-run, a different "Confirmation**"** window appears to ask if you would like to create a duplicate key. If you would, click "Yes." This will now create the isolate with a "/#1" at the end; all the demographic information will stay the same.

5.9.4 Do not upload isolates with a "/#" at the end of them. Pull up all versions of the pattern and choose which one looks the best and should be included in the national database. Double-click on the isolate and click on the "Edit Database Fields" icon (lightning bolt with green "i"). In the "Patterns" box, click on the button that corresponds to the "best pattern." In the PFGE fingerprint box that opens, click on the "Make first pattern…" button (this button will not appear if the pattern you have chosen is already the first pattern). If the duplicate that was just linked is the best pattern, it will no longer have a "/#" behind it and can be uploaded.

5.9.5 Do not link or upload standard/reference lanes. The only exception to this rule is for submission of certification results.

5.9.6 To enter isolate information for a gel lane, double-click on the desired lane in the "Fingerprint file" window. This will open the "Entry edit" window. Click on the "Edit database fields" (green "i") button to open the customized PulseNet "Entry properties" window. Note that data can be entered in the "Entry edit" window; however, the data will be lost if you then open the "Entry Properties" window without first clicking "OK."

5.9.7 Enter the isolate information into data fields in the customized PulseNet "Entry properties" window. Most of the data fields use drop-down menus (pick lists) containing fixed values, as well

as previous entries that were used, however, data can also be typed into these fields. In order to enter correct serotype, ***use only the drop-down menu*** to enter serotype or antigenic formula information for any specific isolate.

5.9.8 Antibiogram types can be entered for an isolate by clicking on the "Antibiotics" button to open the isolate antibiotic screen. Set an antibiotic resistance level using the pick lists. To add an antibiotic to the list click "Add," and enter the new antibiotic name into the text box and click "OK." However, only the antimicrobials on the original list will be uploaded. Click "OK" when done.

5.9.9 Repeat entering information for all isolates on the gel.

5.10 Uploading data

5.10.1 Connecting to the PulseNet server is a two-part process. First, authenticate to the CDC firewall using a SecurID key fob issued by PulseNet. You must pass analysis certification for each organism database in order to have access to each database. If you are certified for analysis, but do not have a SecurID, contact the PulseNet Database Team at the CDC. Next, connect to the server using the BioNumerics software.

5.10.2 After connecting to the server, open the gel with the isolates that need to be uploaded. Click "Submit fingerprint file to server" (lightning bolt with a blue arrow). In the "Submit fingerprint" window, select only the lanes that need to be uploaded. Check or uncheck "Submit antibiotics data" and "Submit biochemical data" as necessary. Then click "OK" to upload the isolates to the PulseNet database.

5.10.3 If you need to resubmit gel information (i.e., you need to update a serotype or demographic information), make sure to select all lanes that were previously submitted to the database. Otherwise, those lanes not selected will become unlinked in the national database.

5.11 Creating comparisons and performing cluster analyses

5.11.1 Select the isolates that you would like to include in your comparison:

5.11.1.1 <Ctrl> + left click; <Shift> + left click; or left click + <space bar>

5.11.2 Or you can select isolates by querying the local database:

5.11.2.1 Several queries can be performed using the script icons on the main BioNumerics screen.

5.11.2.2 There are queries for: isolate information, pattern information, antibiotics resistance, and biochemical information.

5.11.2.3 The binoculars can be used to search for information in any field.

5.11.2.4 Use a wildcard (*) around the search criteria to ensure that all entries with the search string are found.

5.11.3 In the upper toolbar click "Comparison" and select "Create new comparison."

5.11.4 When the comparison screen opens, highlight the enzyme you wish to show by clicking on the picture of the gel located next to the enzyme in the bottom toolbar.

5.11.5 In the upper toolbar click on "Clustering" and select "Calculate," then choose "Cluster analysis (similarity matrix)…"

5.11.6 In the Comparison settings window the Similarity coefficient should be Band based using the Dice coefficient, and the Dendrogram type should be set to UPGMA.

5.11.7 You may have to adjust the Position tolerance settings for your dendrogram. You can do so by clicking on the "Position tolerances…" button; however, PulseNet suggests using an Optimization of 1.50 and a Position tolerance of 1.50. Click "OK" to close the Position tolerance settings window.

5.11.8 Click "OK" in the Comparison settings window to create the dendrogram tree structure in the dendrogram area of the Comparison window.

5.11.9 To compare two isolates, select the entries and click "Comparison" in the upper toolbar. Select "Compare two entries" and highlight the enzyme that you wish to show.

6. FLOW CHART:

7. BIBLIOGRAPHY:

Weinberg, Sandy. GOOD LABORATORY PRACTICE REGULATIONS. Second edition. Marcel Dekker, Inc. USA (1995).

8. CONTACTS:

8.1 PulseNet Database Team
(404) 639-4558
PFGE@cdc.gov

9. AMENDMENTS:

1. PURPOSE: To explain the procedure for obtaining access to and using the PulseNet/OutbreakNet SharePoint site.

2. SCOPE: This procedure applies to all PulseNet participants who need or have access to the PulseNet/OutbreakNet SharePoint site. OutbreakNet participants should contact the CDC Outbreak Response Team outbreakresponse@cdc.gov to obtain access to SharePoint and/or with any questions about OutbreakNet's terms of use.

3. DEFINITIONS/TERMS:

3.1 PulseNet/OutbreakNet SharePoint site: A closed, web-based discussion forum used for communication among PulseNet/OutbreakNet participants. The PulseNet/OutbreakNet SharePoint site is open to all laboratory staff at PulseNet participating laboratories and OutbreakNet Epidemiologists working in collaboration with these laboratories. When approved by PulseNet, U.S. food regulatory staff and PulseNet International representatives may also have access. This SharePoint site is not open to university or private industry personnel unless their inclusion is deemed to be in the interest of public health. Throughout this document, the PulseNet/OutbreakNet SharePoint site will be referred to as "SharePoint." (http://partner.cdc.gov/sites/NCEZID/DFWED/EDLB/PulseNet)

3.2 SharePoint: SharePoint is a web-based tool developed by Microsoft that can be used for many different functions. PulseNet/OutbreakNet use it for document sharing, announcements, a calendar, website links and discussion forums.

3.3 PFGE: Pulsed-field Gel Electrophoresis

3.4 CDC: Centers for Disease Control and Prevention

3.5 BioNumerics: Gel analysis software used by PulseNet, developed by Applied Maths, Belgium

3.6 TIFF: Tagged Image File Format, a file of a gel image that can be analyzed in BioNumerics

3.7 PFGE Mailbox: An e-mail account that is maintained and checked by all database managers at CDC. The address is: PFGE@cdc.gov.

3.8 CDC Outbreak Response Team Mailbox: An e-mail account that is maintained and checked by the outbreak response team at CDC. The address is: outbreakresponse@cdc.gov

3.9 EDLB: Enteric Diseases Laboratory Branch

3.10 Area Laboratory: Laboratory, designated by CDC, which has agreed to assume responsibility for additional PulseNet duties for laboratories within their support zone. The current Area Laboratories include CDC, Massachusetts, Michigan, Minnesota, Texas, Utah, Virginia, and Washington.

3.11 Cluster: A group of isolates, identified within the past 30 days for *Yersinia*; 60 days for *Salmonella*, *E. coli*, Non-O157, *Shigella*, *C.* botulinum, *Campylobacter*, and *Vibrio*; and 120 days for *Listeria*, with the same serotype with indistinguishable PFGE DNA patterns by one or more restriction enzymes

3.12 STEC: Shiga toxin-producing *E. coli*. STECs are the most virulent of the diarrheagenic pathogens under surveillance in PulseNet.

3.13 NDA: A Non-disclosure Agreement is a legal contract between at least two parties that outlines confidential materials or knowledge the parties wish to share with one another for certain purposes, but wish to restrict from generalized use. In other words, it is a contract through which the parties agree not to disclose information covered by the agreement. An NDA creates a confidential relationship between the parties to protect any type of trade secret. As such, an NDA can protect non-public business information.

4. PROCEDURE:

4.1 SharePoint Access

4.1.1 All PulseNet personnel who wish to access SharePoint must send an email to the PFGE Inbox (PFGE@cdc.gov) with "SharePoint Request" in the subject line.

4.1.1.1 The requestor should include the person's first and last name, email address, organization, phone number, job title and work address in the email. Complete information is required to create an account so please include all requested information.

4.1.1.2 The request email will be moved to the "Participants" folder by the database manager who checks the PFGE Inbox.

4.1.2 Federal, state and local epidemiologists must request access to SharePoint through the Outbreak Response Team at CDC outbreakresponse@cdc.gov .

4.1.3 Access is approved if the person is certified and/or in the PulseNet contacts database. If the person is not in the contacts database, contacts from the person's lab will be contacted by the designated database manager to assure the person should obtain access.

4.1.4 If access is disapproved, the designated database manager notifies the requestor immediately.

4.1.5 If access is approved, the PulseNet Database Unit Chief (or other personnel designated by the Unit Chief) will add the participant to CDC Join. For additional information on CDC Join, including how to add new participants please review the training document saved here \\Cdc\project\CCID_NCZVED_DFBMD_PulseNet\SharePoint\CDC Join\CDC Join Tips

4.1.6 Once a new participant has been added to CDC Join, they will receive a system-generated email with instructions to complete registration and to send an email to the sponsor, notifying them that registration has been completed. The PulseNet Database Unit Chief (or other personnel designated by the Unit Chief) will submit an ITSO ticket requesting the participant's access to SharePoint. Make sure the ITSO ticket is sent Attn: [*PulseNet IT Support*, see contacts section below for contact information] and includes the new participant's email address.

4.1.7 PulseNet IT support will be assigned the ITSO ticket and use the participant's contact information in CDC Join, http://externalpartners.cdc.gov, to populate a database. The database will be used to keep track of the accounts, including NDA submission. In case of an emergency and the SharePoint site is not accessible for an extended period of time, the participants can be notified using the information in the database. Then PulseNet IT support will grant access to SharePoint by adding their email to the appropriate group. Once access to SharePoint has been granted, the participant will receive a system-generated email welcoming them to SharePoint, providing login information and requesting that they complete the Non-Disclosure Agreement (NDA). When accessing the SharePoint site, for external partners, the username must include the prefix **cdcpartners** and for CDC staff, the userID must include the prefix **cdc**.

4.1.8 Access to SharePoint will grant the new participant access to the Non-Disclosure Agreement Subsite -. The new subscriber must print, read, sign, scan and email or fax the completed NDA to CDC as described in Appendix PND03-1. Signing the NDA

indicates that the user agrees to follow the SharePoint policy on sharing information (section 4.2 below).

4.1.9 When CDC receives the scanned or faxed copy of the completed NDA, EDLB Administrative Support will add a check mark in the NDA section for the participant and submit an ITSO ticket Attn: [*PulseNet IT Support*, see contacts section below for name] stating the NDA has been received.

4.1.10 PulseNet IT Support will be assigned the ITSO ticket, verifies the account in the database and closes the ITSO ticket.

4.1.11 Passwords must not be shared or disclosed to anyone, for security purposes.

4.1.12 When a participant leaves their current position or is no longer performing PulseNet duties, they or their supervisor are required to notify CDC so that SharePoint access is terminated. CDC may be notified by emailing PFGE@cdc.gov (or outbreakresponse@cdc.gov if epidemiologist) with "SharePoint" in the subject line or by calling (404) 639-4558.

4.1.13 Once CDC is notified of a participant's departure, the PulseNet Database Unit Chief (or other personnel designated by the Unit Chief) submits an ITSO ticket Attn: [*PulseNet IT Support*, see contacts section below for contact information] to have that participant removed from SharePoint.

4.1.14 PulseNet IT Support receives the ITSO ticket, removes the participant from the subscriber database, removes their access to SharePoint and closes the ITSO ticket.

4.2 SharePoint Policy on Sharing Information:

Often the PulseNet/OutbreakNet SharePoint postings contain preliminary information on presumptive disease clusters and ongoing outbreak investigations. Therefore, SharePoint postings are not appropriate for sharing with persons outside public health and food regulatory agencies. If you would like to share SharePoint messages with persons not directly associated with public health or food regulatory agencies, we require that you obtain prior approval from the person or agency that posted the information. We would appreciate your strict compliance with this policy. Violation of this policy will result in loss of access to the PulseNet/OutbreakNet SharePoint website.

4.3 PulseNet/OutbreakNet SharePoint Administrative Roles:

There are four types of SharePoint users, each with a different level of privileges and security. In descending order, these are the SharePoint Site Owner, Moderator, Member and Visitor.

4.3.1 **SharePoint Site Owners** have complete control over the entire SharePoint website. This level of administration is managed by the division SharePoint site collection administrator, with oversight of the CDC Information Technology Services Office. This is not managed by anyone in the PulseNet or Outbreak Units at CDC.

4.3.2 **SharePoint Site Moderators** have full responsibility for maintaining assigned discussion forums by responding to, updating, archiving, and removing information as described in 5.2. Moderators can add, edit or delete postings, retrieve them from the archives, and modify discussion settings. This level of administration is managed by the SharePoint site Owners.

4.3.3 **SharePoint Site Members** can read, post, and respond to messages on the SharePoint site. Members can delete messages that they post. Members do not necessarily have access to all discussions or subsites on the PulseNet/OutbreakNet SharePoint site. This level of administration is managed by the SharePoint site Owners.

4.3.4 **SharePoint Site Visitors** can read messages on the SharePoint site. This level of administration is managed by the SharePoint site Owners.

4.4 SharePoint Setup

4.4.1 SharePoint is divided into several organized content sections: Documents, Announcements, Discussions and Subsites. Topics should be posted within the appropriate content section. Topics may be added or removed at the discretion of the Moderators.

4.4.2 Clicking on a topic within the content section allows the member to see the items and postings within that document library, announcements section, discussion or subsites.

5. RESPONSIBILITIES:

5.1 Posting Cluster or Outbreak-Related Topics and Responses on SharePoint

5.1.1 Cluster and outbreak information should be posted on SharePoint for quicker and more efficient reporting.

5.1.1.1 Indications for when to post a cluster

5.1.1.1.1 When a *Salmonella* or *Shigella* cluster of three or more indistinguishable patterns has an epidemiological connection; state the epidemiological link in the posting

5.1.1.1.2 When a *Salmonella* or *Shigella* cluster of three or more isolates (same serotype) with a pattern that shows an increase in your state or region

5.1.1.1.3 For other organisms, when a cluster of two or more indistinguishable patterns has an epidemiological connection; state the epidemiological link in the posting

5.1.1.1.4 For other organisms, when there is a cluster of two or more isolates (same serotype) with a pattern that shows an increase in your state or region

5.1.1.1.5 When there is a cluster of a common pattern, monitor that cluster to see if there is a significant increase in your laboratory before posting. A significant increase can be determined by graphing out monthly uploads of the pattern/pattern combination over time. Look at the frequency over the past month, 60 or 120 days (depending on the organism) and determine if it is an increase for that same time period, during that time of year over the past several years.

5.1.1.1.6 Pattern and serotype commonality differ within each community, therefore the decision to post a cluster is up to each individual laboratory

5.1.1.1.7 If you have questions about when to post, you can always contact CDC or your PulseNet Area Laboratory

5.1.1.2 Isolate patterns must be uploaded to the PulseNet national databases before posting to SharePoint (if not certified or unable to upload for any reason, the patterns must be submitted via email to the PFGE inbox as soon as possible).

5.1.1.3 The following information must be included in the SharePoint posting and/or sent by email to the PFGE Inbox (see Appendices PND03-2 and PND03-3 for example postings):

5.1.1.3.1 For initial postings, a ***PulseNet*** bundle file containing the cluster or outbreak patterns should be provided (TIFFs are no longer necessary)

5.1.1.3.1.1 A PulseNet bundle file will allow users to compare the isolates in BioNumerics without having to reanalyze the gel image

and/or having to connect to the national database(s). NOTE: If a ***PulseNet*** bundle file is not used, it will be deleted from SharePoint by the discussion moderator and the poster will be asked to post a ***PulseNet*** bundle file.

5.1.1.3.2 A subject line for the posting

5.1.1.3.3 Name of the submitting laboratory

5.1.1.3.4 Which enzyme(s) used—state if second enzyme is pending

5.1.1.3.4.1 Second enzyme should always be run for *E. coli* O157:H7 and other STECs and *Listeria monocytogenes* per the "Second enzyme recommendations" document posted on SharePoint.

5.1.1.3.4.2 Second enzyme should also be run on *Campylobacter* isolates involved in a cluster or outbreak.

5.1.1.3.5 Serotype, if applicable

5.1.1.3.6 Shiga toxin type for *E. coli* O157 and other STECs if available

5.1.1.3.7 Collection and/or isolation date and date received when available

5.1.1.3.8 Information on additional isolates that may be part of the cluster or outbreak

5.1.1.3.9 Additional information about the cluster pattern, such as number of isolates and frequency of pattern in your **local** database

5.1.1.3.10 In an original posting, CDC pattern designations **should not** be posted by anyone other than CDC Database Team members, even if the pattern is listed as confirmed within the national database.

5.1.1.3.11 Postings should only include information pertaining to the laboratory's **local** data; regional data may only be included if a representative from the included labs have been contacted first (additional observances or questions regarding the national database should be limited to emails directly to the PulseNet Database Team, PFGE@cdc.gov)

5.1.1.3.12 Any available epidemiological information

5.1.1.3.13 Personal identifier information such as patient names **should not be posted** to SharePoint

5.1.1.3.14 Please leave the Cluster Status field blank, the CDC Database Team will complete this field. It will either be set to Active or Active (PN). Active is defined as those clusters which are actively being followed by CDC epidemiologists; Active (PN) is defined as those clusters which are not actively being followed by CDC epidemiologists, but are considered by the CDC PulseNet Database Team as worth watching.

5.1.1.3.15 Select an Interest group from the options in the pull down menu (Both, Epi or Lab). This allows users to sort postings based on interest. For example if the posting only contains laboratory data then select "Lab"; if the posting contains information about a potential epidemiological link between cases and/or an update on an epidemiological investigation then select "Epi." If the posting contains information related to both lab and epi then select "Both."

5.1.1.4 For laboratory response postings, the following information must be provided:

5.1.1.4.1 If a laboratory has seen the cluster pattern within the past 30 days for *Y. pestis*, 60 days for *E. coli*, Non-O157, *Shigella*, *Salmonella*, *C. botulinum*, *Campylobacter*, and *Vibrio* or 120 days for *Listeria*, then an isolate number or file name should be provided in the posting.

5.1.1.4.2 It is not necessary for a lab to post indistinguishable isolates that are already included in a CDC cluster/outbreak summary spreadsheet on SharePoint. Please continue to post new isolates that have not been included in these spreadsheets.

5.1.1.4.3 It is mandatory that all patterns reported as indistinguishable be uploaded to the PulseNet national databases or emailed to PFGE@cdc.gov.

5.1.1.4.4 It should be noted that public health epidemiologists should go through their laboratorians when contacting the CDC laboratorians regarding an investigation.

5.1.1.4.5 Select an interest group from the options in the pull down menu (Both, Epi or Lab). This allows users to sort postings based on interest. See section 5.1.1.3.16 above for additional information.

5.1.1.5 Epidemiologist response postings

5.1.1.5.1 Epidemiologists are encouraged to post epidemiological information on SharePoint. We encourage laboratorians and epidemiologists to work together before posting information on SharePoint.

5.1.1.5.2 It should be noted that public health laboratorians should go through their epidemiologists when contacting the CDC epidemiologists regarding an investigation.

5.1.1.5.3 Select an interest group from the options in the pull down menu (Both, Epi or Lab). This allows users to sort postings based on interest.

5.2 SharePoint Management

5.2.1 Thread Removal

5.2.1.1 Once a new thread has been posted to SharePoint, a discussion moderator should respond within two business days of the original posting.

5.2.1.2 Moderator response postings for outbreaks or clusters should include (but are not limited to) an outbreak/cluster code (if applicable), pattern number assignments, frequencies of patterns, any pertinent epidemiological data known, a PulseNet bundle file containing a pattern representative (if one has not already been posted) and a line list.

5.2.1.3 Topics should be renamed with the following format: <Outbreak Code> <(pattern number)>_<LabID of posting lab>_<Organism and/or Serotype>.

5.2.1.4 Only the information posted by the database team should be in dark purple font.

5.2.1.5 Updated information should be posted by the discussion moderator as necessary.

5.2.1.6 The discussion moderators reserve the right to delete all or part of any posting if the criterion in section 5.1 is not met.

5.2.1.7 After 3 weeks of inactivity, if a cluster code was assigned, a cut thread will be posted to the topic indicating that the posting will be removed after a week unless someone responds with a reason why the posting should not be archived (i.e. epi information, local or regional increase, etc.). The original posting and all response postings will be archived according to the “PulseNet Guidelines for Archiving SharePoint Postings” (see Appendix PND03-4).

6. FLOW CHART:

7. BIBLIOGRAPHY:

8. CONTACTS:

8.1 PulseNet Database Team
PFGE@cdc.gov
(404) 639-4558
8.2 OutbreakNet Response Team
outbreakresponse@cdc.gov
8.3 PulseNet Database Unit Chief
Kelley Hise
kpb6@cdc.gov
(404) 639-0704
8.4 EDLB Administrative Support
Mike Korth
mqk8@cdc.gov
(404) 639-2099
8.5 PulseNet IT Support
Brenda Brown
bsb6@cdc.gov
(404) 639-3942

9. AMENDMENTS:

9.1 This SOP replaces the former SOP PND03 PulseNet Listserv Access and Use describing the use of WebBoard as the PulseNet communication listserv. As of February 29, 2008 the contents of WebBoard were moved to the PulseNet workspace on CDC Team. CDC Team is now being used as the PulseNet web-based discussion forum.
9.2 2011-02-22 Appendix PND03-4 was updated
9.3 2011-07-27 Appendix PND03-4 was updated
9.4 2011-08-29 5.2.1.5 and 5.2.1.6 were updated to clarify when postings would be removed from CDC Team
9.5 2011-09-09 Section 5.2.1 and appendix PND03-4 were updated. Previously database managers would post a cut thread to all postings with at least 3 weeks of inactivity and then archive the posting once the timeframe in the cut thread had passed. Please see section 5.2.1 and appendix PND03-4 for updated guidelines for archiving postings.
9.6 This SOP replaces the former SOP PND03 PulseNet Listserv Access and Use describing the use of CDC Team as the PulseNet communication listserv. As of December 19, 2011 the contents of the PulseNet workspace on CDC Team were moved to the PulseNet/OutbreakNet SharePoint site. SharePoint is now being used as the PulseNet web-based discussion forum.

Appendix PND03-1

PulseNet/OutbreakNet SharePoint Site Non-disclosure Agreement

Welcome to the PulseNet/OutbreakNet SharePoint site!

In order to comply with Health and Human Services security requirements, we are required to obtain a signed non-disclosure agreement (NDA) from each PulseNet/OutbreakNet SharePoint site participant.

The following is the PulseNet SharePoint and OutbreakNet site policy on sharing information. It is displayed on the PulseNet/OutbreakNet SharePoint site as a reminder:

PulseNet SharePoint and OutbreakNet site Policy on Sharing Information

Often PulseNet/OutbreakNet SharePoint postings contain preliminary information on presumptive disease clusters and ongoing outbreak investigations. Therefore, SharePoint postings are not appropriate for sharing with persons outside public health and food regulatory agencies. If you would like to share SharePoint messages with persons not directly associated with public health or food regulatory agencies, we require that you obtain prior approval from the person or agency that posted the information.

We would appreciate your strict compliance with this policy. Violation of this policy will result in loss of access to the PulseNet/OutbreakNet SharePoint site.

Thank you for your cooperation

Peter Gerner-Smidt MD, PhD
PulseNet, Centers for Disease Control and Prevention

Ian Williams, PhD, MS
OutbreakNet, Centers for Disease Control and Prevention

***** ***** ***** E-MAIL THIS PAGE ONLY ***** ***** *****

PulseNet/OutbreakNet SharePoint Non-Disclosure Agreement

By signing this non-disclosure agreement, you agree that you will not share information from the PulseNet/OutbreakNet SharePoint site with personnel not associated with the public health community without appropriate approval.

☐ I have read the **PulseNet/OutbreakNet SharePoint Policy on Sharing Information** and agree not to share information from the PulseNet/OutbreakNet SharePoint site with personnel not associated with the public health community without appropriate approval.

Name: _____

PulseNet/OutbreakNet Affiliation: _____

Email Address: _____

Signature: _____ Date: _____

Sign, Scan and email to: Mike Korth at MQK8@cdc.gov

Appendix PND03-2

Example: Correct PulseNet Initial SharePoint Posting

Discussion: *E. coli*

Subject: *E. coli* O157:H7 in GA

Body: GA is experiencing a cluster (5 cases and 1 food) of ***E. coli* O157:H7** in the Atlanta area. The following information pertains to the attached PulseNet bundle file, GA110374PN. The associated TIFF was uploaded to the PulseNet national database for *E. coli* this morning. Both enzymes have been run for these isolates.

Isolate #	Collection date	Date received	Source Type	Source Site
GA1000	01-05-11	01-08-11	Human	Stool
GA1001	01-04-11	01-07-11	Human	Stool
GA1002	01-08-11	01-11-11	Human	Blood
GA1003	01-08-11	01-10-11	Food	Hamburger
GA1004	01-06-11	01-08-11	Human	Stool
GA1005	01-03-11	01-06-11	Human	Blood

[Patient demographics, including age, sex, and county, is not necessary in SharePoint posting, but needs to be included in the email to PFGE@cdc.gov if information has not been uploaded to the PulseNet database.]

Within our local database, this pattern is not common; we have seen it only once before. The isolate number is GA0076, and it was uploaded in January of 2008. As of this date, the cluster is potentially linked to hamburger.

Epi Contact Info: John Doe JDoe@email.org

GA11374PN.BDL (this is the attached PulseNet bundle file)

Cluster Status: Please leave this blank, the CDC database team will add this information.

Interest Group: Both (indicates both lab and epi may be interested in this posting)

Appendix PND03-3

Example: Correct PulseNet Response SharePoint Posting

If lab has seen posted pattern:

Discussion: *E. coli*

Subject: *E. coli* O157:H7 in GA

Body:
AL has two possible matches to the GA patterns. These isolates are AL1234 with an upload date of 1-12-11 and AL1245 with an upload date of 1-13-11.

AL11056PN.bdl (this is the attached PulseNet bundle file)

Interest Group: Lab (indicates lab will be interested in this posting)

Appendix PND03-4

PulseNet Guidelines for Archiving SharePoint Postings

1. If a SharePoint cluster posting does not follow the posting guidelines as stated in section 5.1 of the SOP, the database manager reserves the right to edit or delete the posting. The poster should be notified that the posting is being deleted and why. The poster should also be reminded of when and when not to post a cluster to SharePoint.

2. Depending on the pathogen/database, SharePoint postings that receive cluster codes are archived 4 weeks from the LAST posting of the topic or thread and/or if the epis decide to close the cluster.

3. Postings that do not get a code, may be kept on SharePoint for a longer period of time if the national and/or regional numbers are indicating a potential rise or due to other reasons as determined by the national database managers.

4. A cut thread should be posted for all clusters. If the cluster has been closed for a while, either by the epis or on SharePoint, no cut thread needs to be posted.

5. Only for larger outbreaks, and especially those that resulted in regulatory action--before posting the cut thread, summarize the information from the posting. (i.e., if it was an outbreak, give pattern numbers, numbers seen from which state(s), commonality of the pattern in the database, if the outbreak was ever linked back to a food item and/or resulted in a recall). Or you could simply post the link to the CDC web update if there is one. A template can be found here: \\cdc\project\CCID_NCZVED_DFBMD_PulseNet\SharePoint\admin\Templates

6. Post a cut thread (see below or go to \\cdc\project\CCID_NCZVED_DFBMD_PulseNet\SharePoint\admin) to that posting.

   [title topic] has been closed and will be removed from SharePoint in approximately 7 days. Any additional replies or inquiries should be made before then.

   Thank you,

   PulseNet Database Administration Team
   Phone: (404) 639-4558

7. NOTE: the above can be done automatically from the outbreak log:
   A) Before you close a cluster, click on the “Close Clusters” button in the main organism screen
   B) Choose the cluster(s) you want to close and move them to the Clusters to Close box
   C) Click the button “Close and Generate Cut Thread(s).”

8. After 7 days, if there have been no responses, the topic can be archived.

9. **To archive a posted thread:**

- Highlight the entire topic—make sure to include all postings—and copy

- Go to Edit>Paste Special, and choose “Unformatted Text”, and paste the copied topic into a blank Word document
- Save the word document under: \\Cdc\project\CCID_NCZVED_DFBMD_PulseNet\SharePoint\Forum\topic folder\year
- Use the following format for the filename: ***for coded outbreaks and clusters that do not get codes use:*** Topic name (ex: 0707COEXK-1_(EXKX01.0001)_CO_E. coli O121/O26) or ***for non-outbreaks use:*** YYYY-MM-DD_LabID-Topic (ex: 2002-09-17_CDC-Serotyping and Ribotyping Outbreak.doc); the date is the date of the first posting
- Save only attachments that we would not already have elsewhere (TIFFs and bundles in the database; line lists in the outbreak folder, etc.) into an appropriate “attachments” folder.

10. Once the thread is saved, the thread can be deleted from SharePoint.

1. PURPOSE: To describe the guidelines for standardization of TIFF image analysis in order to accurately compare PFGE patterns between PulseNet participating laboratories.

2. SCOPE: This procedure applies to all analysis submitted to PulseNet, thereby allowing comparisons of results with other PulseNet laboratories.

3. DEFINITIONS/TERMS:

3.1 PFGE: Pulsed-Field Gel Electrophoresis
3.2 TIFF: Tagged Image File Format. A file of a gel image that can be analyzed in BioNumerics
3.3 BioNumerics: Gel analysis software used by PulseNet, developed by Applied Maths, Belgium
3.4 SOP: Standard Operating Procedure

4. BACKGROUND: PulseNet Gel Analysis Guidelines

Computerized gel analysis with BioNumerics facilitates comparison of PFGE patterns from isolates of foodborne bacteria on gels produced by all certified PulseNet laboratories. It is critical that excellent quality gels that have been analyzed accurately are submitted to the PulseNet on-line databases for valid comparison of results.

Computer analyses (from lane definition to normalization to band finding) should always be checked against a copy of the actual gel image being analyzed, as visual (i.e., manual) analysis is the gold standard. Analysis is only as good as the TIFF of the gel image being analyzed. For this reason, each gel image should be of the highest quality possible. Consistency from run to run is critical. Factors such as fuzzy bands, smearing, high background, the presence of shadow bands, and running the gel too short or too long can make TIFFs of the images needlessly difficult to analyze and can compromise comparisons to patterns in the national databases. Gels that do not yield TIFFs with acceptable quality should be rerun.

Sometimes, suggestions to improve gel quality come about after computer analysis of what appears to be a good gel visually. Factors such as consistent slants of lanes and bands, band distortions, light bands at the bottom of the gel or in a few lanes, extreme differences in the band spacing of the standards compared to the global standard, debris or artifacts that affect analysis, and poor band resolution can be more apparent during computer analysis of a TIFF than upon visual inspection. When factors such as these are noted during analysis, the person performing the analysis should suggest and/or implement changes in gel preparation so that gel quality will be of the highest quality possible.

5. RESPONSIBILITIES/PROCEDURE:

5.1 Standards

5.1.1 Standards are very important for normalization. Standards can also be used to gauge consistency from gel to gel and provide clues about how to evaluate the test isolates. Evaluation of the standards may include the following:

5.1.1.1 Are the standard bands consistently the same thickness or intensity from gel to gel?

5.1.1.2 During normalization, note how the standard pattern on the gel matches the band pattern of the reference standard. If BioNumerics must stretch or shrink the pattern to match the reference standard, is the stretching or shrinking the same from gel to gel? If a sudden change occurs, an error might have occurred during the run such as the wrong electrophoresis program used to run the gel. Using the show distortion bars feature will aid in visualizing these types of problems and assist with correct normalization.

5.1.1.3 From gel to gel, is the standard pattern consistently the same or does the gel resolution vary enough to affect the standard pattern (i.e., doublets appear sometimes and single bands appear at other times in certain areas of the pattern)? For normalization purposes on *E. coli* O157:H7 gels (for example), 15 normalization markers on 15 single bands can be placed on the *Salmonella*

Braenderup H9812 standard. However, some laboratories are resolving doublets where single bands appear on the global standard near the bottom of the H9812 pattern. If doublets resolve, mark the normalization bands carefully.

5.1.2 Not all observed bands in a standard lane are used to normalize. If an area of a standard lane resolves as a doublet on a test lane but appears as a single band on the global standard, a single normalization marker should be marked in this area on the test lane. However, for band finding (step 4), a doublet should be marked on the test lane. Normalization (step 3) will not be affected if areas of the standard lanes are normalized correctly as single bands but marked as doublets during band finding.

5.1.3 If shadow bands appear in the standard lanes, shadow bands could be present in test lanes. Ideally, the gel should be repeated. Lanes containing shadow bands are difficult to analyze. Shadow bands probably indicate incomplete restriction and should not be present on a gel. Repeat the gel performing restriction with more units of enzyme, for a longer amount of time and/or with a different lot of enzyme. Wash the plugs at least two more times with TE Buffer before restriction is repeated.

5.2 Before analysis

5.2.1 Consistently taking pictures where the gel image fills almost the entire window on the imaging equipment screen (without cutting off wells or the bottom of the gel) should improve consistency in normalization and band finding. Please see the SOP PNL07 Image Acquisition for instructions and additional information.

5.2.2 Print out or open up on a computer screen the gel image to be analyzed. A printed copy of the gel image can help steer decisions on band finding, such as deciding if an area is a doublet or a single band.

5.2.3 If shadow bands are present or if incomplete restriction is suspected, rerun the sample(s) or, if necessary, the entire gel. Lanes with incomplete restriction of DNA are difficult to analyze and may yield a different pattern when the PFGE is repeated. Results from isolate patterns showing incomplete restriction are not always reproducible and cannot be accurately compared with patterns from other isolates in a local database or with the national database.

5.3 BioNumerics – Step 1 Strips

5.3.1 At the beginning of the analysis, assign the TIFF to the enzyme used on the gel. If two enzymes are used, lanes restricted with secondary enzymes should be changed after analysis but before lane linkage to the local database (see section 5. 7of this document).

5.3.2 To define the gel strips, place the sides of the green box frame on the edges of the first and last lanes of the gel. Place the top of the green box frame directly under the wells. Place the bottom of the green box frame approximately 1-2 cm below the lowest band of the *Salmonella* Braenderup H9812 standard on a full-size image. (On a gel following the PulseNet standardized procedure where the last band of the standard is 1-1.5 cm from the bottom of the gel and the gel image fills the entire window on the imaging equipment screen, 1-2 cm below the last band of the standard **should be at the bottom of the gel**.) This will standardize lane lengths, which will produce more accurate normalizations and improve comparisons with the national database.

5.3.3 After the lanes are defined, adjust the “thickness” under “edit settings” to increase or decrease the thickness of the defined gel strips so that the left and right edges of the strips are just outside the outer edge of the bands. This will ensure that the bands will appear in a size for proper analysis. If the gel strips are defined incorrectly (too wide), the resulting narrow bands with large white patches on either side of the bands are difficult to analyze. Gel strips may need adjustment when switching between 10-well and 15-well gels and also if the gel image is captured differently (i.e., close up versus farther away) from gel to gel. The width of an individual lane can be adjusted independently, if necessary.

5.3.4 Using the “linear adjustment” in the “edit tone curve” feature may provide better band clarity for analysis.

5.3.5 In the edit tone curve feature, enhancing weak bands is sometimes useful to detect bands accurately. However, when using the edit tone curve feature to enhance weak bands (primarily seen at the bottom of the gel), too much enhancement of weak bands may make the stronger stained bands in the top and

middle portions of the pattern appear too thick and make them difficult to analyze. Always compare band markings in the software to a copy of the original gel image.

5.3.6 Increasing contrast is sometimes useful to detect bands accurately.

5.3.7 **Do not** use background reduction or spot removal on gel images submitted to CDC. The different usage of these BioNumerics tools among PulseNet participating laboratories causes problems when performing comparisons within the national databases. Participating laboratories may use background reduction and spot removal in their local databases, but should remove these features before submitting analyses to the national databases. CDC does not recommend the use of spot removal.

5.4 BioNumerics - Step 2 Curves

5.4.1 If bands in the test lanes or the standard lanes are distorted or contain artifacts and the gel image must be analyzed without rerunning the gel, ensure that the curve strips are placed accurately.

5.5 BioNumerics - Step 3 Normalization

5.5.1 If artifacts (e.g., specks or dots) appear near a band on the TIFF image, ensure a normalization marker is not placed on the artifact instead of where the actual band is on the TIFF image.

5.5.2 Check normalization using the "distortion bars" function (under the Normalization menu). The color of the distortion bars should be the same moving across the gel. If a high amount of orange or red distortion bars or distortion bar colors that change as they move horizontally across the gel strips appear, normalization marker placement should be examined. If dark distortion bars are present and uneven across the gel and the normalization marker placement is correct, the gel should be rerun, verifying that the correct running conditions are used.

5.6 BioNumerics - Step 4 Bands

5.6.1 If you use the auto search function, look at the whole gel and delete obvious extra bands first before carefully going through each lane and matching band markings to a copy of the gel image. This will ensure that extra bands, such as those near the top or bottom of a TIFF image, are deleted. If artifacts are present on a TIFF image or the TIFF image is dark, the auto search function may find many extra bands.

5.6.2 Review the bands detected by the auto search function.

5.6.2.1 Toggle between the normalized and un-normalized viewing screens to ensure bands were not accidentally marked above and/or below the standard. Sometimes debris will be marked outside of the normalized viewing screen. This is important to double check.

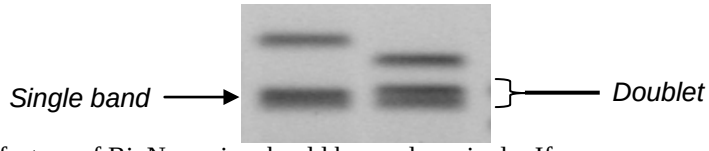
5.6.2.2 Mark bands carefully. Use the peak of the shoulder on the densitometric curve if band placement is in question. If a band is considered a singlet, ensure that the single band is placed correctly. Bands should be marked in the middle of areas considered single, solid bands.

5.6.2.3 Mark bands as they appear on a copy of the actual gel image. Defer to a hard copy of the image when determining if a questionable area should be marked as a doublet or a single band.

5.6.2.4 All lanes containing profiles, which by visual inspection are obviously indistinguishable, should be marked uniformly.

5.6.2.5 If artifacts (e.g., specks or dots) appear near a band on the TIFF image, make sure a band is not placed on the artifact instead of where the actual band is on the gel image.

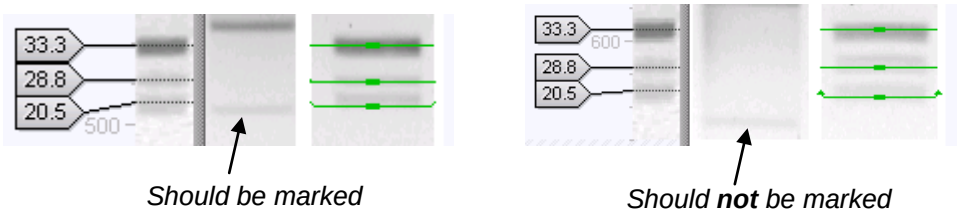
5.6.3 When deciding if a band is a doublet or a single band, look for white space in the middle of the area or indentations (i.e., doughnuts or shoulders) on the sides of the area that separate two bands. If indentations or white space are apparent on a copy of the TIFF, the area is most likely a doublet. If the indentations or white space are questionable, the area probably should be marked as a single band. Separate peaks on the densitometric curve may also provide clues as to the band designation of the area.



5.6.4 The uncertain band feature of BioNumerics should be used sparingly. If an area on an excellent or good gel is either a doublet or a single band and the criteria in 5.6.3 does not aid in band marking, the uncertain band feature may be used. If the gel quality is fair or poor, repeat the gel and produce a good quality pattern before marking bands.

5.6.5 As overall gel quality improves, resolution will become sharper. If an area that previously resolved as a thick singlet becomes a doublet or triplet on subsequent gels, that area should be marked **exactly** as it appears on each gel.

5.6.6 Visible bands of test isolates should be marked down to ~20.5 kb, the bottom band used for normalization in the *Salmonella* Braenderup H9812 standard. If any portion of a band is even with or above the bottom of H9812 it should be marked. If the entire band falls below the last band of the standard, it should not be marked. There will always be some level of variability with PFGE, use your best judgment and please remember to be consistent.



5.6.7 In almost all cases, band intensity differences are not reliable enough for use in assigning pattern numbers. Two patterns that are indistinguishable except for one or more band intensity difference(s) will be assigned the same pattern number. Isolates with only intensity differences should be reported as indistinguishable by the test(s) run and the PulseNet criteria.

5.7 Lane linkage

5.7.1 Before linking a lane to the local database, make sure that the fingerprint type (e.g., PFGE-XbaI, PFGE-BlnI, etc.) is correct. Changing fingerprints is necessary when more than one enzyme is used for restriction on a particular gel. If the lanes are linked with incorrect fingerprint types, possible duplicate entries in the database could occur. At the beginning of the analysis, assign the TIFF to they enzyme used on the gel. Any lanes restricted with secondary should be changed after analysis but before lane linkage to the local database. To change the fingerprint type, right-click on the desired lane and select "Change fingerprint type of lane…" After the fingerprint type is changed, you can link the lane to an entry in the database. Both fingerprint types should be indicated with a green dot next to the one entry in the BioNumerics database.

5.8 Review of analysis and submission to CDC

5.8.1 PulseNet participating laboratories should review their band finding and normalization before submitting to the PulseNet national database, PFGE inbox, or the PulseNet Listserv. After band finding and the linkage of lanes in a TIFF, normalization and band finding can be roughly checked by analyzing a dendrogram showing gel strips and band markings.

5.8.2 Submit your laboratory's best possible gel image and analysis of a particular pattern. If one or more lanes of an otherwise good gel are unsatisfactory, do not upload the unsatisfactory lanes to the national database. Rerun unsatisfactory isolates on another gel.

5.8.4 Within your laboratory, regularly check band markings of all analysis-certified personnel to ensure consistency. This should be included as part of your laboratory's Quality Assurance/Quality Control program.

5.8.5 All TIFF and bundle files must be named according to the PulseNet protocols for naming. TIFFs and bundle files from each PulseNet participating laboratory should have unique names by laboratory and not by species when they are sent to CDC. For example, the first TIFF and bundle from GA in 2011 should be named GA11001.tif and GA11001PN.bdl. This could be a gel containing *E. coli* O157:H7 or another organism. The second TIFF and bundle from GA in 2011 should be named GA11002.tif and GA11002PN.bdl. This could be a gel containing *Salmonella* or another organism.

5.8.6 All bundle files posted to the PulseNet Listserve and/or exchanged with PulseNet participating laboratories must be created using the Create PulseNet Bundle file lightning bolt icon on the left side of the BioNumerics screen. Bundle files created using this icon will contain only PulseNet fields and "PN" will automatically be added to the file name. Do not ever manually add "PN" to the end of a non PulseNet bundle file.

5.9 After submission to CDC

5.9.1.1 All certified PulseNet participating laboratories have the ability to check the on-line databases to see if previously submitted patterns have been assigned pattern numbers by CDC. These pattern names can be downloaded to the local database.

5.9.1.1.1 If a pattern number is followed by a "&" or "@," the number has not been confirmed by the CDC Database Team. These pattern numbers are to be considered preliminary and **must not** be reported.

5.9.1.1.2 If the band marking is different in the local database than it is in the on-line database, the CDC Database Team has changed the participating laboratory's original analysis. The participating laboratory should review the changes made by the Database Team, and if necessary, contact CDC to find out why the changes were made. The participating laboratory can then download the changes to their local database.

5.9.1.1.3 If a pattern status has been marked "unsatisfactory," the submitting laboratory must rerun the isolate on another gel. Contact the CDC Database Team if there is a question about the designation.

5.9.2 If resubmission/reupload of results from the same TIFF is necessary (e.g., for *Salmonella* serotype information), highlight all isolates on that gel before reuploading, even if only one lane has results that are being resubmitted. If all lanes are not highlighted, unlinking could occur.

6. FLOW CHART:

7. BIBLIOGRAPHY:

8. CONTACTS:

9. AMENDMENTS:

9.1. Statements regarding unsatisfactory patterns having "NG" in the pattern names were removed because the CDC Database Team no longer uses 'NG" to denote an unsatisfactory pattern. Please see section 5.9.1.2.3.

9.2. Updated section 5.8.5 and 5.8.6 to include a description of the "PN" for PulseNet bundle files

9.3. References to the BioNumerics Training Manual were removed. Current versions of the training notebooks are posted on the PulseNet Listserv after each CDC BioNumerics Training Course. Please refer to those presentations for step-by-step instructions on using the tools in BioNumerics.

9.4. 2011-07 Section 5.6.6 was updated to clarify verbiage and add example images.

1. PURPOSE: To describe the procedure for setup and use of a SecurID Key Fob.

2. SCOPE: This procedure applies to all PulseNet personnel who are analysis certified and receive a SecurID Key Fob.

3. DEFINITIONS/TERMS:

3.1. SecurID Key Fob: Token that displays a six-digit passcode. When used in combination with a four-digit pin number, allows access through the CDC firewall.

4. RESPONSIBILITIES/PROCEDURE:

4.1 Precautions for Protecting Your SecurID Key Fob. For your own protection and that of the system, always take the following precautions:

4.1.1 Never reveal your PIN or user password to anyone. Do not write them down.

4.1.2 If you think someone has learned your PIN, notify CDC PulseNet to have the PIN cleared immediately. At your next login you will have to create a new PIN to use.

4.1.3 Exercise care not to lose your SecurID Key Fob or to allow it to be stolen. If your key fob is missing, notify CDC PulseNet immediately. They will contact the administrator, who will disable the key fob so that it is useless to unauthorized users.

4.1.4 Do not let anyone access the system under your identity (that is, log in with your PIN and a code from your SecurID token).

4.1.5 It is essential to site security that you follow your system's standard logoff procedures. Failure to log off properly can allow unauthorized access from your workstation by another user.

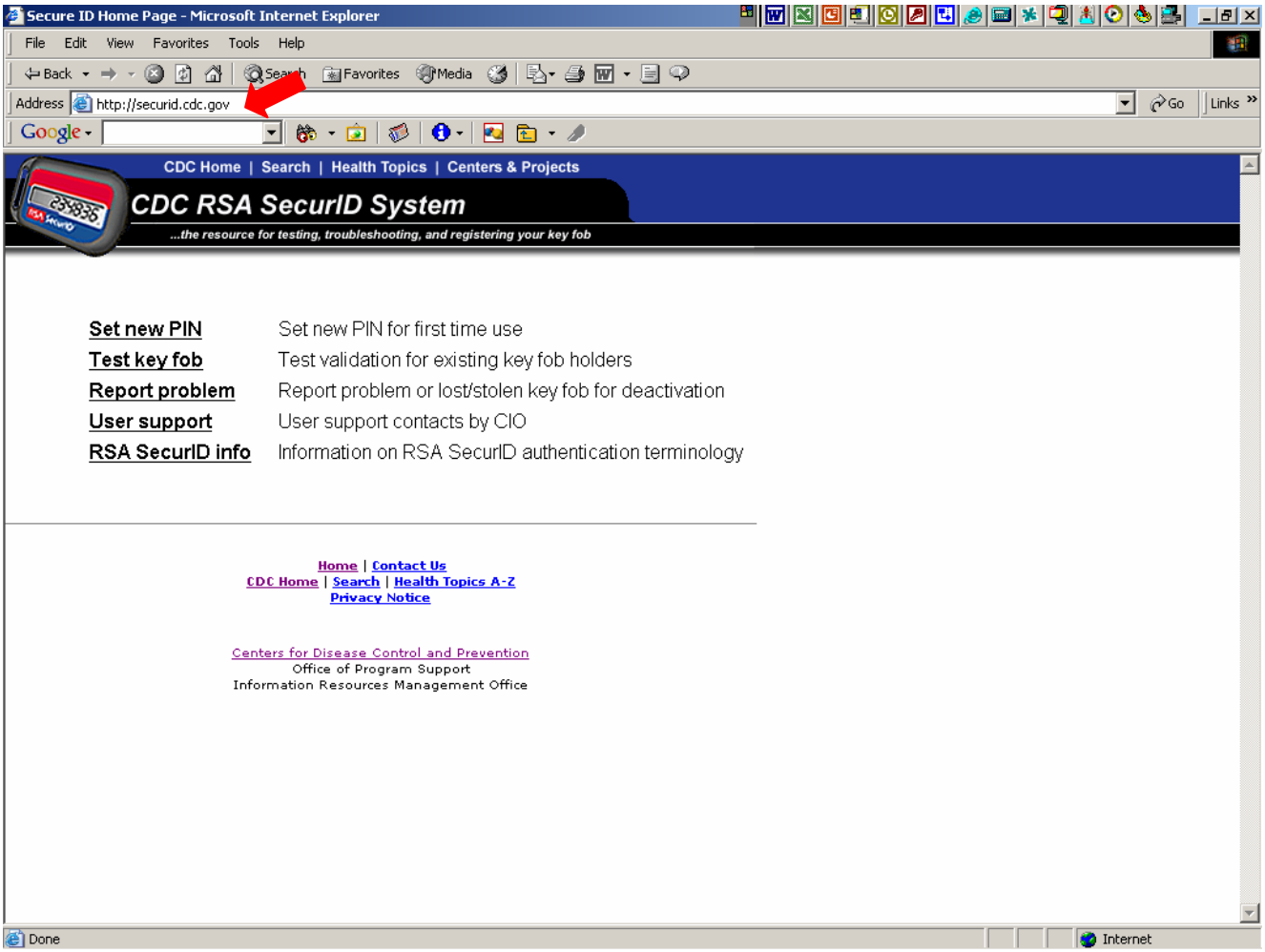
4.1.6 **Protect your key fob from physical abuse. Do not immerse it in liquids or expose it to extreme temperatures.**

Setting Up a New PIN

1. Open a web browser, either **Netscape** or **Explorer**.

2. Access the **CDC SecurID System** website by entering the following address for location: **http://securid.cdc.gov**

3. Select **Set new PIN**.



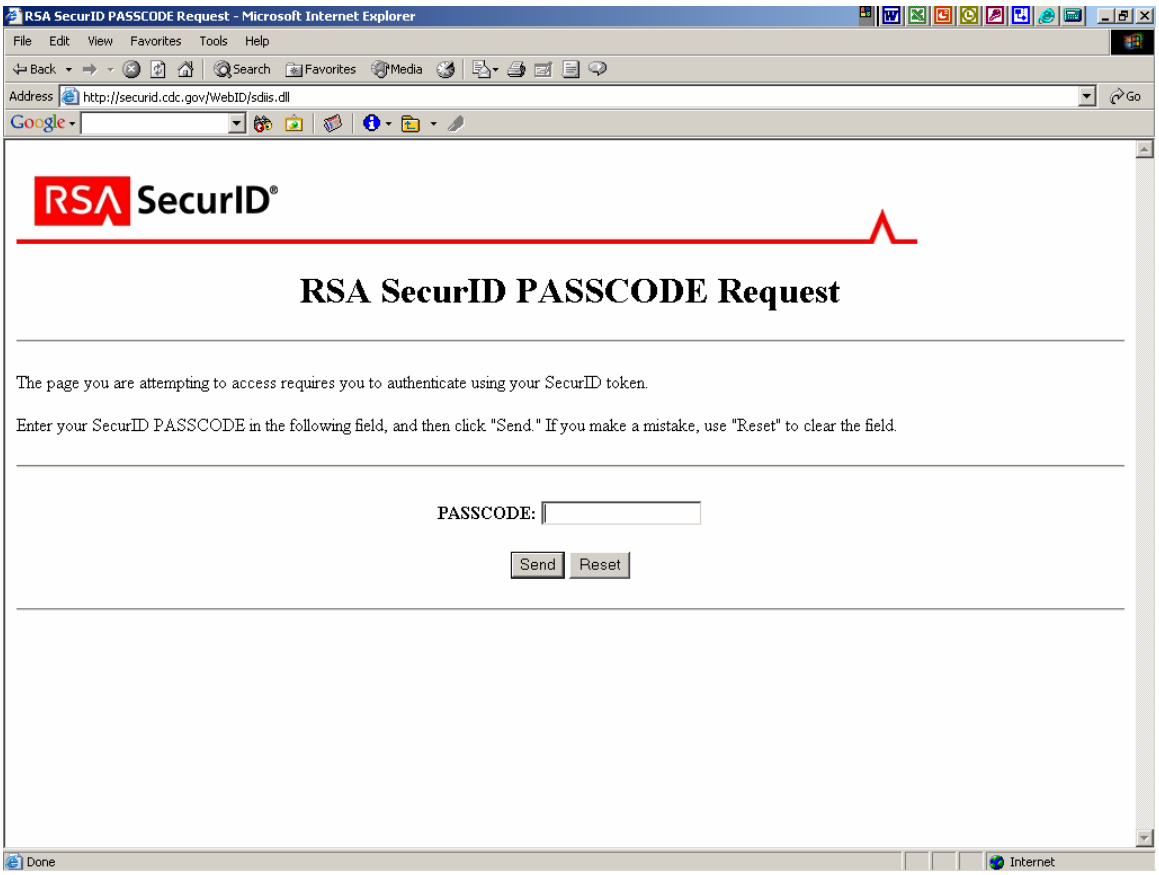
4. Enter your **Username:**

You have received your username in a separate communication.

NOTE: Please be sure there are five or six “ticks” on the left side of the device. This will allow time for synchronization between the device and the server for authentication.

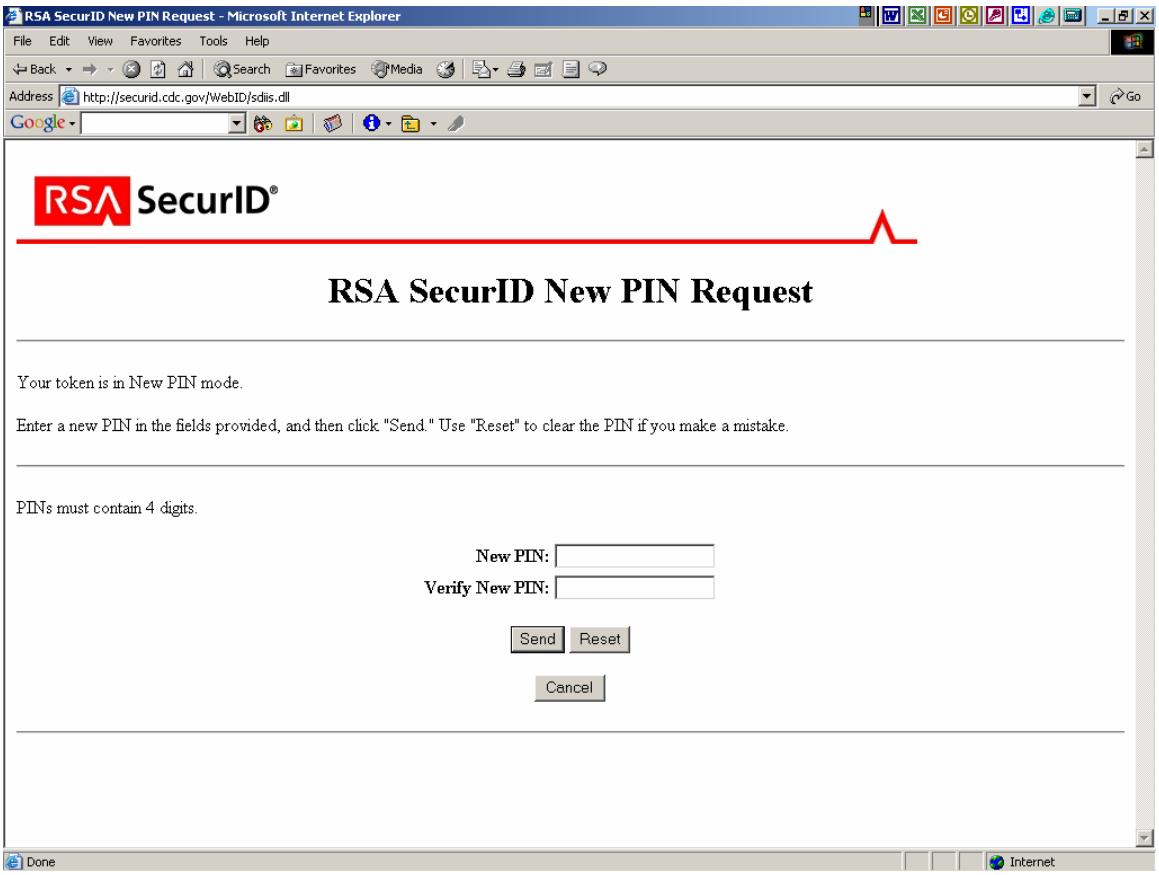
5. Click **Send.**

If you receive this screen again, please wait for the next number on the SecurID Key Fob, then enter the username and number.



6. Enter the **PASSCODE: Initially, this is the six digits displayed on the SecurID Key Fob.**

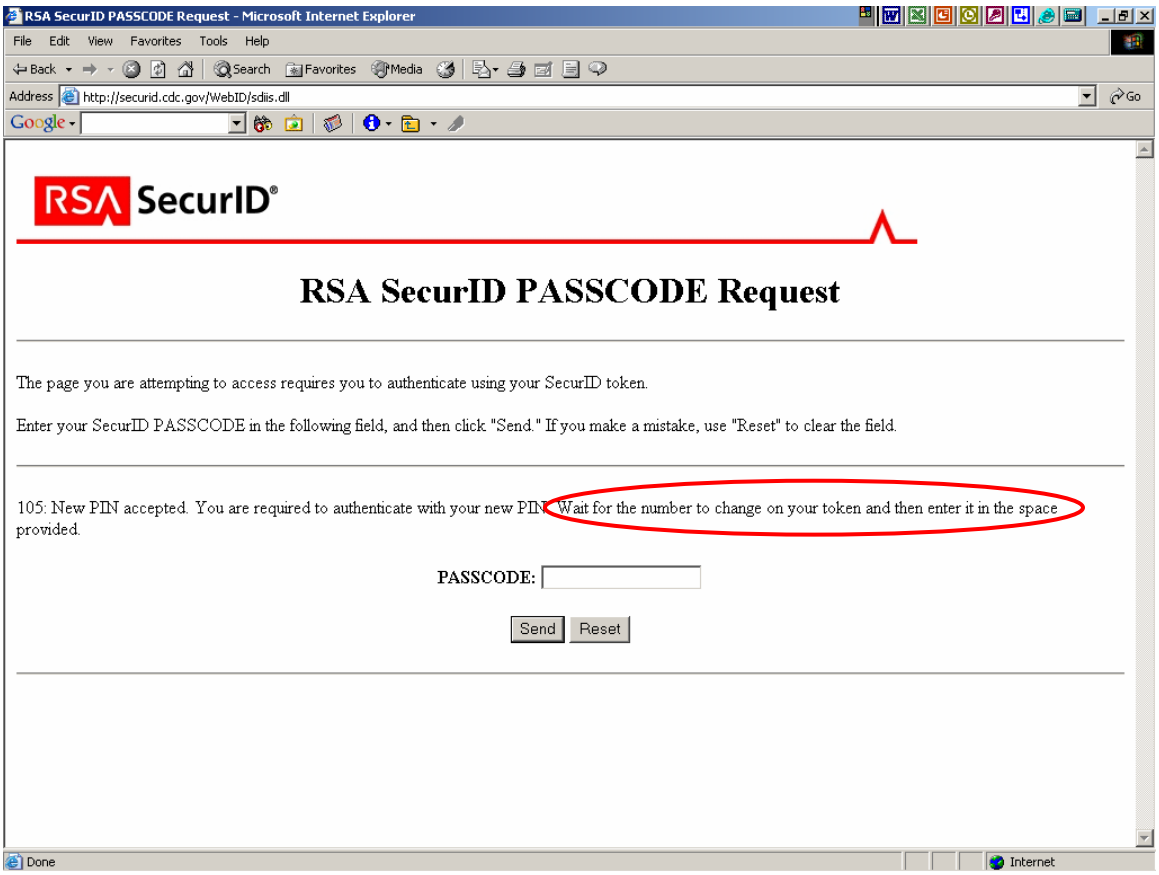
7. Click **Send**.



8. Enter a **four digit number** that will serve as your **PIN.**

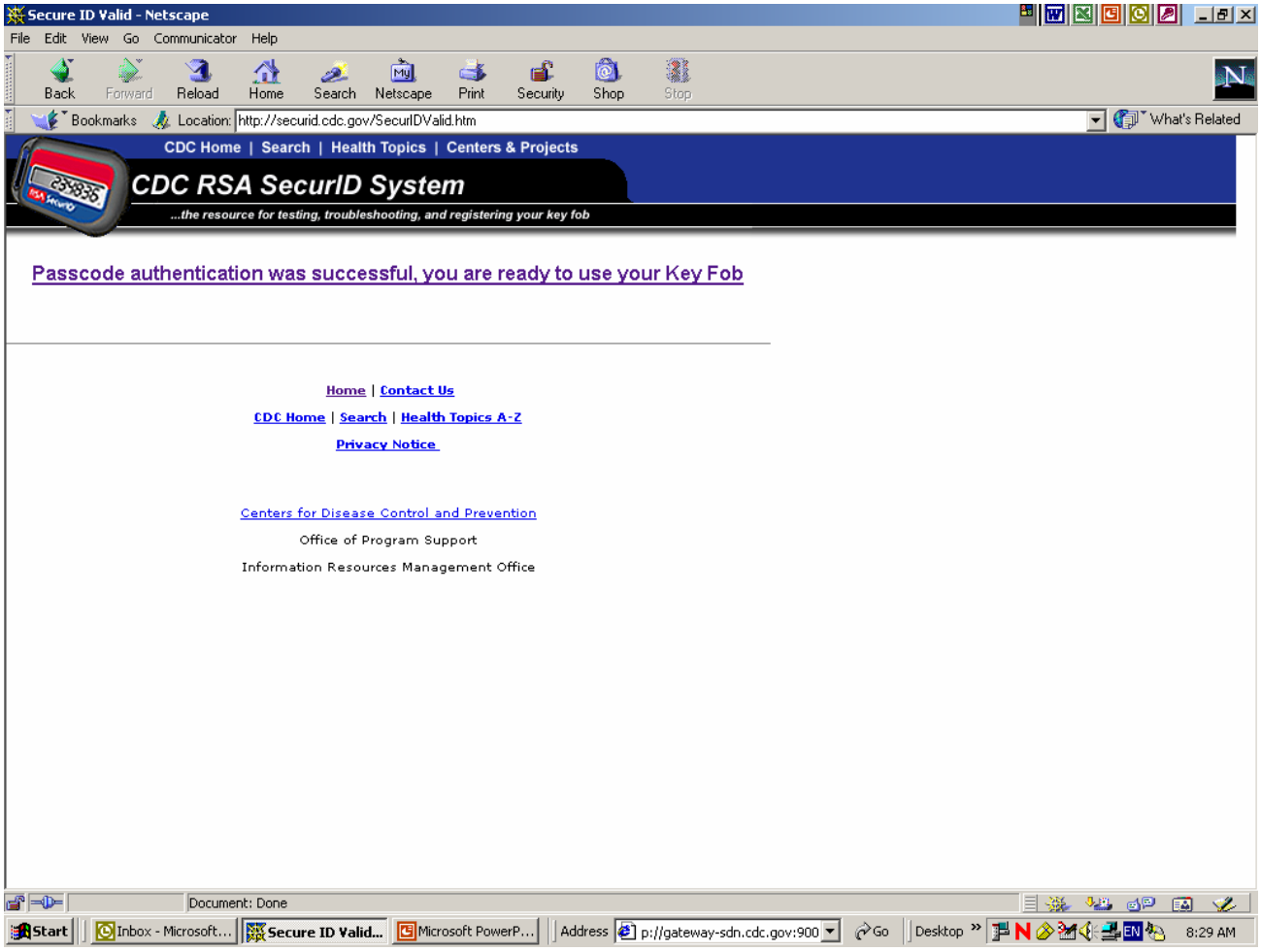
9. Then enter your **PIN** again for verification.

10. Click **Send**.



11. Your **PIN** has been accepted**. Wait for the numbers on the SecurID Key Fob to change**, then enter your **PASSCODE. (Now it is your four digit PIN + six digits displaying on SecurID Key Fob – no spaces.)**

12. Click **Send**.



You have now set up and tested your PIN.

NOTE: Please be sure to read and observe Precautions for Protecting Your SecurID Key Fob and the secured information you will be accessing.

NOTE: If you leave your current position, notify CDC PulseNet and return the SecurID Key Fob to FedEx to the following address:

Attn: Michael Korth
Centers for Disease Control
1600 Clifton Road, NE
MS C03
Atlanta, GA 30333
Phone: (404) 639-3334

5. FLOW CHART:

6. BIBLIOGRAPHY:

7. CONTACTS:

7.1 PulseNet Database Team
Centers for Disease Control and Prevention
1600 Clifton Road NE
MS C03
Atlanta, Georgia 30333
Phone: (404) 639-4558
Email: PFGE@cdc.gov

8. AMENDMENTS:

1. **PURPOSE:** To describe the procedure for authenticating and connecting to the server.

2. **SCOPE:** This procedure applies to all PulseNet personnel who are analysis certified and receive a SecurID Key Fob.

3. **DEFINITIONS/TERMS:**

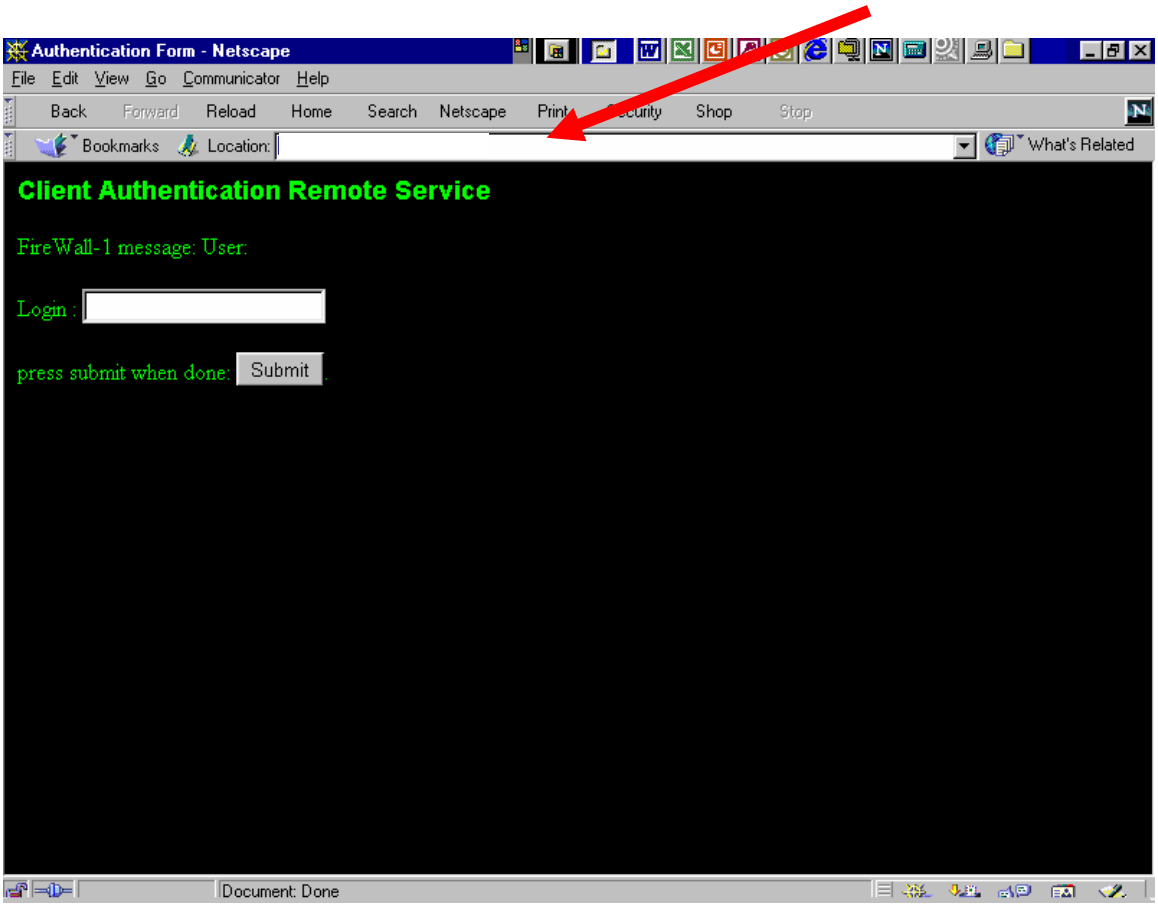
   3.1. SecureID Key Fob: Token that displays a six-digit passcode. When used in combination with a four digit PIN number, allows access through the CDC firewall.
   3.2. BioNumerics: Gel analysis software used by PulseNet, developed by Applied Maths, Belgium.

4. **RESPONSIBILITIES/PROCEDURE:**

Step 1: Authenticate to the Firewall

Open a web browser, either **Netscape** or **Internet Explorer**

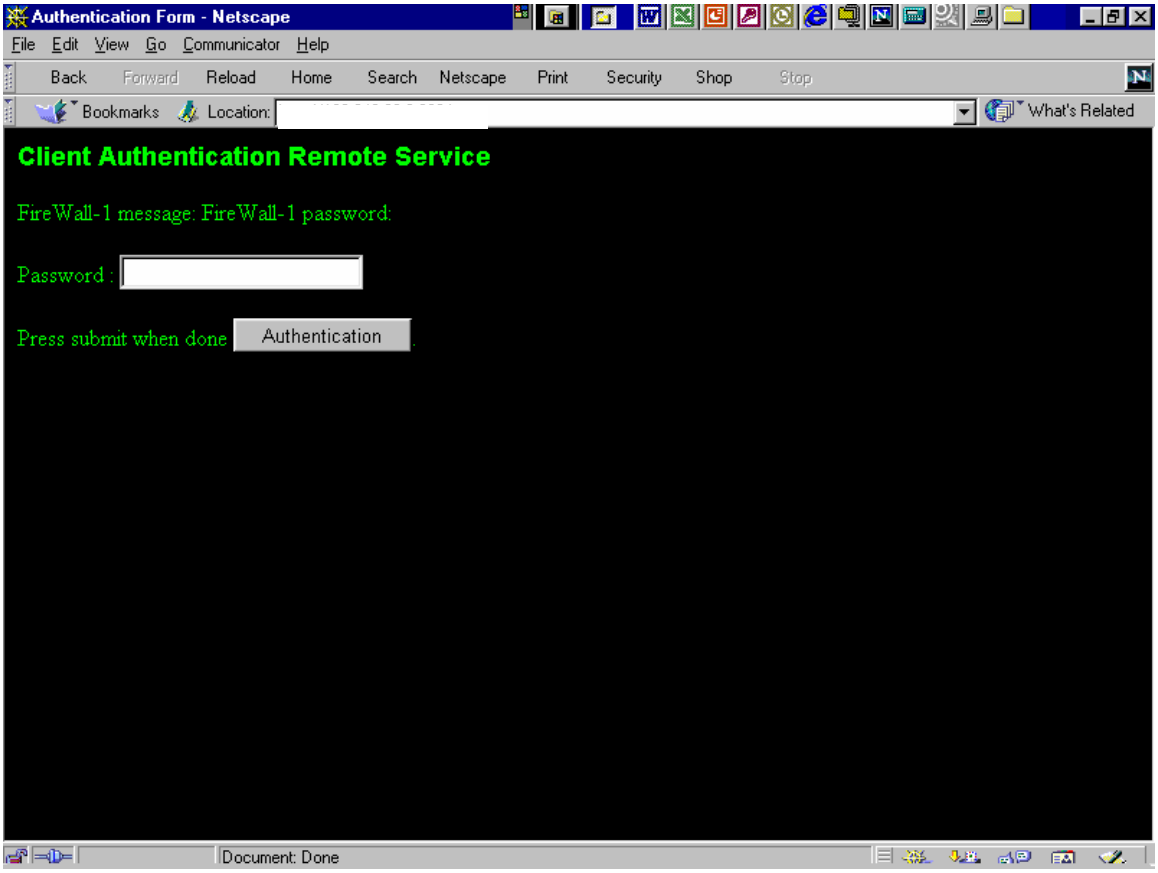
Access your bookmark name **Authentication Form** or enter the following address for location: **http://gateway-sdn.cdc.gov:900**.
You will see this screen: Client Authentication Remote Service



Enter your login. Y***ou will receive your username in a separate communication.***

Click ***Submit.***

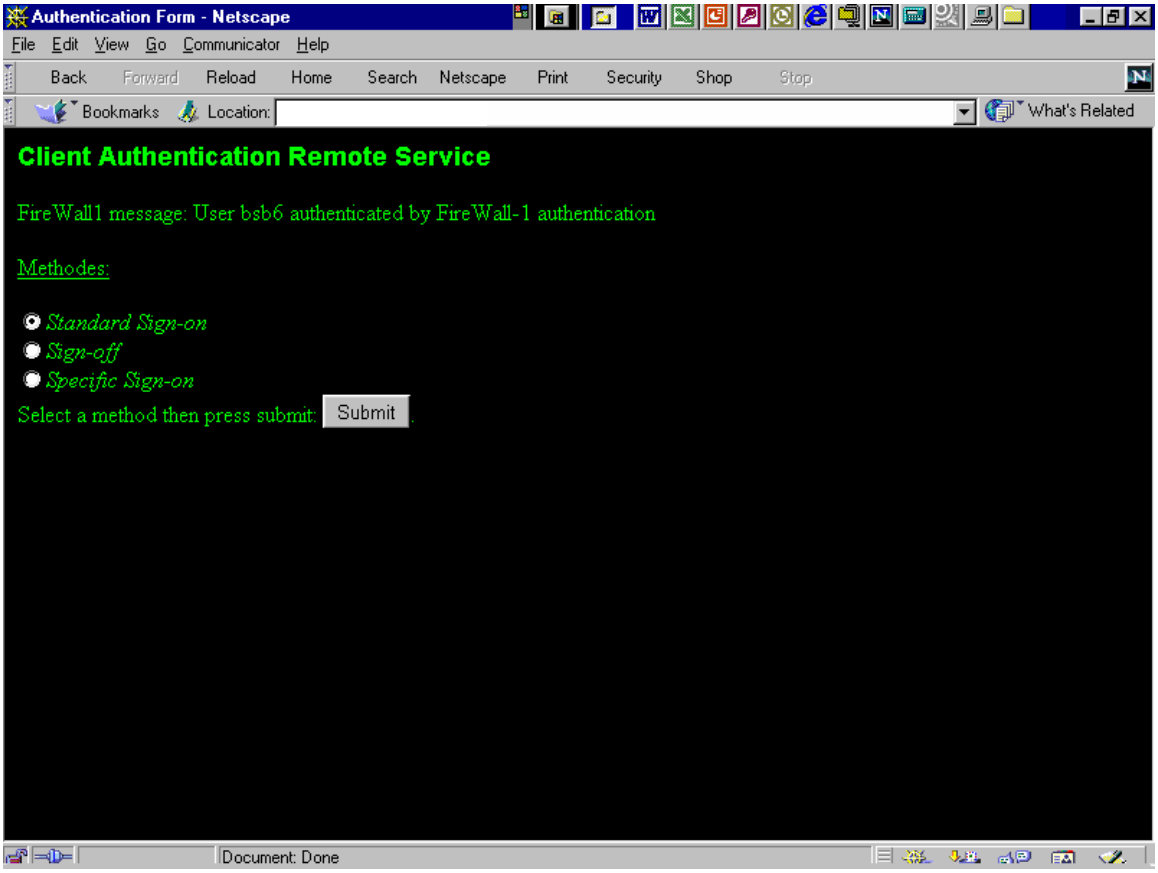
You will see this screen: Client Authentication Remote Service



Enter your 10-diigit password:
four-digit PIN plus six-digit SecurID number

NOTE: Please be sure there are two or three “ticks” on the left side of the device. This will allow time for sychronization between the device and the server for authentication.

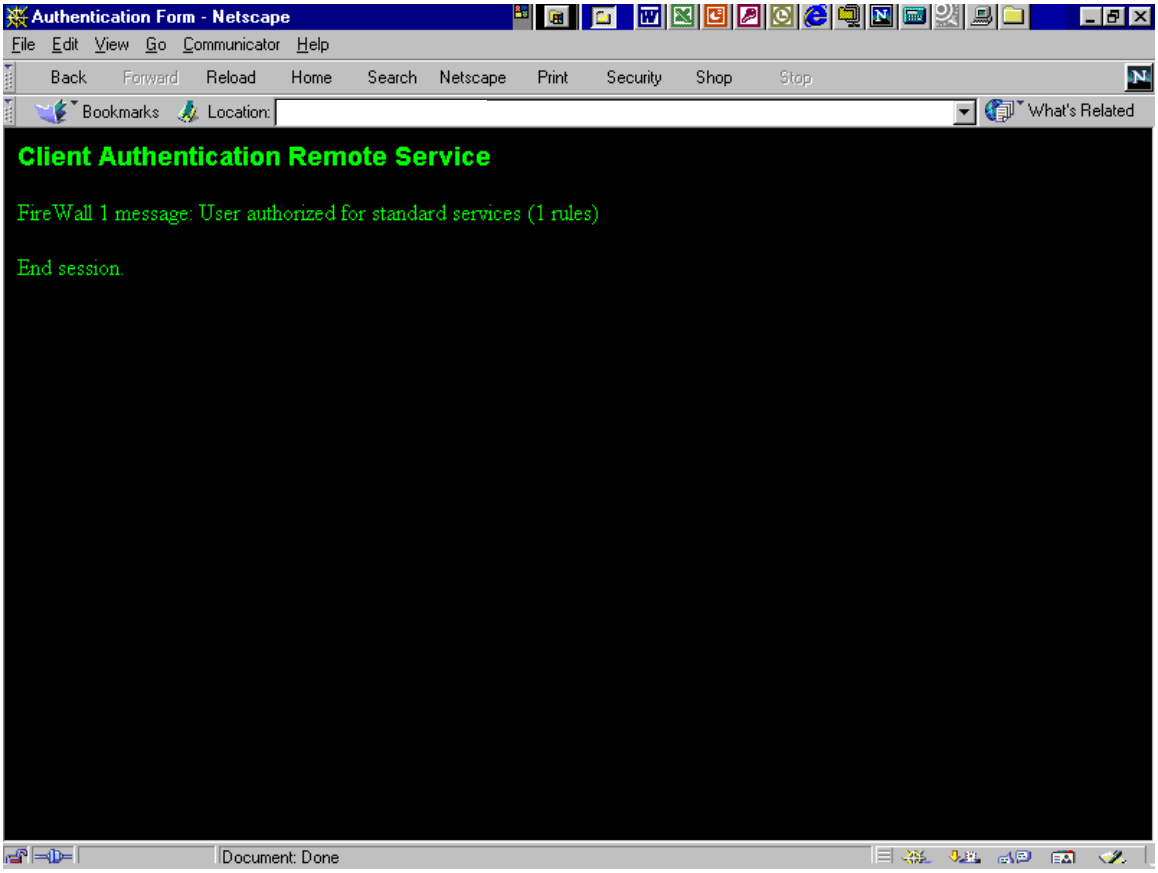
Click ***Authentication.***



You will see this screen: Client Authentication Remote Service

Verify **Standard Sign-on** is selected.

Click ***Submit.***



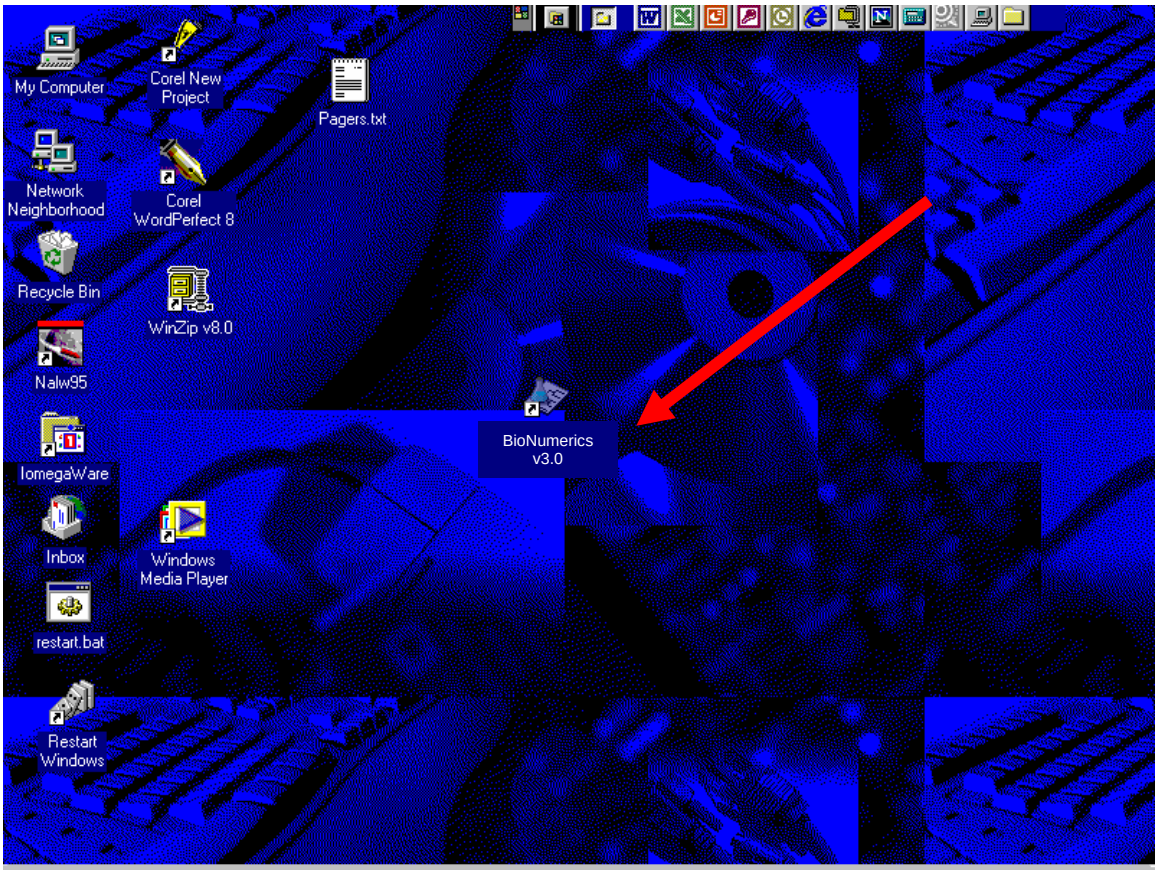
You will see this screen: Client Authentication Remote Service

You should have at least one rule.

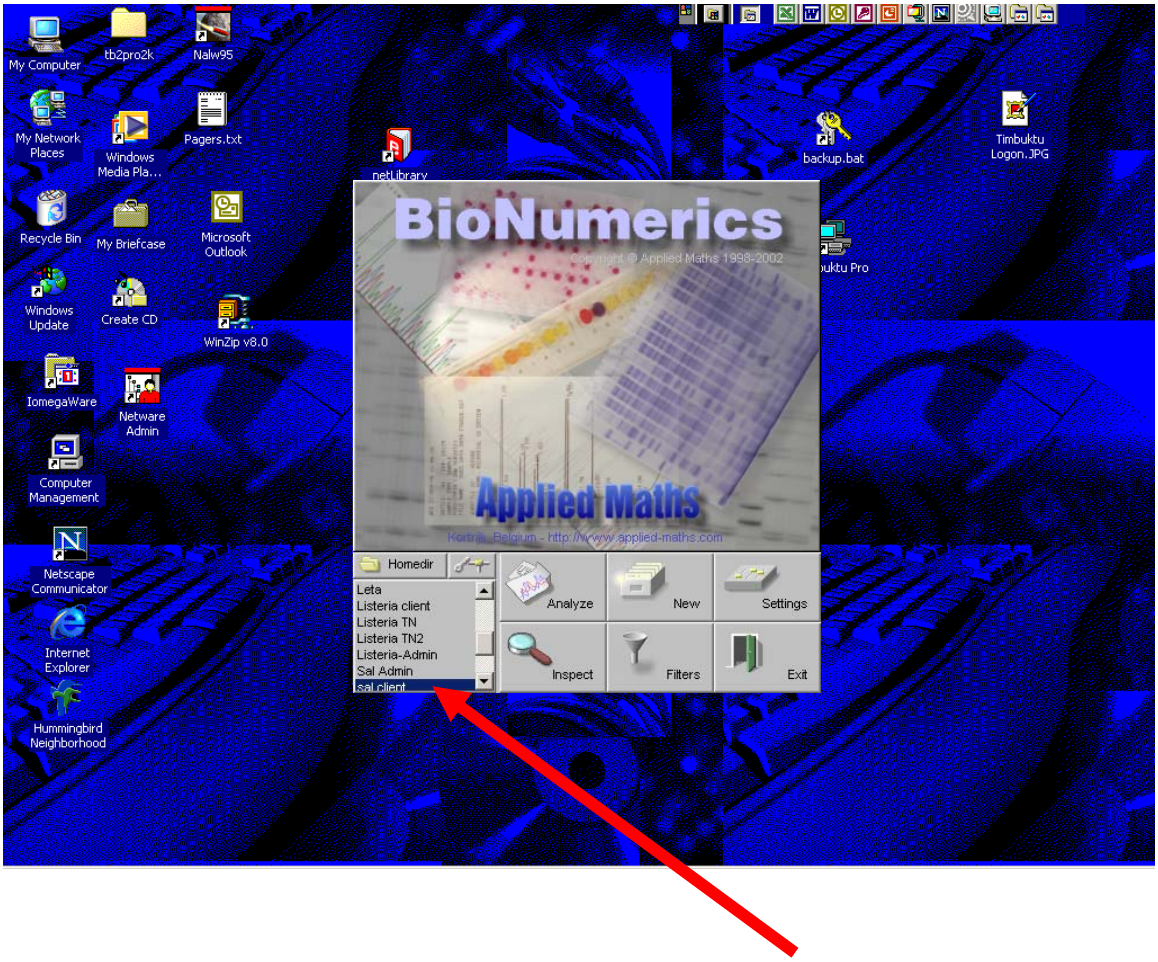
Minimize the screen

NOTE: IF YOU CLOSE THIS SCREEN YOU WILL TERMINATE YOUR CONNECTION TO THE FIREWALL.

Step 2: Using BioNumerics



Open ***BioNumerics***

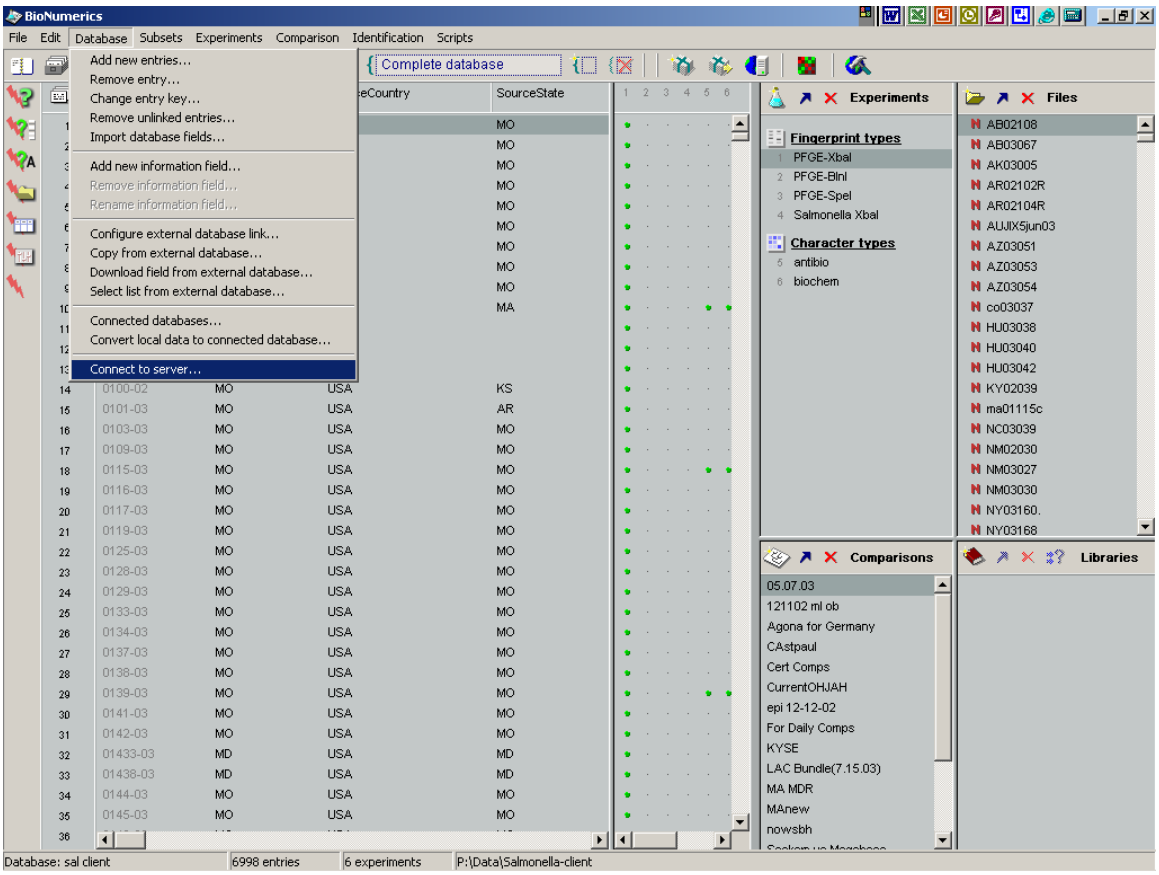


Select the database you want to analyze **(this could be Ecoli-client, Salmonella-client, Listeria-client, Shigella-client, or Campylobacter-client)**.

Click ***Analyze***

The PulseNet logo will appear if you have installed the PulseNet customization files.

This screen tells you the total number of isolates currently in your database, then the individual number, by pattern.

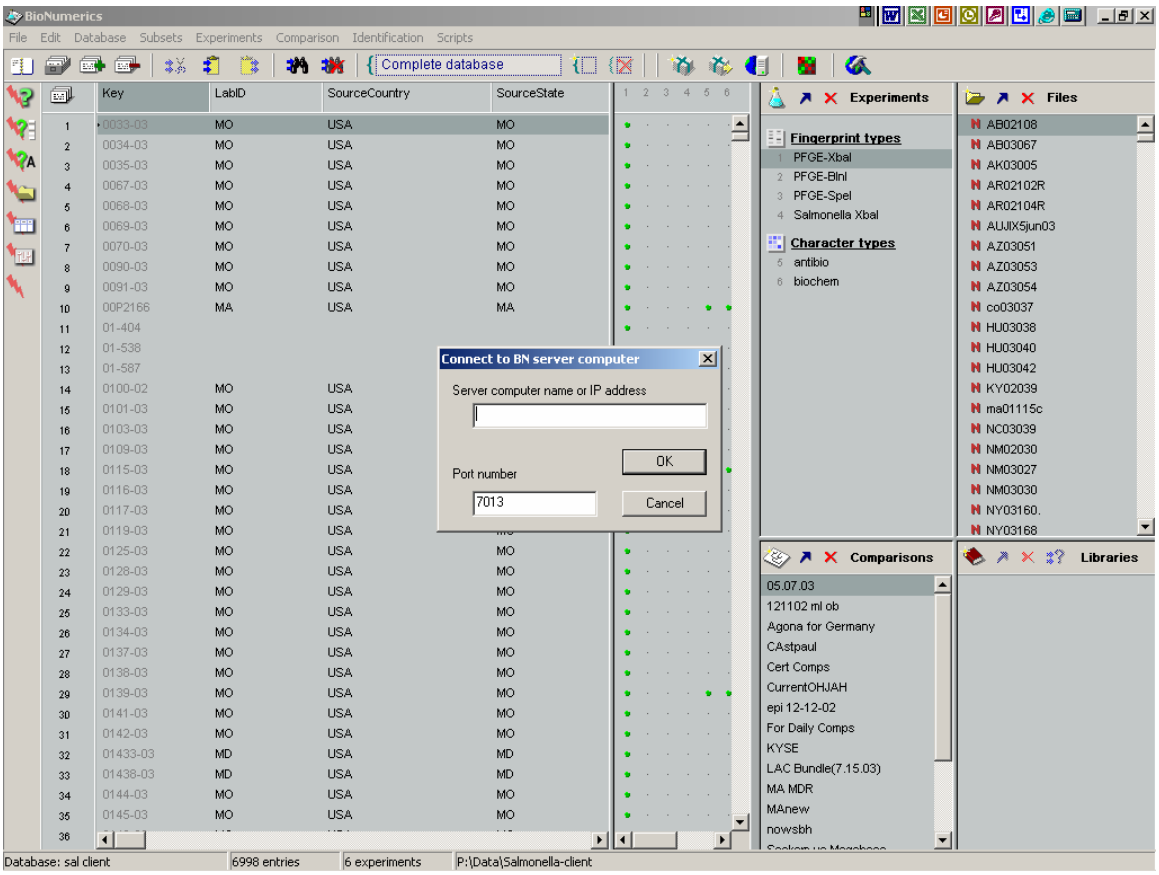


To connect to the Online BioNumerics server:

Click **Database**

Select **Connect to server…**

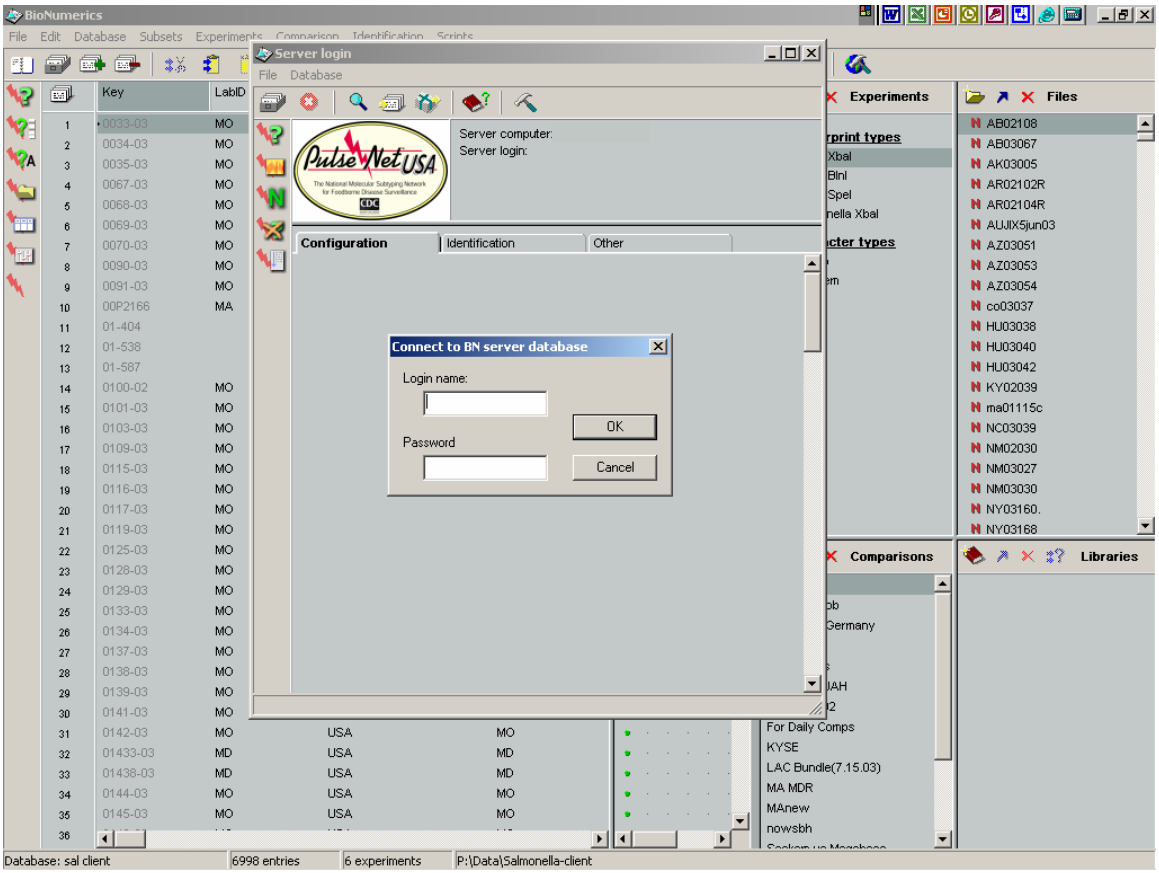
REMINDER: YOU SHOULD STILL HAVE THE AUTHENTICATION SCREEN MINIMIZED.



Enter the **IP address** of the BioNumerics server. You have received this in a separate communication.

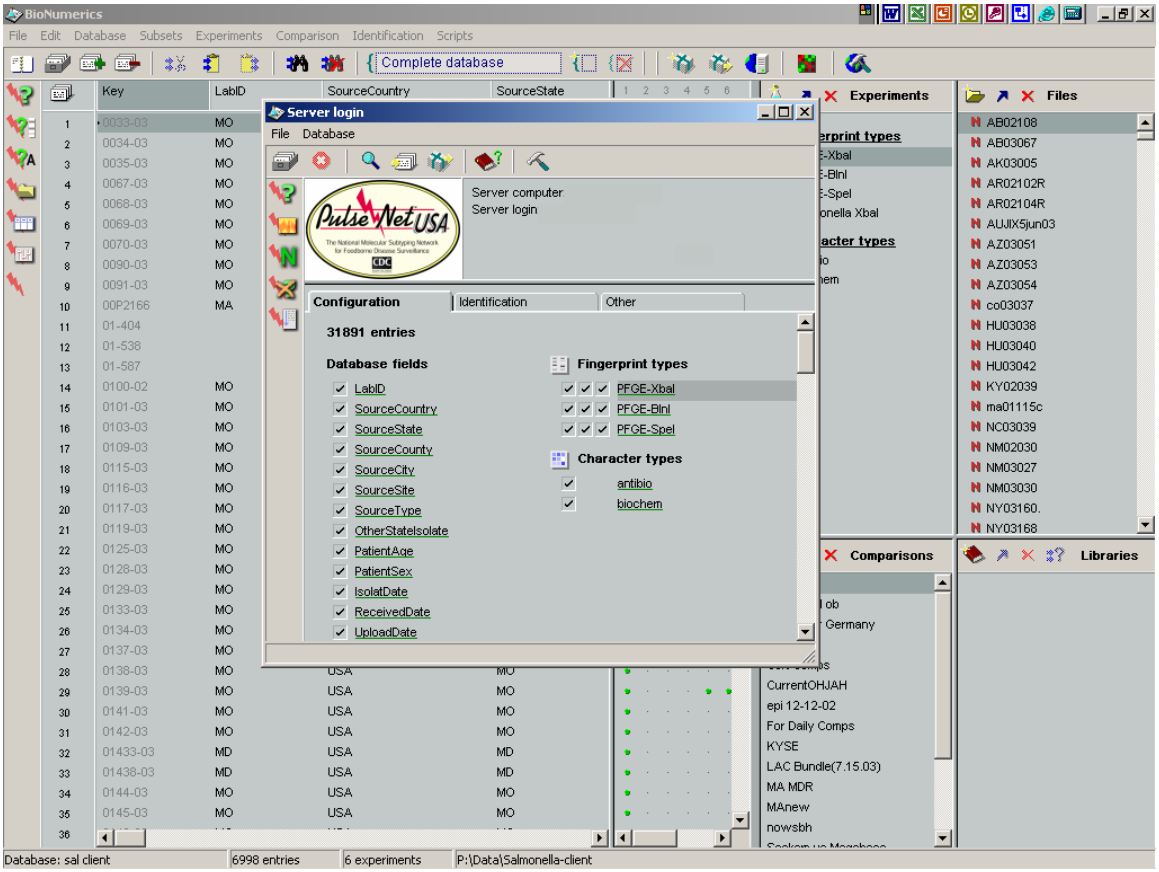
Verify the Port number is **7013**

Click ***OK***



Enter the **Login** and **Password** to access the BioNumerics server. You have received this in a separate communication.

Click ***OK***



You will see the IP address and the server login

You have now successfully connected to the BioNumerics Online server.

5. FLOW CHART:

6. BIBLIOGRAPHY:

7. CONTACTS:

7.1 CDC PulseNet Database Team
Centers for Disease Control and Prevention
1600 Clifton Road NE
Mailstop C03
Atlanta, Georgia 30333
Phone: (404) 639-4558
Email: PFGE@cdc.gov

8. AMENDMENTS:

1. **PURPOSE:** To describe the procedure for training PulseNet personnel in PulseNet database management and communication protocols.

2. **SCOPE:** This procedure applies to all PulseNet participants and hosts of PulseNet database training courses.

3. **DEFINITIONS/TERMS:**

3.1 Host lab: Term used to describe a PulseNet laboratory that has been approved by CDC to host a training course
3.2 Training personnel: Term used to describe PulseNet participant(s) who have been approved by CDC to train other PulseNet participants
3.3 APHL: Association of Public Health Laboratories
3.4 SOP: Standard Operating Procedure
3.5 CDC: Centers for Disease Control and Prevention
3.6 TIFF: Tagged Image File Format. A file of a gel image that can be analyzed in BioNumerics
3.7 BioNumerics: Gel analysis software used by PulseNet, developed by Applied Maths, Belgium

4. **RESPONSIBILITIES:**

4.1 All PulseNet personnel are required to read the PulseNet QA/QC manual and all PulseNet SOPs.
4.2 At least one PulseNet participant from each participating PulseNet laboratory is required to attend annual PulseNet update meetings and regional meetings when they occur.
4.3 All PulseNet personnel submitting TIFFs of PulseNet pathogens for submission to the national databases must have at least a basic level of computer knowledge and be familiar with the BioNumerics analysis software.
4.4 Host lab(s), APHL, and/or CDC will determine training needs of PulseNet participants.
4.5 All PulseNet personnel must be trained by a host lab or by other approved training personnel.
4.5.1 PulseNet database management and communication training covers analysis of PFGE gels using BioNumerics software, how to access the PulseNet National Databases with data-sharing tools, how to upload PFGE data to the national server, and how to communicate with others in the PulseNet network and CDC.
4.6 Host labs and training personnel should adequately prepare participants in the PulseNet database management and communication. When training is finished, the trainee(s) should be able to submit PulseNet gels and bundle files for analysis certification (see SOP PNQ02).
4.7 Host labs and training personnel must be analysis-certified.
4.8 Host labs must put together training materials for the trainees.
4.9 PulseNet participants will then be evaluated through certification (PNQ02) and proficiency testing (PNQ04).

5. **PROCEDURE:**

5.1 APHL and CDC continuously monitor PulseNet participant training needs.
5.2 Host labs and/or training personnel should work with trainees and/or APHL to determine a feasible time and location for the course.
5.3 The following is a recommended procedure for hosting a PulseNet database management course:
5.3.1 The host lab should organize an agenda committee to create an agenda and a timeline of organizational duties, obtain needed training materials, and arrange for IT support.

5.3.2 It is recommended that each participant be provided, but not limited to, the following items:

5.3.2.1 Participant and trainer contact information
5.3.2.2 Handouts of training presentations, preferably in an organized notebook format
5.3.2.3 CD containing the BioNumerics software (for use in class only)
5.3.2.4 CD containing PulseNet masterscripts (for use in class only)
5.3.2.5 Practice exercises and training TIFFs with all PulseNet organisms
5.3.2.6 Course evaluations
5.3.2.7 Course certificates to indicate successful completion of training

5.3.3 The committee may also be responsible for making lodging, transportation, and meal arrangements for course participants.

5.3.4 It is recommended that training be carried out using *E. coli* or *Salmonella* unless another PulseNet organism is specifically requested.

5.3.5 Refer to PND02 (SOP for TIFF Image Analysis) and PND03 (SOP for Accessing and Using the PulseNet Listserv) in the preparation of materials. Host labs can also contact the CDC PulseNet Database Team for training materials.

5.3.6 Refer to Appendix PND10-01 for recommended training topics, and to PND01 (SOP for Computer Equipment and Supplies) for a list of necessary materials.

5.3.7 Assign training responsibilities to trainers.

5.3.8 It is recommended that there be one trainer per five participants and a maximum of two participants per computer.

5.3.9 At the completion of training, participants should fill out evaluations and be awarded certificates of completion.

5.3.10 Summarize evaluations and supply trainers and members of the agenda committee a summary of the training evaluations.

6. FLOW CHART:

7. BIBLIOGRAPHY:

8. CONTACTS:

8.1 PulseNet Computer support CDC contact: Brenda Brown
(404) 639-3942
BLBrown1@cdc.gov

8.2 Training support at CDC: PulseNet Database Administration Team
(404) 639-4558
PFGE@cdc.gov

9. AMENDMENTS:

Appendix PND10-01

Sample BioNumerics and PulseNet Masterscripts Training Course Agenda

SAMPLE AGENDA
Beginning BioNumerics Workshop for PulseNet Participants

General Information about the Workshop

Welcome and Introductions; Overview of Workshop

Overview of BioNumerics—Goals and Objectives

BioNumerics 3.0/3.5 and PulseNet Master Scripts

Beginning BioNumerics: Processing PFGE Gel Images

Exercise 1: Processing a PFGE gel image and linking entries to a database

Review of Morning Sessions: Questions and Answers

How to Setup Your SecurID Key Fob, Authenticating to CDC Firewall, and Using the PulseNet BioNumerics Online Server

Uploading and Accessing Data via the National Server

Exercise 2: Uploading data to the National server

Questions and Answers

Exercise 3: Practice using *Salmonella* or *E. coli* clients to analyze and enter data

Review of Day 1: Questions and Answers

Basics behind Comparisons and Clustering

Creating Comparisons in BioNumerics

Exercise 4: Performing comparisons

Communication with PulseNet and WebBoard

Exercise 5: Pulling it all together: practice scenario

**Questions and Answers, Return Evaluation Form,
Presentation of Certificates**

1. **PURPOSE:** To describe the procedure for beta testing BioNumerics MasterScripts for new PulseNet databases.

2. **SCOPE:** This procedure applies to CDC staff and PulseNet laboratories outside CDC who participate in beta testing exercises.

3. **DEFINITIONS/TERMS:**

3.1. APHL: Association of Public Health Laboratories
3.2. SOP: Standard Operating Procedure
3.3. CDC: Centers for Disease Control and Prevention
3.4. TIFF: Tagged Image File Format. A file of a gel image that can be analyzed in BioNumerics.
3.5. Bundle file: A file generated within BioNumerics for sharing and comparing PFGE patterns and associated demographic and laboratory data from one database to another. The file extension is .bdl and is located in the bundle folder of the corresponding database directory (X://Program files/bionumerics/data/test_*organism database*/bundle)
3.6. BioNumerics: Gel analysis software used by PulseNet, developed by Applied Maths (Sint-Martens-Latem, Belgium.
3.7. Scripts: Macros (computer commands) which allow a repeated task in BioNumerics to be automated.
3.8. MasterScripts: Macros designed specifically for a PulseNet laboratory to submit and query PFGE patterns and demographic data on a national database.
3.9. Database Team Members: personnel at CDC who have been designated to manage a particular PulseNet database; these people manage all administrative aspects of the databases, including being involved in script creation, testing, naming patterns, and correcting band markings.
3.10. On-line: Connected to the PulseNet server. Certified individuals may access the information within the particular on-line database.
3.11. Test database: Term used to describe the new database that has been created in which to test the MasterScripts for the new database.
3.12. Beta testing: Testing a pre-release version of the MasterScripts by making it available to selected users.
3.13. Screen dump: By pressing the “print screen” key on a computer keyboard, a user captures an image of whatever is currently displayed on the monitor. A user may then paste this image into another program such as Microsoft PowerPoint.
3.14. PFGE inbox: An email account that is maintained and checked by all database team members at CDC. The address is PFGE@cdc.gov.
3.15. Remote testing lab: A PulseNet-participating lab other than CDC that has been designated to perform testing on new scripts.

4. **RESPONSIBILITIES AND PROCEDURE:**

4.1. Internal Testing to be completed at CDC by Database Team Member(s).
    4.1.1. The Database Team Member(s) will use the Testing Results_Client and Testing Results _Admin spreadsheets (excel files found in P:\BioNumerics\Scripts\Testing\Testing Templates) as guidelines to complete the beta testing and record any errors or comments as necessary. Any errors should be captured in a screen dump and explained in a PowerPoint slide.

4.1.2. The Testing Results_Client and Testing Results_Admin spreadsheets and the PowerPoint slides will be saved in P:\BioNumerics\Scripts\Testing*MasterScripts v## testing* and/or *Organism* Testing.

4.1.3. The Database Team Member(s) will notify the PulseNet Database Unit Chief and PulseNet Computer Support once testing has been completed and the spreadsheets and PowerPoint slides are ready for review.

4.1.4. PulseNet Computer Support will review the spreadsheets and address any script errors or comments as necessary and fill in any corrective measures taken. Once this is completed, the Database Team Member and Database Unit Chief are notified for additional testing as necessary.

4.2. External testing is to be completed by a remote testing lab. This can be a laboratorian from a state or local health department who has been analysis-certified by CDC for at least one organism (see Certification SOP PNQ02).

4.3. At least one PulseNet participant from a remote testing lab and a Database Team Member should beta test the MasterScripts before new MasterScripts are distributed to PulseNet participants and the new database is considered "on-line" (if a new database was added to the MasterScripts) and accessible to analysis-certified personnel.

4.4. Those performing testing at CDC will need to have login access to the test database. This is accomplished by:

4.4.1. The Database Team Member(s) responsible for the test database initiates an e-mail to PulseNet Computer Support or other responsible individual for request to access the on-line test database for beta testing of new scripts.

4.4.2. PulseNet Computer Support will initiate access to the test database.

4.4.3. Login and password information will be given to the individual(s) who will be completing the beta testing once approval for access has been granted.

4.5. The Database Unit Chief will forward all necessary documents and files needed for beta testing to the remote testing lab. These documents and files include:

4.5.1. Cover letter (see Appendix PND11-1)

4.5.2. Instructions for setting up a new database in BioNumerics (taken from BioNumerics manual section "Setup")

4.5.3. Instructions for installing new scripts from the MasterScripts CD

4.5.4. CD containing the newest beta version of the MasterScripts

4.5.5. File(s) of testing PFGE gel(s) (.tif)

4.5.5.1. PFGE gel file name is BETA TEST GEL_*organism*.tif

4.5.6. PFGE report containing necessary demographic and laboratory data for testing PFGE gel(s)

4.5.7. Checklist for beta testing new MasterScripts (see Appendix PND11-2)

4.6. PulseNet participants in the remote testing lab should read and become familiar with instructions for creating a new database and installing scripts prior to the start of beta testing.

4.7. The following procedure for beta testing scripts for a new PulseNet national database is as follows:

4.7.1. Follow instructions to create a new PulseNet database in BioNumerics. The name of the database should be test_*organism initials* (i.e. test_EC).

4.7.2. Follow instructions to install MasterScripts from CD in BioNumerics

4.7.3. Analyze the test PFGE gel(s) in BioNumerics

4.7.4. Link lanes and enter demographic and laboratory data as described in the PFGE report(s)

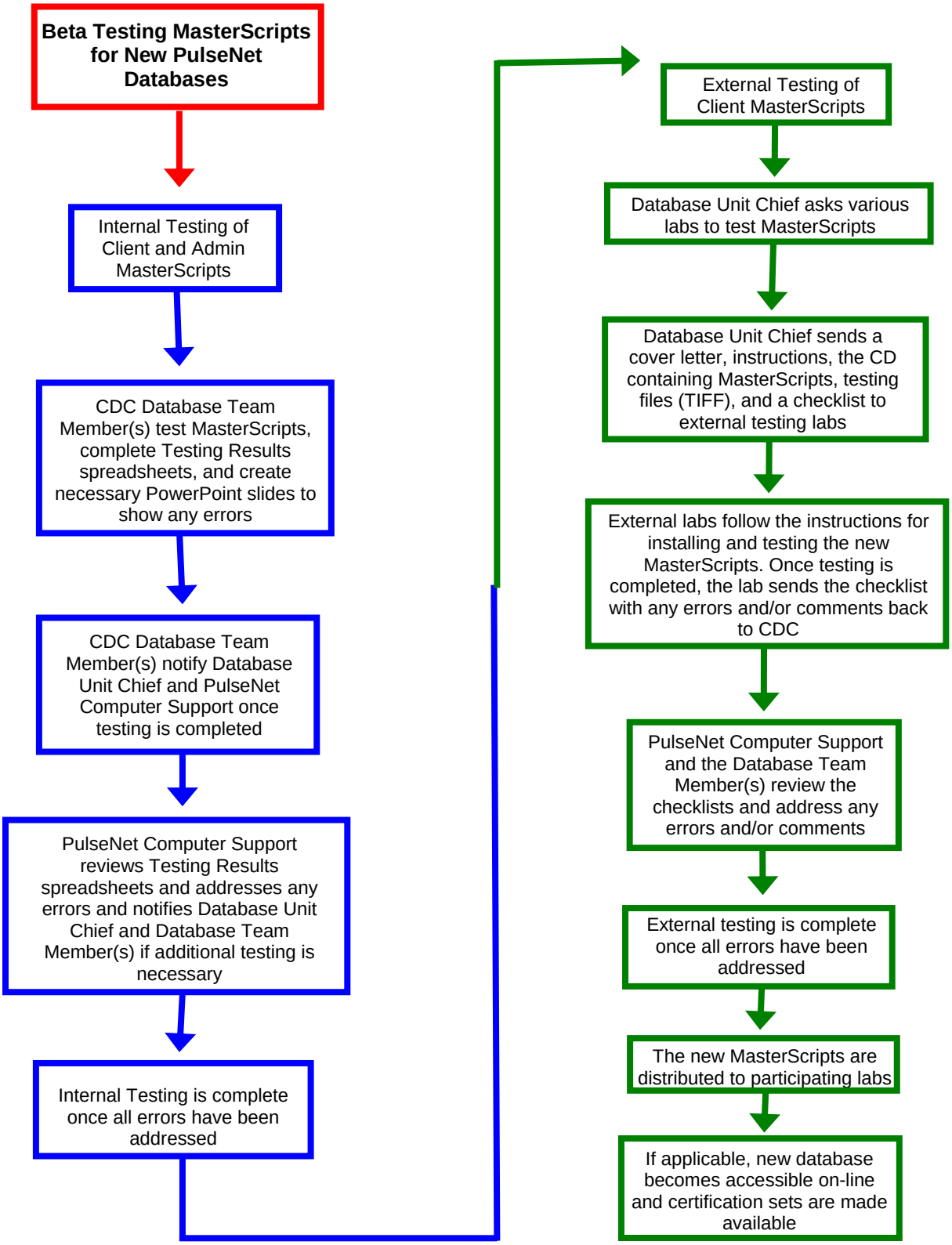
4.7.5. Create a bundle file (.bdl) and e-mail it to the PFGE inbox with "Test Bundle" in the subject line of the email.

4.7.5.1. This step ensures that the reference standard and entry property fields have been installed correctly in the client database.

4.7.6. Once the bundle file has been sent, await confirmation from the PulseNet Database Team

4.7.7. Create a comparison of all entries in the local database
4.7.8. Select the experiment type icon (e.g. PFGE-XbaI) on the bottom of the comparison window to visualize the PFGE patterns. Note: All patterns should be visible and no remapping errors should be shown.
4.7.9. Perform queries on your local test *organism* database. Make note of any errors or anything that does not perform correctly.
4.7.10. If a new database has been added to the MasterScripts, look over the fields, pick lists, and organization of the *organism* database. Make note of anything that appears incorrect and/or list any ideas you have to improve the look or organization of the new database.
4.7.11. Perform any other queries or actions as directed by the PulseNet Database Team. This will include any new items or tools added to the MasterScripts.
4.7.12. The checklist (Appendix PND11-2) should be completed and comments added and sent back to the PFGE inbox.
4.7.13. The Database Team Member responsible for beta testing of the database should give feedback and results to all participants involved with beta testing of MasterScripts within 2 weeks of the testing date.
4.7.14. If errors are found, the PulseNet Database Team will communicate those errors to PulseNet Computer Support or Applied Maths for resolution.
4.7.15. Once all testing is successfully completed, the MasterScripts can be distributed and any new databases should then become accessible on-line to all analysis-certified participants.
4.7.16. The certification process should be initiated for PulseNet laboratories interested in submitting data.

5. FLOW CHART:


Beta Testing MasterScripts for New PulseNet Databases
Internal Testing of Client and Admin MasterScripts
CDC Database Team Member(s) test MasterScripts, complete Testing Results spreadsheets, and create necessary PowerPoint slides to show any errors
CDC Database Team Member(s) notify Database Unit Chief and PulseNet Computer Support once testing is completed
PulseNet Computer Support reviews Testing Results spreadsheets and addresses any errors and notifies Database Unit Chief and Database Team Member(s) if additional testing is necessary
Internal Testing is complete once all errors have been addressed
External Testing of Client MasterScripts
Database Unit Chief asks various labs to test MasterScripts
Database Unit Chief sends a cover letter, instructions, the CD containing MasterScripts, testing files (TIFF), and a checklist to external testing labs
External labs follow the instructions for installing and testing the new MasterScripts. Once testing is completed, the lab sends the checklist with any errors and/or comments back to CDC
PulseNet Computer Support and the Database Team Member(s) review the checklists and address any errors and/or comments
External testing is complete once all errors have been addressed
The new MasterScripts are distributed to participating labs
If applicable, new database becomes accessible on-line and certification sets are made available

6. BIBLIOGRAPHY:

7. CONTACTS:

7.1 CDC PulseNet Computer Support
Brenda Brown
(404) 639-3942
BLBrown1@cdc.gov

7.2 CDC PulseNet Database Team
(404) 639-4558
PFGE@cdc.gov

7.3 PulseNet Database Team Unit Chief
Kelley Hise
(404) 639-0704
KHise@cdc.gov

8. AMENDMENTS:

Appendix PND11-1

Beta Testing MasterScripts Cover Letter Template

Beta testing *organism* MasterScripts v*XX* Beta

Scripts for PulseNet participating laboratories enable users to share and compare patterns within the PulseNet community. Reference systems (H9812), pick lists, and other functions within BioNumerics are standardized using scripts so that data is comparable among participating laboratories. These scripts are developed by CDC and Applied Maths and are highly customized for each national database.

Before a set of scripts are implemented for use by the PulseNet community, a series of beta testing is done at both CDC and remote PulseNet laboratories. These testing procedures test the functionality of the database (i.e. ability to analyze and enter demographic/laboratory data, queries, etc.) as well as the overall layout and look of the database. This testing is greatly needed and participation from PulseNet laboratories is always appreciated.

This packet contains all the necessary information for remote PulseNet laboratories to beta test scripts for new PulseNet national databases. This beta testing will be for the *organism* national database which is considered online and in the production phase of testing. If at any time you have questions, please do not hesitate to call or e-mail the PulseNet Database Team at 404-639-4558 or pfge@cdc.gov.

Below are the following documents and CD that are included in this packet:

1. CD containing 2 folders:
   a. **MasterScripts v*XX* Beta:** has the files for testing *organism* scripts
   b. **Testing:** has PFGE TIFFs of *organism* and TIFF demographic information, electronic copy of checklist and procedures
2. Laboratory and demographic data for PFGE TIFFs
3. Checklist for testing the MasterScripts for the *organism* database

Thank you again for testing the MasterScripts v*XX* Beta. Your participation in PulseNet and in the testing has been greatly appreciated.

PulseNet Database Team

Appendix PND11-2

Checklist for testing MasterScripts v*XX* Beta for PulseNet *organism* database

LabID_____ Date Testing Completed_____

Insert an "X" when completed	Item to be Tested for *organism*
	Create a local test *organism* database
	Install MasterScripts v*XX* Beta for the test *organism* database
	Analyze a *organism* PFGE gel **(test of gel analysis)**
	Add demographic information for gel **(test of pick lists, etc)**
	Create bundle file and e-mail to: pfge@cdc.gov with "Test Bundle" in subject line **(test of bundle file function)**
	Perform queries on your local test *organism* database
	Create comparison and pull up patterns to view in comparison window
	Look over the fields, pick lists and organization of the *organism* database **(please comment below)**

Comments:

After completing, either fax [404-639-3333 (ATTN: PulseNet Database Team)] or email to pfge@cdc.gov and put "Testing" in the subject line. Please don't forget to fill in the LabID information at the top.

Thank you.

1. PURPOSE: To describe the PulseNet standardized nomenclature for outbreaks and clusters.

2. SCOPE: This procedure applies to all CDC PulseNet Database personnel entering outbreak and cluster information into the "Outbreak" field in BioNumerics.

3. DEFINITIONS/TERMS:

3.1 TIFF: Tagged Image File Format. A file of a gel image that can be analyzed in BioNumerics.
3.2 BioNumerics: Gel analysis software used by PulseNet, developed by Applied Maths, Belgium
3.3 CDC: Centers for Disease Control and Prevention
3.4 PFGE: Pulsed-field Gel Electrophoresis
3.5 Cluster: A group of isolates with the same serotype determined to possess indistinguishable PFGE DNA patterns using one or more enzyme restrictions.
3.6 DNA: Deoxyribonucleic acid
3.7 LITS: Laboratory Information Tracking System
3.8 LabID: Unique identification assigned to each PulseNet participating laboratory, usually two-four letters that correspond with the postal code that is assigned by CDC.
3.9 PulseNet Listserv: A closed, moderated, electronic web conference used for communication among PulseNet participants. The PulseNet Listserv is open to all laboratory staff at PulseNet participating laboratories. Epidemiologists at these laboratories, when approved by the PulseNet laboratory contact, U.S. food regulatory staff, and PulseNet International representatives may also have access. The PulseNet Listserv is not open to university or private industry personnel unless their inclusion is deemed to be in the interest of public health.
3.10 Outbreak: A cluster of cases of infections with a common epidemiological exposure, e.g. to a specific food product.
3.11 Cluster: A group of isolates with the same serotype with indistinguishable PFGE DNA patterns by one or more restriction enzyme

4. RESPONSIBILITIES/PROCEDURE:

4.1 Basic code: A code consisting of the date of recognition of the cluster in the YYMM (2-digit year and 2-digit month) format is created, followed by the LabID abbreviation where it was first recognized (via PulseNet Listserv or other such posting).
<Date><LabID><LITS code for organism>-<number of cluster in month>
4.1.1 If a cluster is recognized at more than one lab simultaneously, the letter code should be "ml" for multi location.
4.1.2 The reason for this order of characters in the basic code is so it will be possible to query and sort the result of a query by year using only this single field.
4.2 The first characters identify the cluster/outbreak in a unique way. A dash ("-") plus a digit should follow the initial 9-11 characters (accounting for LabIDs with >2 characters) to indicate:
4.2.1 The number of the cluster/outbreak for the month. There can be different clusters/outbreaks with different serotypes occurring simultaneously in the same state; hence, to avoid the risk of mixing information on separate clusters/outbreaks together in the databases, the three-letter code for the serotype used in the naming of the PFGE-profiles should also be added after the date information.
4.2.1.1 Example: 0805MAJEG-2 is the second *Salmonella* Enteritidis outbreak in Massachusetts, first seen in May 2008. "0805" indicates the cluster was first noticed in May of 2008; "MA" indicates that it was first seen in MA; "JEG" represents *Salmonella* Enteritidis; the "-2" indicates that this is the second outbreak of this serotype for this month recognized in MA.
4.2.2 A "?" indicating that the status of the profile/case has not been firmly established as being associated with the cluster
4.2.3 An "x" indicating isolates that are being used as controls in a study
4.2.4 Examples:

4.2.4.1 **0805mlGX6-1** (the first *Listeria monocytogenes* multi-state outbreak recognized simultaneously in two or more states in May 2008)

4.2.4.2 **0210MSJPX-3** (a cluster of cases associated with *Salmonella* Typhimurium discovered in October 2002 following two other clusters/outbreaks in Mississippi that month

4.2.4.3 **0207FLJ16-2?** The second *Shigella sonnei* Florida outbreak turns out to be a big one and there are a lot of isolates that may be related to the outbreak. It takes time to sort out whether they are part of the outbreak (the database manager has received a lot of TIFFs or they have done a search on the profile but haven't analyzed the results, yet). It could also be a single unconfirmed isolate from a patient in a different state in a presumably single state outbreak.

4.2.4.4 **0309WADBR-1?** This is a big one, recognized as a cluster of *Campylobacter jejuni* in Washington in September of 2003. Before they get interrupted, the database manager names all the possible matches they have found so that they may find them again easily.

4.2.4.5 **0307GAJJP-1x** This is the first outbreak of *Salmonella* Newport that began in July of 2003 in Georgia; however, the "x" on the end denotes that these were considered to be controls in the outbreak, and do not have the outbreak pattern. This simplifies being able to pull these up at a later date.

5. FLOW CHART:

6. BIBLIOGRAPHY:

7. CONTACTS:

8. AMENDMENTS:

8.1 2009-01-01: Removed the "c" that used to be placed at the end of the basic outbreak code indicating the cluster was not yet an outbreak. Also removed the "ml" that used to be placed at the end of the basic outbreak code indicating the cluster was a multi-state cluster.

1. **PURPOSE:** to describe the standardized protocol for analysis of MLVA data of Shiga toxin-producing *Escherichia coli* O157 (STEC O157) and *Salmonella enterica* serotypes Typhimurium and Enteritidis in BioNumerics.

2. **SCOPE:** to provide PulseNet participants with a single protocol for analyzing MLVA data of STEC O157 and *Salmonella* serotypes Typhimurium and Enteritidis, thus ensuring inter-laboratory comparability of the generated results.

3. **DEFINITIONS:**
   3.1.MLVA: Multiple-locus variable-number tandem repeat analysis
   3.2.VNTR: Variable-number tandem repeat
   3.3.CDC: Centers for Disease Control and Prevention
   3.4.SOP: Standard Operating Procedure

4. **RESPONSIBILITIES/PROCEDURE:**
   4.1 Software needed for data analysis
   4.1.1. BioNumerics version 4.5 or higher
   4.1.2. Customized scripts for data import (*VNTRImport_v4.bns*), copy number calculation (= allele assignment) (*VNTRCalc_v4.bns*), troubleshooting (*VNTRReport.bns*) and support functions (*VNTRDetails_v2.bns*).
   4.1.3 Look-up tables (*BeckmanEcoli.txt, BeckmanST.txt, BeckmanSE.txt*) for allele size ranges
   4.2 General overview
   4.2.1 The analysis process consists of three major steps:
   4.2.1.1 Exporting the appropriate data file from the Beckman system (refer to SOP PNL19, step 4.10.1.6)
   4.2.1.2 Importing this file into BioNumerics (using the script *VNTRImport_v4.bns*)
   4.2.1.3 Determining the copy numbers (assigning alleles) for each VNTR (using the script *VNTRCalc_v4.bns* and the organism-specific look-up table *BeckmanEcoli.txt, BeckmanST.txt, BeckmanSE.txt*)
   4.2.1.3.1 *VNTRCalc_v4.bns* script facilitates the allele assignment in two different ways:
   4.2.1.3.1.1 "BeckmanEcoli, BeckmanST and BeckmanSE" – assign the copy number based on the look-up table (PND14-1a-c). This method should be used in all routine analysis.
   4.2.1.3.1.2 "Predicted" – calculates the copy number based on the mathematical formula: (Observed fragment size – offset)/repeat size. This method should only be used when a new allele (not specified in the look-up table) is identified
   4.3 Required import format
   4.3.1 The data should be exported from the Beckman CEQ/GeXP software as CSV (comma-delimited) text files containing the fragment length information. Multiple columns will be present in the file but only four columns are of interest:

"RN," "dye," "est size (nt)" and "pk height (rfu)". All other columns will be ignored by the script during import. The field "RN" should contain information designating the BioNumerics key number (isolate identifier) loaded in each well of the sequencer plate run, as well as which VNTR mastermix or reaction was loaded. The import script will ignore any text that appears beyond the dot "**.**" in the RN field.

4.3.1.1 **NOTE:** no spaces or underscores are allowed between the strain ID and the reaction ID or between the reaction ID and the dot "." in the RN field.

4.4 Setting up a new database

4.4.1 Click "Create New Database" icon to set up a new database

4.4.2 Type in the name (for example "O157MLVA") of your new database and click "Next"

4.4.3 Select the directory (default on CDC training laptops is [HOMEDIR]\O157MLVA; this will save the data on the G-drive) in which you want to set up the new database. Choose the default "Yes(recommended)" when asked "Do you want to automatically create the required directories?". Click "Next".

4.4.4 Choose "Yes" to enable creation of log files and click "Finish"

4.4.5 In the appearing "Set up new database" pop-up window, choose "Local database (single user only)" and click "Proceed"

4.4.6 Click "Yes" in the next pop-up confirmation window ("Choosing 'local database' will restrict some functionality of the software. Are you sure you want to continue?") and "Proceed" in the following "Plug-in" pop-up window

4.4.7 Close the newly created database.

4.4.8 Go to the directory in which you placed your database at step 4.4.3 (on CDC training laptops: C:\Program files → BioNumerics → Data), open folder "O157MLVA" and create two subfolders named "Scripts" and "VNTRtables".

4.4.9 Save the four MLVA specific scripts (*VNTRImport_v4.bns*, *VNTRCalc_v4.bns, VNTRReport.bns, VNTRDetails_v2.bns*) in the "Scripts" folder and the organism specific assay look-up table (e.g. *BeckmanEcoli.txt*) in the "VNTRtables" folder

4.4.10 Open the database. The scripts (except the VNTRDetails script) should now appear under the "Scripts" drop-down menu

4.5 Importing a peak file for the first time

4.5.1 Review the peak file: make sure that the observed size for the D1 labelled molecular size standard peaks is within ± 1 bp from the expected size. Remove any data (failed reactions, controls, internal ladder) that you don't want to import in the BioNumerics from the CSV file. Re-name and re-save the CSV file either on your hard drive or on the flash drive.

4.5.2 In BioNumerics, run the script *VNTRImport_v4* from the "Scripts" drop-down menu. The "Import VNTR peak data" dialog box will appear. From the "Peak file format" drop-down menu, select "Beckman peak file". The script pops up a file dialog box, prompting for the name of the file to import. Select the appropriate

file and click "OK". A second dialog box pops up, prompting for a variety of other information:

4.5.2.1 *Fingerprint file name*. This is the name of the fingerprint file that will be used in BioNumerics. Leave this unchanged, unless a file has already been imported with the same name. In this case, change the file name into a new, unique and informative name.

4.5.2.2 *Dyes to import*. The first dye (D1) contains only reference markers and does not need to be imported. Use this list to select what dyes will be imported; they are D2, D3 and D4. Each dye will be stored in a different file, resulting from appending the dye name to the fingerprint file name.

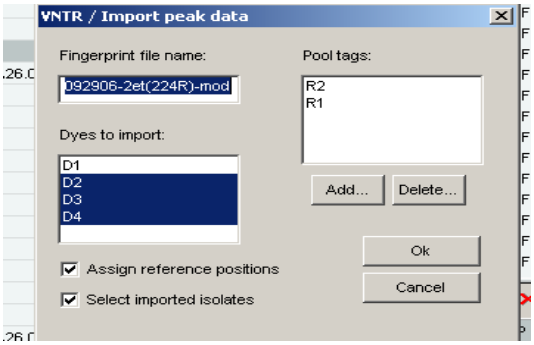
4.5.2.3 *Assign reference positions*. Leave this option checked.

4.5.2.4 *Select imported isolates*. Leave this option checked.

4.5.2.5 *Pool tags*. For this protocol, there are two pool tags representing the two PCR reactions for each isolate (all loaded on different wells) and tagged with names "R1" and "R2". The two tags should appear in the list. If they do not, they can be added by selecting "Add" to update.

4.5.2.6 If everything is filled appropriately, click "OK" to start the import.

4.5.2.7 **NOTE**: These settings are automatically saved and reloaded the next time this script is run.



4.5.3 When the script is finished, it has:

4.5.3.1 Created a fingerprint type "*VNTRFpr*" with appropriate settings

4.5.3.2 Created fingerprint types with appropriate settings for each dye and each pool used. The names of these fingerprint types are "VNTRFpr"+"pool name"+"dye" (for example: VNTRFprR1_D2).

4.5.3.3 Created fingerprint files for each dye in the fingerprint type "*VNTRFpr*," and imported the band information in these lanes. The individual lanes are assigned to their appropriate pool fingerprint type, according to the known pool tags found in the "RN" field.

4.5.3.4 Created new database entries for all isolates found

4.5.3.5 Linked the fingerprints to their corresponding entries

4.5.3.6 Selected all isolates for which fingerprint data was imported

4.6 Determining the copy numbers for the first time (allele assignment)

4.6.1 Run the script *VNTRCalc_v4* from the scripts menu. The first time this script is run, it will automatically create a new character set called "*VNTR_vals*". This character set will hold the copy numbers for each VNTR. The script will also create a second character set called "*VNTR_frags*" which holds the fragment size with the highest fluorescence for each VNTR. Click "OK" on the two pop-up windows notifying that these two character types will be created.

4.6.2 Before the script can determine copy numbers from the fragment length information, it should have sufficient information about what VNTRs can be found in which pool and dye, what are the repeat lengths, etc. To this end, the script pops up a dialog box called "VNTR assign". This dialog box contains a list of defined VNTRs that is initially empty. To enter the parameters of the first VNTR, click on "Add". This brings up a second dialog box, prompting for all properties of this first VNTR. Refer to the appendices PND14-2a-c for all necessary information to load the specifications for each VNTR.

4.6.3 The VNTR information includes:

4.6.3.1 *Name*. This name will be used for further reference and for the character name in the character type *VNTR_vals*. Each VNTR should have a unique name (for example VNTR_3, VNTR_34, etc).

4.6.3.2 *Offset*. Each fragment consists of a repeat portion and a constant portion, due to the fact that the primers do not occur exactly at the start and the end of the repeat region. This parameter specifies the size (in base pairs) of the constant portion.

4.6.3.3 *Repeat length*. This parameter specifies the size of a unit repeat block (in base pairs).

4.6.3.4 *Copy range*. These parameters specify the minimum and maximum number of copies that the script will consider during the copy number determination.

4.6.3.5 *Tolerance*. This specifies the maximum difference between the expected fragment length (calculated by the software) and the actual length estimated by the sequencer.

4.6.3.6 *Take from fingerprint type*. This drop-down list should be used to indicate on what fingerprint type this VNTR was run. The fingerprint type is determined by the dye and the pool tag.

4.6.4 If all parameters are filled in appropriately, click OK to add the VNTR and return to the previous dialog box ("VNTR assign"). Repeat the same actions to enter the information for all VNTRs used for this protocol. When all VNTRs are entered, make sure the box for "Code absent as negative" is checked. Select the appropriate assay specific look-up table (for example "*BeckmanEcoli*" for STEC O157) from the "Fragment ranges" drop-down menu and press the "Save&Assign" button to let the script assign copy numbers for the currently selected entries.

4.6.4.1 **NOTE 1:** pressing "Save&Quit" would store the information without calculating copy numbers for the current selection.

4.6.4.2 **NOTE 2**: all information regarding the VNTRs is stored with the database. The next time the script is run, the VNTR definitions will be loaded automatically.

4.6.4.3 For every fragment with the highest fluorescence level in the fingerprint type (= the combination of the reaction and the dye), the script will assign an allele type (=a copy number) based on the fragment size ranges specified in the appropriate look-up table (appendices PND14-1a-c). Note that, within the same fingerprint type, more than one VNTR can be loaded if there is no overlap within the fragment size ranges.

4.6.5 When the script has completed, all copy numbers for all VNTRs for the currently selected entries are determined.

4.6.5.1 **Note**: Two types of problems may arise during the process (if one or more such errors were encountered during the calculations, an error report is displayed listing all the problems):

4.6.5.1.1 *None of the peaks present in a fingerprint are compatible with the fragment size ranges in the look-up table.* In this case, the corresponding character value will be scored "-2.0". In this situation, run the "*VNTRReport*" script from the "Scripts" drop-down menu to verify the reason for the problem:

4.6.5.1.1.1 No amplification (a null allele): mutations, insertions or deletions in the primer annealing region, or locus located on a plasmid that was lost. PCR needs to be repeated only if the null allele occurred in a locus in which null alleles have not been previously detected (For STEC O157: VNTR34, VNTR25, VNTR17; and in the case of VNTR19 for which null alleles are extremely rare, and hence should always be confirmed. For *Salmonella* serotype Typhimurium: ST2, ST3, ST6, ST7 and in the case of ST8 for which null alleles are extremely rare, and hence should always be confirmed. For *Salmonella* serotype Enteritidis: VNTR5, VNTR8, VNTR9; and in the case of SE6 for which null alleles are extremely rare and hence always need to be confirmed).

4.6.5.1.1.2 Fragment size outside the acceptable range (either slightly outside of range for a previously detected allele or a possible new allele). Rerun the fragment analysis reaction to confirm the accuracy and reproducibility of the sizing. The isolate should be submitted to CDC for confirmation only if a possible new allele is detected. If the sizing is reproducibly slightly outside the range for an existing allele, the CDC database managers will adjust the look-up table based on the evidence the submitting laboratory provides.

4.6.5.1.2 *More than one peak in a fingerprint is compatible with an acceptable fragment size range.* In this case, the script will use the solution that corresponds to the peak with the largest "peak height" value. Possible causes for multiple peaks:

4.6.5.1.2.1 Primer stutter: multiple peaks with sizes 1-2 bp from each other. No further action required.

4.6.5.1.2.2 Double alleles: two peaks differing by one or more full repeats from each other. Further actions required for all other loci except *Salmonella* serotype Enteritidis locus SE8 (in a subset of strains double alleles are known to occur and the major (larger) allele always has the highest fluorescence):

4.6.5.1.2.2.1 Repeat the PCR with a freshly made template. If two peaks still observed and the same peak consistently has the highest fluorescence intensity, report the predominant peak only. If the same peak does not consistently have the highest fluorescence intensity, proceed to the next step.

4.6.5.1.2.2.2 Test ten single colony picks from the culture. Report the peak that has the highest fluorescence intensity in the majority of the picks.

4.7 Analyzing a peak file on a routine basis

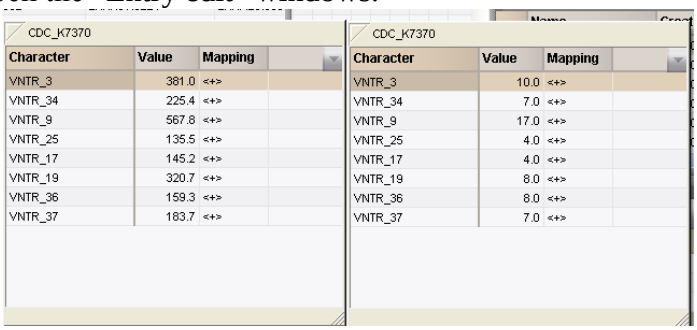
4.7.1 Review the peak file: make sure that the observed size for the D1 labelled molecular size standard peaks is within ± 1 bp from the expected size. Remove any data (failed reactions, controls, internal ladder) that you don't want to import in the BioNumerics from the CSV file. Re-name and re-save the CSV file either on your hard drive or on the flash drive.

4.7.2 In BioNumerics, run the script *VNTRImport_v4* from the "Scripts" drop-down menu. The "Import VNTR peak data" dialog box will appear. From the "Peak file format" drop-down menu, select "Beckman peak file". The script pops up a file dialog box, prompting for the name of the file to import. Select the appropriate file and click "OK". "VNTR/Import peak data" dialog box will appear. De-select "D1" from the "Dyes to import" and click "OK".

4.7.3 Run the script *VNTRCalc_v4* from the scripts menu. "VNTR assign" dialog box will appear. Select the appropriate look-up table (for example "*BeckmanEcoli*" for STEC O157)from the "Fragment ranges" drop-down menu and click "Save&Assign" to assign allele numbers.

4.8 Verifying the allele assignment

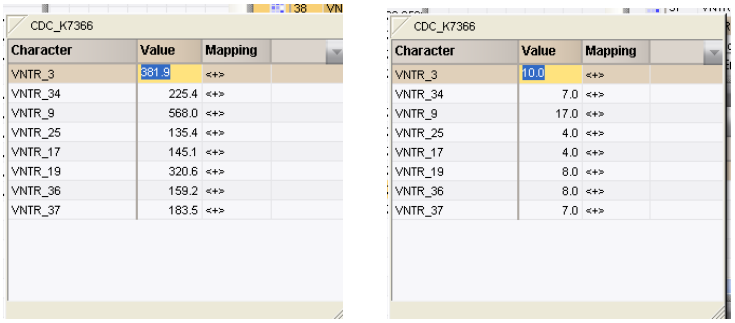
4.8.1 For each isolate in the database that has some VNTR data associated, you can click on the "VNTR frags" and "VNTR vals" entries in the list of yellow buttons to open the "Entry edit" windows.



4.8.2 If the *VNTRCalc_v4* script did not detect a fragment for a VNTR, refer to the step 4.6.5.1.1 of this protocol for details on how to proceed. If the fragment size is slightly outside the range specified in the look-up table it is possible to manually assign a temporary allele size and type for that VNTR until an official confirmation has been performed by CDC.

4.8.2.1 **Note:** only the CDC database managers are allowed to modify the look-up table txt-files. Once a modification has been made, the modified file will be posted on the PulseNet SharePoint site under QA/QC manual. An automatic e-mail notification will be sent about the change in the SOP**.**

4.8.2.2 To manually assign an allele size and type click on the fragment size in the "VNTRfrags" entry edit window or the allele type in the "VNTR vals" entry edit window. This will open the "Change character value" window in BioNumerics lower than 5.0. In BioNumerics 5.0 the allele size or type can be directly highlighted and changed in the entry edit window (Screen shots below)



4.9 Creating a composite data set to visualize the allele types

4.9.1 In order to display copy numbers next to a dendrogram in a comparison, first create a "*composite data set*" that holds the VNTR data.

4.9.1.1 **NOTE:** The created composite dataset will be automatically saved in the database, and hence only need to be created once before the first analysis in the database.

4.9.1.2 From the "Experiments" drop-down menu, select the option "Create new composite data set…", enter a name (e.g. *VNTR_cmp*), and click the "OK" button. The "Composite data set 'VNTR_cmp'" window will appear.

4.9.1.3 Highlight the experiment *VNTR_vals* and from the "Experiment" drop-down menu, select the option "Use in composite data set". Close the window.

4.9.2 Next time a comparison window is opened (see below step 4.10), there will be a new experiment *VNTR_cmp* listed in the bottom of the window (BioNumerics versions lower than 5.0) or in the top left corner of the window (BioNumerics version 5.0). This experiment will facilitate the display of a spreadsheet-like view of the copy numbers (note that it may be necessary to scroll the experiment list to the right with the arrow button to bring *VNTR_cmp* in display in BioNumerics versions lower than 5.0). This can be shown next to a dendrogram analysis of the data set.

4.10 Performing comparisons based on the VNTR data

4.10.1. The VNTR data contained in the character set *VNTR_vals* can be analysed in BioNumerics with all the tools that are available for character data. That includes cluster analysis with a variety of methods and similarity coefficients. For VNTR data, the coefficients that make most sense are:

4.10.1.1 *Categorical*: preferred if differences in copy numbers should be treated in a qualitative way. This is the only option for creating dendrograms using MLVA data.

4.10.1.2 *Manhattan*: preferred if differences in copy numbers should be treated in a quantitative way (i.e. a larger difference means more distantly related organisms). This coefficient can be used to construct minimum spanning trees.

4.10.2 In order to create a dendrogram:

4.10.2.1 Select the isolates to be included in the dendrogram

4.10.2.2 From the "Comparison" drop-down menu, select the option "Create new comparison" and a "Comparison" window will appear

4.10.2.3 Select "*VNTR_cmp"* from the bottom of the window in BioNumerics versions lower than 5.0 or from the top left "Experiments" window in BioNumerics 5.0.

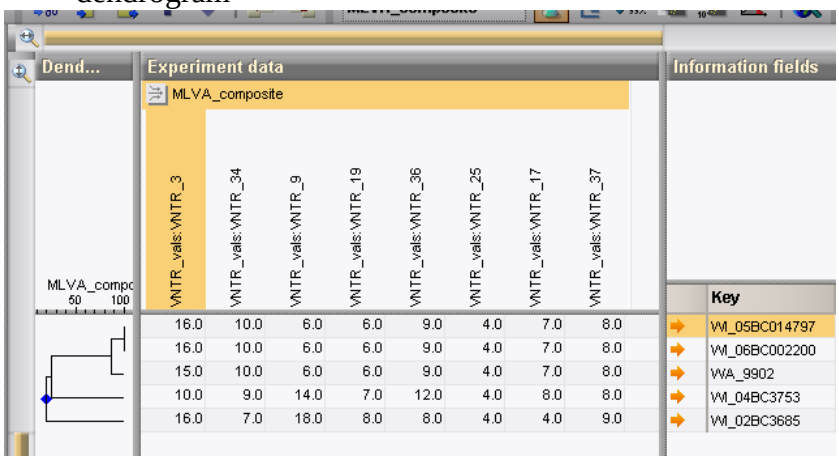
4.10.2.4 From the "Clustering" drop-down menu, select the option "Calculate…Cluster analysis (similarity matrix)" and a "Composite data set comparison" dialog box will appear

4.10.2.5 Select "Categorical" for "Multi-state coefficient" and "UPGMA" for "Dendrogram type"

4.10.2.6 Click on "OK" button to finish the calculations and the "Comparison" window with the dendrogram will reappear

4.10.2.7 From the "Layout" drop-down menu, select the option "Show image"

4.10.2.8 From the "Composite" drop-down menu, select the option "Show quantification (values)" and the copy numbers will appear next to the dendrogram



4.10.3 In order to create a minimum spanning tree:

4.10.3.1 Select the isolates to be included in the spanning tree

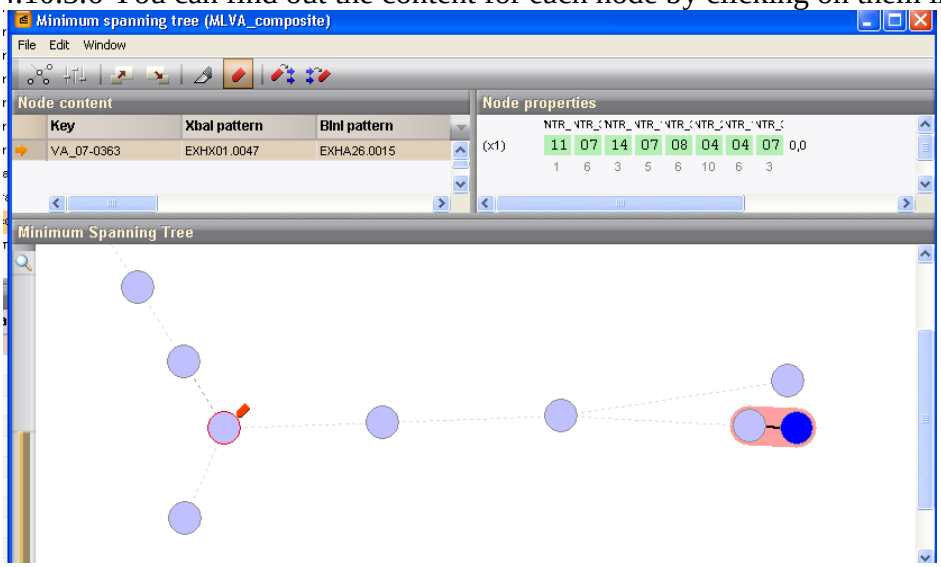
4.10.3.2 From the "Comparison" drop-down menu, select the option "Create new comparison" and a "Comparison" window will appear

4.10.3.3 Select "*VNTR_cmp"* from the bottom of the window in BioNumerics versions lower than 5.0 or from the top left "Experiments" window in BioNumerics 5.0.

4.10.3.4 From the "Clustering" drop-down menu, select the option "Calculate…Minimum spanning tree (population modeling)" and a "Minimum spanning tree" dialog box will appear

4.10.3.5 Make sure the default "Manhattan" is checked for "Coefficient" and click "OK" and the "Minimum spanning tree" window with the tree will appear.

4.10.3.6 You can find out the content for each node by clicking on them individually.



5. FLOW CHART:

6. BIBLIOGRAPHY:

7. CONTACTS:

7.1. Eija Trees, D.V.M., Ph.D.
PulseNet Next Generation Subtyping Unit, EDLB, DFWED, CDC
(404) 639-3672
EHyytia-Trees@cdc.gov

7.2. Patti Lafon
PulseNet Next Generation Subtyping Unit, EDLB, DFWED, CDC
(404) 639-2828
PLafon@cdc.gov

7.3. Ashley Sabol
PulseNet Next Generation Subtyping Unit, EDLB, DFWED, CDC
(404) 639-2947
ASabol@cdc.gov

8. AMENDMENTS:

1/27/2009: the allele (= copy number) assignment will be based on a look-up table listing the actual observed fragment size ranges instead of using the mathematical formula that assigns alleles based on predicted fragment sizes. The look-up table approach in allele assignment will facilitate the comparison of fragment size data from the Beckman Coulter CEQ 8000/8800/GeXP and the Applied Biosystems Genetic Analyzer 3130xl in the same database.
4/8/2013: appendix PND14-2 (BioNumerics specifications for the STEC O157 VNTR loci) was added.
4/8/2013: Additions to step 4.6.5: more clarification for reasons for error messages that occur upon allele assignment and guidance for further follow-up for these errors.
2/26/2014: Merged the SOPs for STEC O157 (PND14), *Salmonella* serotypes Typhimurium (PND15) and Enteritidis (PND19) Analysis for Beckman Coulter CEQ8000/8800/GeXP Data into one protocol.

Appendix PND14-1a
STEC O157 VNTR Allele List and Corresponding Observed Fragment Sizes in the Beckman Coulter CEQ 8000/8800/GeXP (*BeckmanEcoli* look-up table)

Note: This is posted on the PulseNet SharePoint site under the QA/QC manual as a TXT-file so users may save it locally and use it with the BioNumerics MLVA scripts. Every time the table is updated, the latest version is posted on SharePoint site.

Count	VNTR_3	VNTR_34	VNTR_9	VNTR_25	VNTR_17	VNTR_19	VNTR_36	VNTR_37
1			471-472	116-119				
2	333-334			122-124	130-133	282-284		
3	339-340	153-154	482-484	128-131	137-140	291-292	123-125	159-161
4	345-346	170-172	488-490	134-136	143-146	296-299	130-132	164-166
5	350-353	188-190	494-497	141-143	149-152	302-304	136-139	170-173
6	356-359	206-209	499-503	146-148	156-159	307-311	143-146	176-179
7	362-365	224-227	506-509	153-154	162-165	314-316	150-153	181-185
8	368-371	241-245	512-515	158-160	169-172	320-322	158-161	187-191
9	373-377	260-262	518-521	165-166	175-178	325-328	165-168	194-197
10	380-383	278-280	524-528	170-171	182-184	332-334	172-175	200-203
11	386-389	295-298	530-534		188-190	337-339	179-182	207-209
12	392-396	313-314	536-540		195-197	343-345	186-189	213-215
13	398-401		543-546	188-190	199-201	349-351	193-196	219-221
14	404-407		549-552			356-357	200-202	225-227
15	410-413		555-558		208-209	361-363	207-210	231-233
16	417-419		561-564				214-216	237-238
17	423-425		566-570				222-223	241-244
18	429-431		572-576		227-228		228-230	249-251
19	435-437		579-582				235-237	255-256
20	442-444		584-587				242-243	261-262
21	448-449		590-594		246-247		250-255	
22	454-456		597-599					273-274
23	460-464		603-605					
24	466-468		608-611					
25	473-474		614-615					
26	476-477							

Appendix PND14-1b

S. enterica serotype Typhimurium VNTR Allele List and Corresponding Observed Fragment Sizes in the Beckman Coulter CEQ 8000/8800/GeXP (*BeckmanST* look-up table)

Note: This is posted on the PulseNet SharePoint site under the QA/QC manual as a TXT-file so users may save it locally and use it with the BioNumerics MLVA scripts. Every time the table is updated, the latest version is posted on the SharePoint site.

Count	ST3	ST5	ST7	ST10	ST2	ST6	ST8
0			113-115				
1			124-126			202-203	
2	164-171	146-149	130-136	322-323		207-209	
3		151-153	139-145			213-215	
4	174-182	162-164	151-154	335-336		219-221	
5	187-191	168-170	162-164	341-343		225-227	
6		172-176	172-173	347-349		231-233	
7		179-182	178-183	354-357		234-239	
8		186-188	187-191	360-362	167-168	242-245	
9		191-194	200-201	366-369		247-250	294-296
10		196-199	209-210	373-375		254-257	
11		203-205		379-381	207-209	259-263	326-328
12		208-211	218-219			264-268	
13		214-217	227-229	385-388		272-274	
14		220-223	232-237	392-394	248-252	278-280	
15		226-228	246-247	398-400		284-287	359-360
16		232-234	255-256	404-406	264-266	290-292	373-375
17		238-239	264-265	411-413	279-281	296-298	384-385
18		243-245	273-274	417-419	289-292	302-303	
19		249-251	282-284	424-426	312-316	305-310	399-40
20		255-257	291-293	430-433	319-326	314-315	417-419
21		260-262	301-302	437-439	341-343	320-322	425-426
22		266-268		443-446	351-357	325-327	432-435
23		272-274	319-322	450-452	358-363	331-333	448-452
24		278-279		456-459	365-369	336-339	460-464
25		284-286		463-465	387-392	343-344	471-475
26		290-292			397-407	349-350	485-488
27				470-472		355-357	493-497
28				476-479	425-427	361-362	501-508
29		312-314		483-485	433-438	367-368	512-513
30				489-492		373-374	526-531
31				496-498			532-537
32				503-504		385-386	547-552

33	509-511	553-558
34	516-518	560-565
35	523-526	580-584
36	530-532	587-592
37		599-609
38		610-618
39		622-624
40	551-552	631-636

Appendix PND14-1c

S. enterica serotype Enteritidis VNTR Allele List and Corresponding Observed Fragment Sizes in the Beckman Coulter CEQ 8000/8800/GeXP (*BeckmanSE* look-up table)

Note: This is posted on the PulseNet SharePoint site under QA/QC manual as a TXT-file so users may save it locally and use it with the BioNumerics MLVA scripts. Every time the table is updated, a latest version of the look-up table is posted on the SharePoint.

Count	SE1	SE2	SE8	SE6	SE9	SE3	SE5
1			344-347		175-176		162-164
2	166-168		433-435		183-186		
3	172-174	295-296			192-195	197-201	171-172
4	179-182	299-303			199-200	209-214	177-179
5	185-189	305-309				224-225	183-185
6	193-196	312-316					189-191
7	200-202	318-322					195-197
8	207-209	325-329		410-413		258-259	201-203
9	214-217	332-335		443-445			207-209
10	222-223	338-342		476-481		280-282	213-215
11	229-230	344-349					219-221
12	236-237	353-354		534-535			225-227
13	244-245	360-365		569-570			231-233
14				593-594			237-239
15	256-257	372-374					243-245
16							249-250
17	270-271						255-257
18							260-263
19							266-268
20							272-274
21							
22							284-285
23							
24	317-318						
24							
25							
26							
27							
28							
29							
30	361-362						

Appendix PND14-2a
BioNumerics specifications for STEC O157 VNTR loci

Reaction	R1	R2
Locus	VNTR_3	VNTR_17
Dye	D2	D2
Offset	321	120
Repeat length	6	6
Copy range	0-25	0-30
Tolerance	2	2
Fragment size in EDL933	373-377	156-159
Copy number in EDL933	9	6
Locus	VNTR_34	VNTR_19
Dye	Quas 670 (D4)	Quas 670 (D4)
Offset	100	272
Repeat length	18	6
Copy range	0-20	0-25
Tolerance	3	2
Fragment size in EDL933	278-280	307-311
Copy number in EDL933	10	6
Locus	VNTR_9	VNTR_36
Dye	Quas 670 (D4)	Quas 670 (D4)
Offset	465	102
Repeat length	6	7
Copy range	0-50	0-20
Tolerance	2	2
Fragment size in EDL933	530-534	158-161
Copy number in EDL933	11	8
Locus	VNTR_25	VNTR_37
Dye	Quas 705 (D3)	Quas 705 (D3)
Offset	110	142
Repeat length	6	6
Copy range	0-20	0-25
Tolerance	2	2
Fragment size in EDL933	134-136	187-191
Copy number in EDL933	4	8

Fragment size ranges based on independent runs on multiple instruments at CDC and PulseNet Participating Laboratories

Appendix PND14-2b
BioNumerics specifications for *S. enterica* serotype Typhimurium VNTR loci

Reaction	**R1**	**R2**
Locus	**ST3**	**ST2**
Dye	Quas705 (D3)	Quas670 (D4)
Offset	156	62
Repeat length	6*	13**
Copy range	0-20	0-40
Tolerance	2	3
Fragment size in LT2	174-182	358-363
Copy number in LT2	4*	23**
Locus	**ST5**	**ST6**
Dye	Quas670 (D4)	D2
Offset	139	191
Repeat length	6	6
Copy range	0-60	0-50
Tolerance	2	2
Fragment size in LT2	220-223	264-268
Copy number in LT2	14	12
Locus	**ST7**	**ST8**
Dye	D2	Quas705 (D3)
Offset	115	196
Repeat length	9	11***
Copy range	0-30	0-50
Tolerance	3	3
Fragment size in LT2	151-154	553-558
Copy number in LT2	4	33***
Locus	**ST10**	
Dye	Quas705 (D3)	
Offset	311	
Repeat length	6	
Copy range	0-50	
Tolerance	2	
Fragment size in LT2	373-375	
Copy number in LT2	10	

* A half of a repeat difference considered significant. Actual repeat length 12 bp and actual copy number in LT2 2.5
** A third of a repeat difference considered significant. Actual repeat length 39 bp and actual copy number in LT2 8.0
*** A third of a repeat difference considered significant. Actual repeat length 33 bp and actual copy number in LT2 11.0

Fragment size ranges are based on independent runs on multiple instruments at CDC and in PulseNet Participating Laboratories.

Appendix PND14-2c

BioNumerics specifications for *S. enterica* serotype Enteritidis VNTR loci

Reaction	**R1**	**R2**
Locus	**SE1**	**SE9**
Dye	Quas670 (D4)	Quas670 (D4)
Offset	152	166
Repeat length	7	9
Copy range	0-20	0-5
Tolerance	2	2
Fragment size in K1891	193-196	183-186
Copy number in K1891	6	2
Locus	**SE2**	**SE3**
Dye	D2	D2
Offset	271	165
Repeat length	7	12
Copy range	0-20	0-10
Tolerance	2	3
Fragment size in K1891	325-329	209-214
Copy number in K1891	8	4
Locus	**SE8**	**SE5**
Dye	Quas705 (D3)	Quas705 (D3)
Offset	260	155
Repeat length	86	6
Copy range	0-3	0-25
Tolerance	4	2
Fragment size in K1891	433-435	201-203
Copy number in K1891	2	8
Locus	**SE6**	
Dye	Quas670 (D4)	
Offset	148	
Repeat length	33	
Copy range	0-15	
Tolerance	3	
Fragment size in K1891	476-481	
Copy number in K1891	10	

Fragment size ranges are based on independent runs on multiple instruments at CDC and in PulseNet Participating Laboratories

1. **PURPOSE:** To describe the standardized protocol for analysis of MLVA data of Shiga toxin-producing *Escherichia coli* O157 (STEC O157) and *Salmonella enterica* serotypes Typhimurium and Enteritidis in BioNumerics.

2. **SCOPE**: To provide the PulseNet participants with a single protocol for analyzing MLVA data of STEC O157 and *Salmonella enterica* serotypes Typhimurium and Enteritidis, thus ensuring inter-laboratory comparability of the generated results.

3. DEFINITIONS:

3.1 **MLVA:** Multiple-locus variable-number tandem repeat analysis
3.2 **VNTR:** Variable-number tandem repeat
3.3 **CDC:** Centers for Disease Control and Prevention
3.4 **SOP:** Standard Operating Procedure

4. RESPONSIBILITIES/PROCEDURE

4.1 **Software needed for data analysis**

4.1.1 BioNumerics version 4.5 or higher

4.1.2 Customized scripts for data import (*VNTRImport_v4.bns*), copy number calculation (= allele assignment) (*VNTRCalc_v4.bns*), troubleshooting (*VNTRReport.bns*) and support functions (*VNTRDetails_v2.bns*).

4.1.3 Organism specific assay look-up tables (*ABIEcoli.txt, ABIST.txt, ABISE.txt*) for allele size ranges

4.2 **General overview**

4.2.1 The import process consists of three major steps:

4.2.1.1 Exporting the appropriate data file from the Genetic Analyzer system (see laboratory protocols PNL23 and PNL28 step 4.10.1.15).

4.2.1.2 Importing this file into BioNumerics (using the script *VNTRImport_v4.bns*).

4.2.1.3 Determining the copy numbers (assigning alleles) for each VNTR (using the script *VNTRCalc_v4.bns* and the organism specific look-up table *ABIEcoli.txt, ABIST.txt, or ABISE.txt*).

4.2.1.3.1 *VNTRCalc_v4.bns* script facilitates the allele assignment in two different ways:

4.2.1.3.1.1 "ABIEcoli, ABIST, and ABISE" – assign the copy number based on the look-up tables (PND16-1a-c, respectively).

4.2.1.3.1.2 "Predicted" – calculates the copy number based on the mathematical formula: (Observed fragment size – offset)/repeat size (not an option for the ABI data)

4.3 **Required import format**

4.3.1 The data should be exported from the GeneMapper software as a txt file (tab-delimited text file) containing the fragment length information. The following columns should appear in the exported table in the following order from left to right: "Dye/Sample peak", "Sample File Name", "Marker", "Allele", "Size", "Height", "Area", "Data point". The field "Sample File Name" should contain information designating the BioNumerics key number (isolate identifier) loaded in each lane of the sequencer, as well as which VNTR mastermix (R1 or R2) was loaded. The import script will ignore any text that appears beyond the dot "." in the "Sample File Name" field.

4.3.1.1 **NOTE:** no spaces or underscores are allowed between the strain ID and the reaction ID (R1 or R2) or between the reaction ID and the dot "." in the RN field.

4.4 **Setting up a new database**

4.4.1 Click "Create New Database" icon to set up a new database.

4.4.2 Type in the name (for example "O157MLVA" for STEC O157, "STMLVA" for *Salmonella* Typhimurium or "SEMLVA" for *Salmonella* Enteritidis) of your new database and click "Next".

4.4.3 Select the directory (default on CDC training laptops is [HOMEDIR]\O157MLVA for STEC O157; this will save the data on the C-drive) in which you want to set up the new database. Choose the default "Yes (recommended)" when asked "Do you want to automatically create the required directories?" and then click "Next".

4.4.4 Choose "Yes" to enable creation of log files and click "Finish".

4.4.5 In the appearing "Set up new database" pop-up window, choose "Local database (single user only)" and click "Proceed".

4.4.6 Click "Yes" in the next pop-up confirmation window ("Choosing 'local database' will restrict some functionality of the software. Are you sure you want to continue?") and "Proceed" in the following "Plug-in" pop-up window.

4.4.7 Close the newly created database.

4.4.8 Go to the directory in which you placed your database at step 4.4.3 (on CDC training laptops: C:\Program Files\BioNumerics\Data), open folder for organism specific assay (for example, "O157MLVA" for STEC O157) and create two subfolders named "Scripts" and "VNTRtables".

4.4.9 Save the four MLVA specific scripts (*VNTRImport_v4.bns*, *VNTRCalc_v4.bns, VNTRReport.bns, VNTRDetails_v2.bns*) in the "Scripts"

folder and the organism specific assay look-up table (e.g. *ABIEcoli.txt*) in the "VNTRtables" folder.

4.4.10 Open the database. The scripts (except the VNTRDetails script) should now appear under the "Scripts" drop-down menu.

4.5 **Importing a peak file for the first time**

4.5.1 Review the peak file in Excel: make sure that the ROX labelled molecular size standard ran properly without skipping any peaks. Remove any data (failed reactions, controls, internal ladder) that you don't want to import in the BioNumerics from the .txt file. Re-name and re-save the modified .txt file with correct naming format either on your hard drive or on a flash drive. For example, a modified file of GA140120 would be saved as GA140120-mod.

4.5.2 In BioNumerics, run the script *VNTRImport_v4* from the "Scripts" drop-down menu. The "Import VNTR peak data" dialog box will appear. From the "Peak file format" drop-down menu, select "ABI GeneMapper peak" file. The script pops up a file dialog box, prompting for the name of the file to be imported. Select the appropriate file and click "OK". A second dialog box pops up, prompting for a variety of other information:

4.5.2.1 *Fingerprint file name*. This is the name of the fingerprint file that will be used in BioNumerics. Leave this unchanged, unless a file has already been imported with the same name. In this case, change the file name into a new, unique and informative name.

4.5.2.2 *Dyes to import*. The dye R (Rox) contains only reference markers and does not need to be imported. Use this list to select what dyes will be imported; they are B (FAM), G (HEX) and Y (Calred 590). Each dye will be stored in a different file, resulting from appending the dye name to the fingerprint file name.

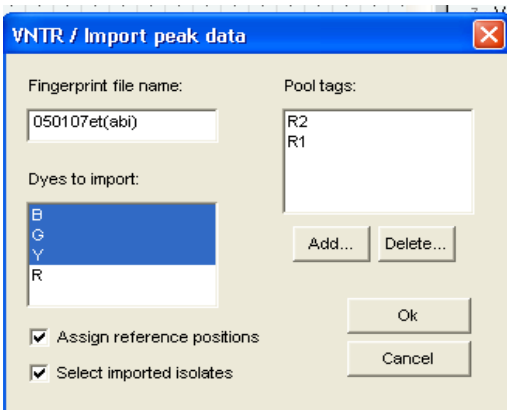
4.5.2.3 *Assign reference positions*. Leave this option checked.

4.5.2.4 *Select imported isolates*. Leave this option checked.

4.5.2.5 *Pool tags*. For this protocol, there are two pool tags representing the two PCR reactions for each isolate (each loaded in different wells) and tagged with names "R1" and "R2". The two tags should appear in the list. If they do not, they can be added by selecting "add" to update.

4.5.2.6 If everything is filled in appropriately, click "OK" to start the import.

4.5.2.7 **NOTE:** These settings are automatically saved and reloaded the next time this script is run.



4.5.3 When the script is finished, it has:

4.5.3.1 Created a fingerprint type "*VNTRFpr*" with appropriate settings.

4.5.3.2 Created fingerprint types with appropriate settings for each dye and each pool used. The names of these fingerprint types are "VNTRFpr"+"pool name"+"dye" (for example: VNTRFprR1_Y).

4.5.3.3 Created fingerprint files for each dye in the fingerprint type "*VNTRFpr*", and imported the band information in these lanes. The individual lanes are assigned to their appropriate pool fingerprint type, according to the known pool tags found in the "Sample File Name" field.

4.5.3.4 Created new database entries for all isolates found.

4.5.3.5 Linked the fingerprints to their corresponding entries.

4.5.3.6 Selected all isolates for which fingerprint data was imported.

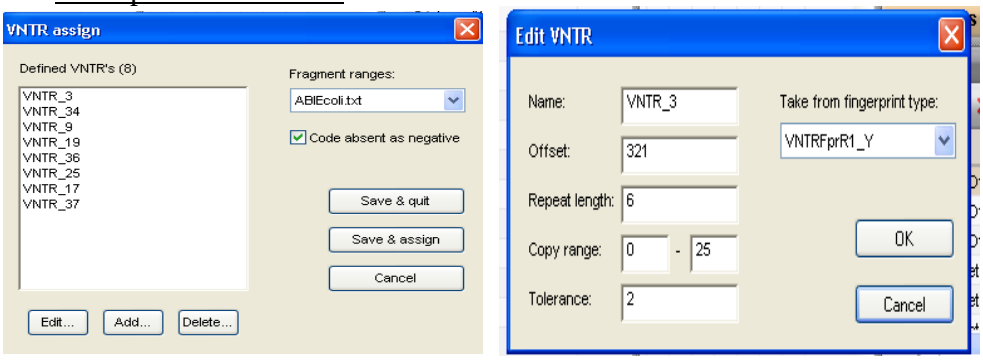
4.6 Determining the copy numbers for the first time (allele assignment)

4.6.1 Run the script *VNTRCalc_v4* from the scripts menu. The first time this script is run, it will automatically create a new character set called "*VNTR_vals*". This character set will hold the copy numbers for each VNTR. The script will also create a second character set called "*VNTR_frags*" which holds the fragment size with the highest fluorescence for each VNTR. Click "OK" on the two pop up windows notifying that these two character sets will be created.

4.6.2 Before the script can determine copy numbers from the fragment length information, it should have sufficient information about what VNTRs can be found in which pool and dye, what are the repeat lengths, etc. To this end, the script pops up a dialog box called "VNTR assign". This dialog box contains a list of defined VNTRs that is initially empty. To enter the parameters of the first VNTR, click on "Add". This brings up a second dialog box, prompting for all properties of this first VNTR. Refer to

Appendices PND16-2a-c for all necessary information to load the specifications for each organism specificVNTR.

Example: STEC O157



4.6.3 The VNTR information includes:

4.6.3.1 *Name*. This name will be used for further reference and for the character name in the character type *VNTR_vals*. Each VNTR should have a unique name (for example VNTR_3, VNTR_34, etc).

4.6.3.2 *Offset*. Each fragment consists of a repeat portion and a constant portion, due to the fact that the primers do not locate exactly at the start and the end of the repeat region. This parameter specifies the size (in base pairs) of the constant portion.

4.6.3.3 *Repeat length*. This parameter specifies the size of the repeat block unit (in base pairs).

4.6.3.4 *Copy range*. These parameters specify the minimum and maximum number of copies that the script will consider during the copy number determination.

4.6.3.5 *Tolerance*. This specifies the maximum difference between the expected fragment length (calculated by the software) and the actual length estimated by the sequencer.

4.6.3.6 *Take from fingerprint type*. This drop-down list should be used to indicate on what fingerprint type this VNTR was run. The fingerprint type is determined by the dye and the pool tag.

4.6.4 If all parameters are filled in appropriately, click OK to add the VNTR and return to the previous dialog box ("VNTR assign"). Repeat the same actions to enter the information for all VNTRs used for the organism specific database. When all VNTRs are entered, make sure the box for "Code absent as negative" is checked. Select appropriate assay specific look-up table (For example, *ABIEcoli*" for STEC O157) from the "Fragment ranges" drop-down menu and press the "Save&Assign" button to let the script assign copy numbers for the currently selected entries.

4.6.4.1 **NOTE 1:** Pressing "Save&Quit" would store the information without assigning copy numbers for the current selection.

4.6.4.2 **NOTE 2**: All information regarding the VNTRs is stored with the database. The next time the script is run, the VNTR definitions will be loaded automatically.

4.6.4.3 For every fragment with the highest fluorescence level in the fingerprint type (= the combination of the reaction and the dye), the script will assign an allele type (copy number) based on the fragment size ranges specified in the appropriate look-up table (appendices PND16-1a-c). Note that, within the same fingerprint type, more than one VNTR can be loaded if there is no overlap within the fragment size ranges.

4.6.5 When the script has completed, all copy numbers for all VNTRs for the currently selected entries are determined.

4.6.5.1 **NOTE:** Two types of problems may arise during the process (if one or more such errors were encountered during the calculations, an error report is displayed listing all the problems):

4.6.5.1.1 *None of the peaks present in a fingerprint are compatible with the fragment size ranges in the look-up table.* In this case, the corresponding character value will be scored "-2.0". In this situation, run the "*VNTRReport*" script from the "Scripts" drop-down menu to verify the reason for the problem:

4.6.5.1.1.1 No amplification (a null allele): mutations, insertions or deletions in the primer annealing region, or locus located on a plasmid that was lost. PCR needs to be repeated only if the null allele occurred in a locus in which null alleles have not been previously detected (For STEC O157: VNTR34, VNTR25, VNTR17; and in the case of VNTR19 for which null alleles are extremely rare, and hence should always be confirmed. For *Salmonella* serotype Typhimurium: ST2, ST3, ST6, ST7 and in the case of ST8 for which null alleles are extremely rare, and hence should always be confirmed. For *Salmonella* serotype Enteritidis: VNTR5, VNTR8, VNTR9; and in the case of SE6 for which null alleles are extremely rare and hence always need to be confirmed).

4.6.5.1.1.2 Fragment size outside the acceptable range (either slightly outside of range for a previously detected allele or a possible new allele). Rerun the fragment analysis reaction to confirm the accuracy and reproducibility of the sizing. The isolate should be submitted to CDC for

confirmation only if a possible new allele is detected. If the sizing is reproducibly slightly outside the range for an existing allele, the CDC database managers will adjust the look-up table based on the evidence the submitting laboratory provides.

4.6.5.1.2 *More than one peak in a fingerprint is compatible with an acceptable fragment size range.* In this case, the script will use the solution that corresponds to the peak with the largest "peak height" value. Possible causes for multiple peaks:

4.6.5.1.2.1 Primer stutter: multiple peaks with sizes 1-2 bp from each other. No further action required.

4.6.5.1.2.2 Double alleles: two peaks differing by one or more full repeats from each other. Further actions required for all other loci except *Salmonella* serotype Enteritidis locus SE8 (in a subset of strains double alleles are known to occur and the major (larger) allele always has the highest fluorescence):

4.6.5.1.2.2.1 Repeat the PCR with a freshly made template. If two peaks still observed and the same peak consistently has the highest fluorescence intensity, report the predominant peak only. If the same peak does not consistently have the highest fluorescence intensity, proceed to the next step.

4.6.5.1.2.2.2 Test ten single colony picks from the culture. Report the peak that has the highest fluorescence intensity in the majority of the picks.

4.7 Analyzing a peak file on a routine basis

4.7.1 Review the peak file: make sure that the ROX labelled molecular size standard ran properly without skipping any peaks. Remove any data (failed reactions, controls, internal ladder) that you don't want to import in the BioNumerics from the txt file. Re-name and re-save the txt file either on your hard drive or on the flash drive.

4.7.2 In BioNumerics, run the script *VNTRImport_v4* from the "Scripts" drop-down menu. The "Import VNTR peak data" dialog box will appear. From the "Peak file format" drop-down menu, select "ABI GeneMapper peak file". The script pops up a file dialog box, prompting for the name of the file to be imported. Select the appropriate file and click "OK". "VNTR/Import peak data" dialog box will appear. De-select "R" from the "Dyes to import" and click "OK".

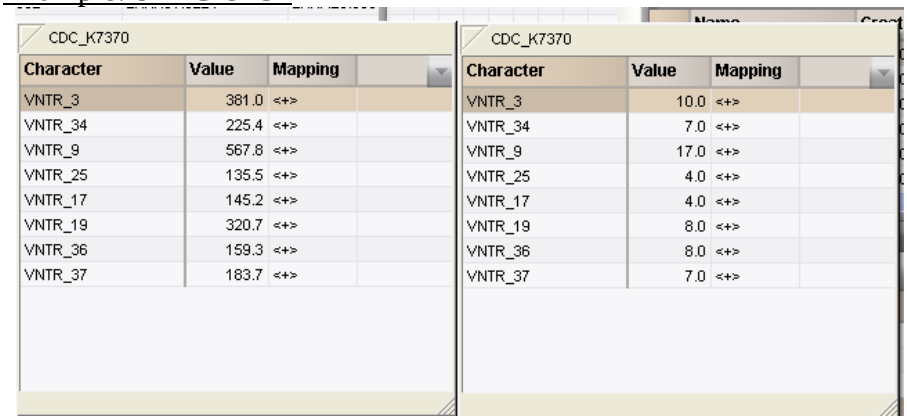
4.7.3 Run the script *VNTRCalc_v4* from the scripts menu. "VNTR assign" dialog box will appear. Select appropriate look-up table (For example, "*ABIEcoli*"

for STECO157) from the "Fragment ranges" drop-down menu and click "Save&Assign" to assign allele numbers.

4.8 Verifying the allele assignment

4.8.1 For each isolate in the database that has some VNTR data associated, you can click on the "VNTR frags" and "VNTR vals" entries in the list to open the "Entry edit" windows.

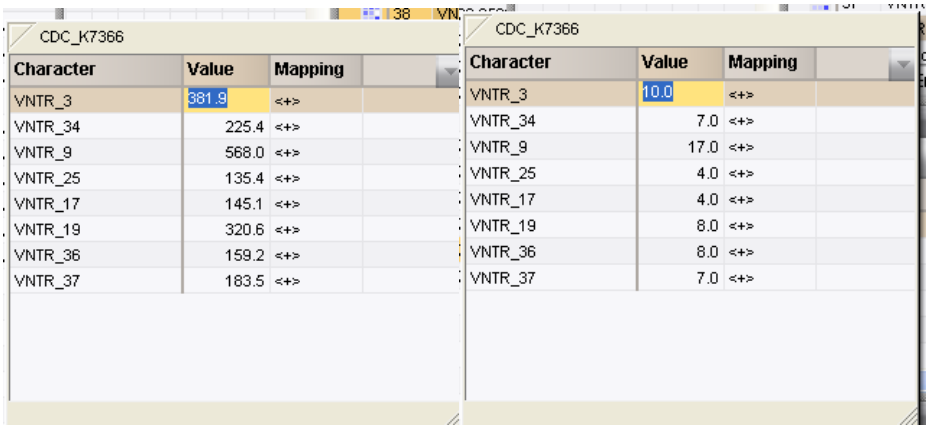
Example: STEC O157



4.8.2 If the *VNTRCalc_v4* script did not detect a fragment for a VNTR, refer to the step 4.6.5.1.1 of this protocol for details on how to proceed. If the fragment size is slightly outside the range specified in the look-up table it is possible to manually assign a temporary allele size and type for that VNTR until an official confirmation has been performed by CDC.

4.8.2.1 **Note:** only the CDC database managers are allowed to modify the look-up table *.txt* files. Once a modification has been made, the modified file will be posted on the PulseNet SharePoint site under the QA/QC manual. An automatic e-mail notification will be sent about the change in the SOP**.**

4.8.2.2 To manually assign an allele size and type, click on the fragment size in the "VNTRfrags" entry edit window or the allele type in the "VNTR vals" entry edit window. This will open the "Change character value" window in BioNumerics lower than v5.0. In BioNumerics v5.0 the allele size or type can be directly highlighted and changed in the entry edit window (STEC O157 screen shots below).



4.9 Creating a composite data set to visualize the allele types

4.9.1 In order to display copy numbers next to a dendrogram in a comparison, first create a "*composite data set*" that holds the VNTR data.

4.9.1.1 **NOTE:** The created composite dataset will be automatically saved in the database, and hence only need to be created once before the first analysis in the database.

4.9.1.2 From the "Experiments" drop-down menu, select the option "Create new composite data set…", enter a name (e.g. *VNTR_cmp*), and click the "OK" button. The "Composite data set 'VNTR_cmp'" window will appear.

4.9.1.3 Highlight the experiment *VNTR_vals* and from the "Experiment" drop-down menu, select the option "Use in composite data set". Close the window.

4.9.2 Next time a comparison window is opened (see below step 4.10), there will be a new experiment *VNTR_cmp* listed in the bottom of the window (BioNumerics versions lower than 5.0) or in the top left corner of the window (BioNumerics version 5.0). This experiment will facilitate the display of a spreadsheet-like view of the copy numbers (note that it may be necessary to scroll the experiment list to the right with the arrow button to bring *VNTR_cmp* in display in BioNumerics versions lower than 5.0). This can be shown next to a dendrogram analysis of the data set.

4.10 Performing comparisons based on the VNTR data

4.10.1. The VNTR data contained in the character set *VNTR_vals* can be analysed in BioNumerics with all the tools that are available for character data. That includes cluster analysis with a variety of methods and similarity coefficients. For VNTR data, the coefficients that make most sense are:

4.10.1.1 *Categorical*: preferred if differences in copy numbers should be treated in a qualitative way. This is the only option for creating dendrograms using MLVA data.

4.10.1.2 *Manhattan*: preferred if differences in copy numbers should be treated in a quantitative way (a larger difference means more distantly related organisms). This coefficient can be used to construct minimum spanning trees.

4.10.2 In order to create a dendrogram:

4.10.2.1 Select the isolates to be included in the dendrogram.

4.10.2.2 From the "Comparison" drop-down menu, select the option "Create new comparison" and a "Comparison" window will appear.

4.10.2.3 Select "*VNTR_cmp"* from the bottom of the window in BioNumerics versions lower than 5.0 or from the top left "Experiments" window in BioNumerics 5.0.

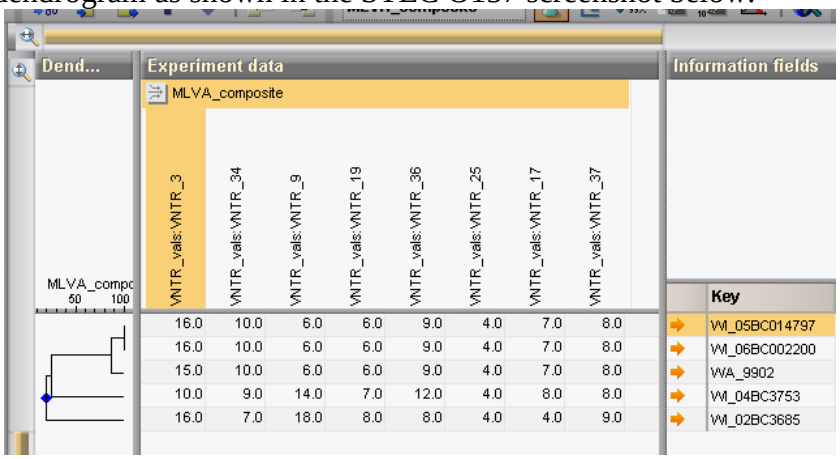
4.10.2.4 From the "Clustering" drop-down menu, select the option "Calculate…Cluster analysis (similarity matrix)" and a "Composite data set comparison" dialog box will appear.

4.10.2.5 Select "Categorical" for "Multi-state coefficient" and "UPGMA" for "Dendrogram type".

4.10.2.6 Click on "OK" button to finish the calculations and the "Comparison" window with the dendrogram will reappear.

4.10.2.7 From the "Layout" drop-down menu, select the option "Show image".

4.10.2.8 From the "Composite" drop-down menu, select the option "Show quantification (values)" and the copy numbers will appear next to the dendrogram as shown in the STEC O157 screenshot below.



4.10.3 In order to create a minimum spanning tree:

4.10.3.1 Select the isolates to be included in the spanning tree.

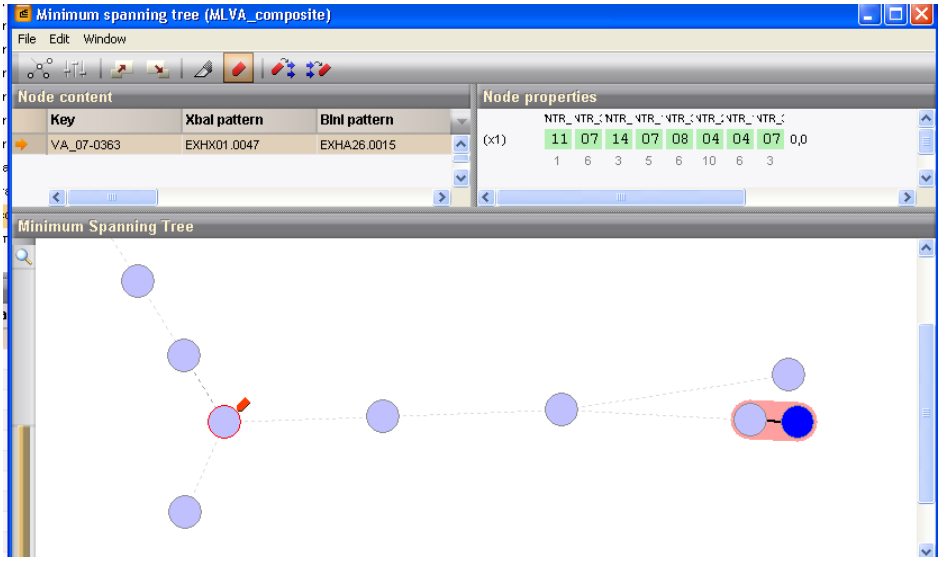
4.10.3.2 From the "Comparison" drop-down menu, select the option "Create new comparison" and a "Comparison" window will appear.

4.10.3.3 Select "*VNTR_cmp"* from the bottom of the window in BioNumerics versions lower than 5.0 or from the top left "Experiments" window in BioNumerics 5.0.

4.10.3.4 From the "Clustering" drop-down menu, select the option "Calculate…Minimum spanning tree (population modeling)" and a "Minimum spanning tree" dialog box will appear.

4.10.3.5 Make sure the default "Manhattan" is checked for "Coefficient" and click "OK" and the "Minimum spanning tree" window with the tree will appear.

4.10.3.6 You can find out the content for each node by clicking on them individually.



5. FLOW CHART:

6. REFERENCES:

6.1 Hyytia-Trees, E., Lafon, P., Vauterin, P., and Ribot, E. (2010) Multi-laboratory validation study of standardized multiple-locus VNTR analysis (MLVA) protocol for Shiga toxin-producing E*scherichia coli* O157 (STEC O157): a novel approach to normalize fragment size data between capillary electrophoresis platforms. Foodborne Path. Dis. 7, 129-136.

7. CONTACTS:

7.1 Eija Trees, D.V.M., Ph.D.
PulseNet Next Generation Subtyping Methods Unit, EDLB, CDC
(404) 639-3672
EHyytia-Trees@cdc.gov

7.2 Patti Lafon
PulseNet Next Generation Subtyping Methods Unit, EDLB, CDC
(404) 639-2828
PLafon@cdc.gov

7.3 Ashley Sabol
PulseNet Next Generation Subtyping Methods Unit, EDLB, CDC
(404) 639-2947
ASabol@cdc.gov

8. AMENDMENTS:

4/9/2013: appendix PND16-2 (BioNumerics specifications for the STEC O157 VNTR loci) was added.
4/9/2013: Additions to step 4.6.5: more clarification for reasons for error messages that occur upon allele assignment and guidance for further follow-up for these errors.
2/26/2014: Merged the SOPs for STEC O157 (PND16), *Salmonella* serotypes Typhimurium (PND17) and Enteritidis (PND18) Analysis for Applied Biosystems Genetic Analyzer 3130/3500 Data into one protocol.

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Appendix PND16-1a

STEC O157 VNTR allele list and corresponding observed fragment size ranges in the Applied Biosystems Genetic Analyzer 3130/3500 (*ABIEcoli* look-up table)

Note: This table is posted on the PulseNet SharePoint under the QA/QC manual as a TXT-file so users may save it locally and use it with the BioNumerics MLVA scripts. Every time the table is updated, the latest version is posted on SharePoint site.

	R1				R2			
Count	VNTR_3	VNTR_34	VNTR_9	VNTR_25	VNTR_17	VNTR_19	VNTR_36	VNTR_37
1			474-475	122-124				
2	339-340			127-128	135-136	283-284		
3	343-346		485-486	132-134	140-142	291-292	123-124	157-158
4	349-352	169-171	491-492	138-140	146-148	296-298	129-131	163-165
5	355-358	187-188	495-498	144-146	153-155	302-304	135-137	169-171
6	361-364	205-206	502-504	150-152	159-161	308-310	141-144	175-176
7	367-370	222-224	508-510	156-158	165-167	314-316	150-151	181-183
8	373-376	242-244	514-516	162-164	172-173	320-322	157-158	187-189
9	379-382	260-262	518-522	168-169	177-179	326-328	163-165	192-194
10	385-388	278-280	525-528	174-175	184-185	332-334	170-172	198-200
11	391-394	295-298	530-534		190-191	338-340	177-179	205-206
12	397-401	315-316	536-540		196-197	344-346	184-185	210-212
13	404-406		543-545	191-192	201-203	350-351	191-192	217-218
14	409-412		548-551			356-357	197-199	223-224
15	415-418		554-557		208-209	362-363	204-206	229-231
16	421-426		559-563				211-213	236-237
17	428-430		565-569				218-219	240-244
18	433-435		572-575		225-228		225-226	249-250
19	440-442		578-581				233-234	255-256
20	447-448		584-586				240-242	262-263
21	453-455		590-591		247-248			
22	459-461		596-597					273-274
23	466-467		601-603					
24	472-473		607-608					
25	478-479		613-614					

Appendix PND16-1b

S. enterica serotype Typhimurium VNTR allele list and corresponding observed fragment size ranges in the Applied Biosystems Genetic Analyzer 3130/3500 (*ABIST* look-up table)

Note: This table is posted on the PulseNet SharePoint under the QA/QC manual as a TXT-file so users may save it locally and use it with the BioNumerics MLVA scripts. Every time the table is updated, the latest version is posted on SharePoint site.

	R1				R2		
Count	ST3	ST5	ST7	ST10	ST2	ST6	ST8
0			121-122				
1			128-130			205-206	
2	177-180	146-147	137-139	322-323			
3		149-153	147-151			216-217	
4	188-192	160-164	157-158	340-341		222-226	
5	200-202	166-168	166-167	345-347		229-231	
6		172-174	175-176	351-354		235-236	
7		178-179	184-185	358-360		241-243	
8		184-185	193-194	364-366	169-170	247-250	
9		190-191	202-203	370-372		253-255	298-299
10		195-197	211-212	376-378		259-261	
11		201-203		382-384	210-211	265-267	331-332
12		207-208	220-221			271-273	
13		213-214	229-230	389-390		277-279	
14		219-220	239-240	394-396	256-257	283-285	
15		225-226	250-251	401-402		289-290	364-365
16		231-233	258-259	407-409	271-272	295-296	377-378
17		237-239		413-415	286-287	300-302	391-392
18		244-245		420-421	298-299	306-308	
19		249-251	286-287	424-427	324-325	312-314	403-405
20		256-257	295-296	431-433	327-334	318-320	424-426
21		261-263	304-305	438-439	351-352	324-326	
22		268-269		444-446	362-366	330-332	435-438
23		273-275	321-322	451-453	370-373	336-337	452-458
24		280-281		458-460	377-380	342-343	464-466
25		286-287		464-466	399-401	348-349	471-474
26		292-293			409-410	354-355	491-492

27				470-472		360-361	497-499
28				477-478	437-439	366-367	503-504
29		315-316		483-484	448-449	372-373	517-518
30				489-491	469-470		530-532
31				495-496		377-378	537-538
32				501-502		389-390	550-552
33				507-508			556-558
34				514-517			561-565
35				520-521			582-584
36				526-527			589-591
37							609-610
38							615-617
39							622-623
40				545-546			

Appendix PND16-1c

S. enterica serotype Enteritidis VNTR allele list and corresponding observed fragment size ranges in the Applied Biosystems Genetic Analyzer 3130/3500 (*ABISE* look-up table)

Note: This table is posted on the PulseNet SharePoint under the QA/QC manual as a TXT-file so users may save it locally and use it with the BioNumerics MLVA scripts. Every time the table is updated, the latest version is posted on SharePoint site.

	R1				R2		
Count	SE1	SE2	SE8	SE6	SE9	SE3	SE5
1			346-350		173-175		160-161
2	163-166		433-436		181-184		
3	170-172	300-303			190-192	199-203	172-174
4	177-179	308-309.5			199-201	211-214	177-180
5	184-186	310.4-316.2				224-225	184-185
6	190-193	317.5-324.0					189-191
7	198-199	324.9-331.5					195-197
8	204-206	335-339		408-413		259-260	201-203
9	211-213	342-345		446-447			207-209
10	218-219	348-351		479-482		283-284	213-215
11	225-226	356-358					218-221
12	232-234	363-364		533-534			225-228
13	240-241	370-373		566-567			232-234
14				588-589			238-240
15	255-256	384-385					244-247
16							249-253
17	269-270						258-259
18							264-265
19							268-271
20							274-281
21							
22							287-288
23							
24	317-319						
25							
26							
27							

28							
29							
30	359-360						

Appendix PND16-2a

BioNumerics specifications for STEC O157 VNTR loci

Reaction	R1	R2
Name	**VNTR_3**	**VNTR_17**
Dye	CalRed590 = Y	CalRed590 = Y
Offset	321	120
Repeat length	6	6
Copy range	0-25	0-30
Tolerance	2	2
Fragment size in EDL933	379-382	159-161
Name	**VNTR_34**	**VNTR_19**
Dye	FAM = B	FAM = B
Offset	100	272
Repeat length	18	6
Copy range	0-20	0-25
Tolerance	3	2
Fragment size in EDL933	278-280	308-310
Name	**VNTR_9**	**VNTR_36**
Dye	FAM = B	FAM = B
Offset	465	102
Repeat length	6	7
Copy range	0-50	0-20
Tolerance	2	2
Fragment size in EDL933	530-534	157-158
Name	**VNTR_25**	**VNTR_37**
Dye	HEX = G	HEX = G
Offset	110	142
Repeat length	6	6
Copy range	0-20	0-25
Tolerance	2	2
Fragment size in EDL933	138-140	187-189

Fragment size ranges are based on independent runs on multiple instruments at CDC and in PulseNet Participating Laboratories.

Appendix PND16-2b

BioNumerics specifications for *S. enterica* serotype Typhimurium VNTR loci

Reaction	R1	R2
Name	ST3	ST2
Dye	HEX = G	FAM = B
Offset	156	62
Repeat length	6*	13**
Copy range	0-20	0-40
Tolerance	2	3
Fragment size in LT2	188-192	370-373
Copy number in LT2	4*	23**
Name	ST5	ST6
Dye	FAM = B	CalRed590 = Y
Offset	139	191
Repeat length	6	6
Copy range	0-60	0-50
Tolerance	2	2
Fragment size in LT2	219-220	271-273
Copy number in LT2	14	12
Name	ST7	ST8
Dye	CalRed590 = Y	HEX = G
Offset	115	196
Repeat length	9	11***
Copy range	0-30	0-50
Tolerance	3	3
Fragment size in LT2	157-158	556-558
Copy number in LT2	4	33***
Name	ST10	
Dye	HEX = G	
Offset	311	
Repeat length	6	
Copy range	0-50	
Tolerance	2	
Fragment size in LT2	376-378	
Copy number in LT2	10	

* A half of a repeat difference considered significant. Actual repeat length 12 bp and actual copy number in LT2 2.5
** A third of a repeat difference considered significant. Actual repeat length 39 bp and actual copy number in LT2 8.0
*** A third of a repeat difference considered significant. Actual repeat length 33 bp and actual copy number in LT2 11.0

Fragment size ranges are based on independent runs on multiple instruments at CDC and in PulseNet Participating Laboratories.

Appendix PND16-2c

BioNumerics specifications for *S. enterica* serotype Enteritidis VNTR loci

Reaction	R1	R2
Name	SE1	SE9
Dye	B (FAM)	B (FAM)
Offset	149	165
Repeat length	7	9
Copy range	0-10	0-5
Tolerance	2	2
Fragment size in K1891	190-193	181-184
Copy number in K1891	6	2
Name	SE2	SE3
Dye	Y (Calred 590)	Y (Calred 590)
Offset	280	165
Repeat length	7	12
Copy range	0-20	0-10
Tolerance	2	3
Fragment size in K1891	335-339	211-215
Copy number in K1891	8	4
Name	SE8	SE5
Dye	G (HEX)	G (HEX)
Offset	261	154
Repeat length	86	6
Copy range	0-3	0-25
Tolerance	4	2
Fragment size in K1891	433-436	201-203
Copy number in K1891	2	8
Locus	SE6	
Dye	B (FAM)	
Offset	149	
Repeat length	33	
Copy range	2-20	
Tolerance	3	
Fragment size in K1891	479-482	
Copy number in K1891	10	

Fragment size ranges are based on independent runs on multiple instruments at CDC and in PulseNet Participating Laboratories.

READING CONTROL SHEET FOR:
STANDARD OPERATING PROCEDURES FOR THE PULSENET DATABASES (PND)

NAME	DATE	COMMENTS	SIGNATURE

By signing above, you are indicating that you have read and understood all SOPs included in the PND section of this manual.

1. PURPOSE: To describe guidelines for the quality of TIFF images submitted to the PulseNet national databases.

2. SCOPE: This applies to all TIFF images submitted to PulseNet, thereby allowing comparison of results with other PulseNet laboratories.

3. DEFINITIONS/TERMS:

3.1 TIFF: Tagged Image File Format
3.2 TIFF Quality: The grading of the appearance and ease of analysis of a TIFF, according to the TIFF Quality Grading Guidelines within this SOP. This is a main component of the evaluation of a TIFF submitted for certification or proficiency testing.
3.3 SOP: Standard Operating Procedure

4. RESPONSIBILITIES/PROCEDURE:

Parameter	TIFF Quality Grading Guidelines			
	Excellent	**Good**	**Fair**	**Poor**
Image Acquisition and Running Conditions	By protocol, for example: - Gel fills whole TIFF - Wells included on TIFF - Bottom band of standard 1-1.5 cm from bottom of gel	- Gel doesn't fill whole TIFF but band finding is not affected	Not protocol; for example, one of the following: - Gel doesn't fill whole TIFF and band finding is affected - Wells not included on TIFF - Bottom band of standard not 1-1.5 cm from bottom of gel - Band spacing of standards doesn't match global standard	Not protocol; for example, >1 of the following: - Gel doesn't fill whole TIFF and this affects band finding - Wells not included on TIFF - Bottom band of standard not 1-1.5 cm from bottom of gel - Band spacing of standards doesn't match global standard
Cell Suspensions	The cell concentration is approximately the same in each lane	1-2 lanes contain darker or lighter bands than the other lanes	- >2 lanes contain darker or lighter bands than the other lanes, or - At least 1 lane is much darker or lighter than the other lanes, making the gel difficult to analyze	The cell concentrations are uneven from lane to lane, making the gel impossible to analyze
Bands	Clear and distinct all the way to the bottom of the gel	- Slight band distortion in 1 lane but doesn't interfere with analysis - Bands are slightly fuzzy and/or slanted - A few bands (e.g., ≤3) difficult to see clearly (e.g., DNA overload), especially at bottom of gel	- Some band distortion (e.g., nicks) in 2-3 lanes but still analyzable - Fuzzy bands - Some bands (e.g., 4-5) are too thick - Bands at the bottom of the gel are light, but analyzable	- Band distortion that makes analysis difficult - Very fuzzy bands. - Many bands too thick to distinguish - Bands at the bottom of the gel too light to distinguish
Lanes	Straight	- Slight smiling (higher bands in the outside lanes vs. the inside) - Lanes gradually run longer toward the right or left - Still analyzable	- Significant smiling - Slight curves on the outside lanes - Still analyzable	- Smiling or curving that interferes with analysis

Restriction	Complete restriction in all lanes	- One to two faint shadow bands on gel	- One lane with many shadow bands - A few shadow bands spread out over several lanes	- Greater than 1 lane with several shadow bands - Lots of shadow bands over the whole gel
Gel Background	Clear	- Mostly clear background - Minor debris present that doesn't affect analysis	- Some debris present that may or may not make analysis difficult (e.g., auto band search finds too many bands) - Background caused by photographing a gel with very light bands (image contrast was "brought up" in photographing gel-makes image look grainy)	- Lots of debris present that may or may not make analysis difficult (i.e., auto band search finds too many bands)
DNA Degradation (smearing in the lanes)	Not present	- Minor background (smearing) in a few lanes but bands are clear	- Significant smearing in 1-2 lanes that may or may not make analysis difficult - Minor background (smearing) in many lanes	- Significant smearing in >2 lanes that may or may not make analysis difficult - Smearing so that a lane is not analyzable (except if untypeable [thiourea required])

5. FLOW CHART:

6. BIBLIOGRAPHY:

7. CONTACTS:

8. AMENDMENTS:

1. **PURPOSE:** To describe the procedure for certifying PulseNet personnel to enable full participation in PulseNet activities, including on-line access to the PulseNet National Databases.

2. **SCOPE:** This procedure applies to all PulseNet personnel performing PFGE, image acquisition, and/or BioNumerics gel analysis.

3. DEFINITIONS/TERMS:

3.1. PFGE: Pulsed-field Gel Electrophoresis
3.2. CDC: Centers for Disease Control and Prevention
3.3. QA/QC: Quality Assurance/Quality Control
3.4. SOP: Standard Operating Procedure
3.5. BioNumerics: Gel analysis software used by PulseNet, developed by Applied Maths, Belgium
3.6. TIFF: Tagged Image File Format. A file of a gel image that can be analyzed in BioNumerics
3.7. Bundle file: A file with a .bdl extension that is produced in BioNumerics and contains the analysis of at least one lane of a gel image
3.8. Gel-certified or TIFF-certified: An individual or laboratory that is certified in laboratory methods for PFGE and image acquisition
3.9. Analysis-certified: An individual who is certified in BioNumerics gel analysis
3.10. Certification files: TIFF and/or bundle files submitted by PulseNet participants for certification evaluation.
3.11. Certification file evaluator: An individual who evaluates certification files
3.12. PulseNet Area Laboratory: Laboratory, designated by CDC, which has agreed to assume responsibility for additional PulseNet duties for laboratories within their support zone. The current Area Laboratories include MA, MN, WA, TX, VA, UT, MI, and CDC.

4. RESPONSIBILITIES:

4.1 Individuals performing PulseNet-related work (i.e., preparing PFGE gels and/or analyzing TIFFs of PFGE gels) must submit certification file(s) and have them reviewed before being able to submit TIFF images and BioNumerics analyses to the PulseNet National Databases.
- 4.1.1 Submitted certification files must document the submitter's highest level of competence in producing and imaging PFGE gels and/or in analyzing TIFFs of PFGE gels.
- 4.1.2 Individuals can be certified for each PulseNet organism in one of three ways:
  - 4.1.2.1 Gel only (i.e., gel-certified). An individual can submit TIFFs to an analysis-certified person for subsequent analysis and uploading to the national databases.
  - 4.1.2.2 Analysis only (i.e., analysis-certified). An individual can analyze TIFFs of gels generated by TIFF-certified individuals and upload those analyzed images to the national databases.
  - 4.1.2.3 Both gel and analysis (i.e., gel and analysis-certified). An individual can perform PFGE, image acquisition, and BioNumerics analysis of TIFFs, uploading the analyses to the national databases.

4.1.3 For each PulseNet organism, at least one person from each PulseNet participating laboratory should be gel-certified and one person should be analysis-certified. One person can be both gel and analysis-certified. Laboratories cannot have analysis-certified personnel without gel-certified personnel; gel certification must occur before or concurrent with analysis certification.

4.2 Individuals performing PulseNet-related work at CDC must submit certification file(s) and have them reviewed before being able to submit TIFF images and BioNumerics analyses to the PulseNet National Databases.

4.2.1 All PulseNet CDC patterns must be uploaded directly from a client database that is housed on the PulseNet network drive. Those at CDC without access to a client database on the PulseNet network drive may not upload PFGE patterns.

5. PROCEDURE:

5.1 PulseNet participants request certification set(s) from CDC (via pfge@cdc.gov) if they do not already have them.

5.1.1 Currently, certification sets are available for *E. coli* O157:H7, *E. coli* Non O157 STEC (analysis-only), *Salmonella*, *Shigella*, *Listeria monocytogenes*, *Campylobacter jejuni*, *Vibrio cholerae*, *Vibrio parahaemolyticus* and *C.botulinum*.

5.2 CDC sends the requested certification set(s) and detailed instructions for the gel preparation and analysis of the certification set organisms. See Appendices PNQ02-1 through PNQ02-9.

5.3 Individuals in each laboratory who wish to be gel-certified make plugs for each sample and run gel(s) with the proper controls.

5.4 Individuals in the laboratory who wish to be analysis-certified analyze TIFF(s) produced using the certification set instructions and create a bundle file of their analyses, according to the certification set instructions.

5.5 TIFF file(s) are submitted to CDC for review. See Appendices PNQ02-1 through PNQ02-9 for submission instructions.

5.6 Bundle files are submitted to CDC for review. See Appendices PNQ02-1 through PNQ02-9 for submission instructions.

5.7 Submitters are notified in writing of the results of their certification file(s) evaluation. (See PNQ03 for more information on the evaluation of PulseNet certification files.)

5.7.1 If the submitted certification files pass the certification evaluation, the submitter is considered certified as long as they remain in their current laboratory and that laboratory successfully completes annual proficiency testing (See PNQ04 for more information on the PulseNet proficiency testing program). If a person relocates to a different PulseNet laboratory, they must be recertified.

5.7.2 If the submitted certification files do not pass the certification evaluation

5.7.2.1 The individual will need to review the troubleshooting comments received from the evaluator and resubmit once results have improved

5.7.2.2 At any point, if the evaluator and/or CDC feels that the individual needs additional troubleshooting and/or training before resubmission, the Area Laboratory responsible for the individual's region will be notified

5.7.2.3 If the submitter fails certification three times, the individual will not be allowed to submit again for six months. Before resubmitting, the individual will be expected to work with CDC and/or their PulseNet Area Laboratory until satisfactory results are achieved. This includes, but is not limited to troubleshooting and training in the PulseNet PFGE protocols, BioNumerics and the PulseNet masterscripts.

6. FLOW CHART:

7. BIBLIOGRAPHY:

8. CONTACTS:

8.1 PulseNet Database Unit Chief: Kelley Hise, MPH
(404) 639-0704
KHise@cdc.gov

8.2 CDC PulseNet Database Administration Team
(404) 639-4558
PFGE@cdc.gov

9. AMENDMENTS:

9.1 Appendix PNQ02-6 added on 5/2/2007
9.2 Appendix PNQ02-7 added on 11/18/2008
9.3 Appendix PNQ02-8 added on 10/6/2009
9.4 As of 10/6/2009 the *Shigella* certification set (Appendix PNQ02-3) no longer includes isolates 92-01, 93-01 or 96-01.
9.5 As of 10/6/2009 the *Campylobacter jejuni* certification set (Appendix PNQ02-5) no longer includes isolates D996 or D2253.
9.6 1/5/2010 Appendix PNQ02-6 section 4 was updated to correct lane information for the test strains
9.7 Appendix PNQ02-9 for *C. botulinum* certification added on 7/15/2010
9.8 July 2010 added « Images that have a file size that is not within the range of ~300-400 kb will be deemed unsatisfactory and certification file evaluators will ask for a resubmission." to all appendices.
9.9 July 2010 added section 4.2.
9.10 November 2010 typos for H9812 were corrected in all the lane assignment tables.
9.11 Appendix PNQ02-8 was updated on 3/4/2013 to reflect changes to the normalization step during analysis of Non O157 images. Please mark standard lanes as shown in the reference lane (top of 668.9 Kb and the top band of 167.1 Kb).
9.12 Appendix PNQ02-10 was added for *Shigella flexneri* analysis certification instructions

Appendix PNQ02-1

Laboratory Protocol to Reconstitute Lyophilized (Freeze-Dried) *E. coli* O157:H7 Cultures

Biological Safety Warning: *E. coli* O157:H7 strains are considered Level 2 biological agents by the U.S. Department of Health and Human Services. Use appropriate precautions when handling the vial or culture. Carry out laboratory work in a biological safety cabinet when applicable to ensure aseptic conditions and personal safety.

Note: Store the lyophilized cultures at 4°C in the dark until they are reconstituted.

Materials Needed:

Sturdy sterile forceps
1 ml pipetman
1 ml sterile pipet tips
1 ul sterile inoculating loop

Reagents Needed:

Trypticase Soy + 5% Sheep Blood Agar plates (BAP) or equivalent media
Sterile grade reagent water
70% isopropyl alcohol

Procedure for Reviving Cultures:

Day 1

1. Document the isolate number(s) and the date(s) lyophilized for your records. Wipe the aluminum cover and outside of the vial with isopropyl alcohol. Using sturdy forceps, aseptically remove the aluminum cover and rubber stopper from the vial containing the lyophilized culture. Wipe the outside of the rubber stopper and neck of the vial with isopropyl alcohol before removing the stopper.

2. Re-suspend the lyophilized cells with 1ml of sterile grade reagent water. Allow to stand for a few minutes and/or mix gently to produce a uniform suspension. With an inoculating loop, streak a small amount of this suspension onto a blood agar plate (BAP) and incubate at 37ºC overnight.

Days 2 and 3

Check the BAP; if the culture appears pure, pick an isolated colony, and inoculate a fresh BAP for heavy growth; incubate at 37°C overnight. Use the growth from this plate to make PFGE plugs of the standard strains. Before making the plugs, transfer culture to fresh medium and incubate at 37°C overnight; this will ensure that the same culture can be retested, if necessary.

After the strains have been reconstituted according to the above directions, streak each culture to agar plates, pick an isolated colony, and subculture to another plate. Use the growth from the second plate to make the PFGE plugs. Please let me know if the package does not arrive in satisfactory condition, or if the cultures are not viable. **Freeze (-70°C) or stock these strains according to your laboratory's policy within one week of receiving them. Then your laboratory will have stock cultures of this PulseNet certification set for future use, including the PulseNet certification of additional personnel.**

The strain numbers of the *E. coli* cultures are as follows:

CDC16-98 CDC20-98 CDC24-98 CDC68-98

Please follow these supplemental instructions for making the PFGE plugs and running the gels for these strains. Refer to the "One-Day (24-28 h) Standardized Laboratory Protocol for Molecular Subtyping of *Escherichia coli*, non-typhoidal *Salmonella* serotypes and *Shigella sonnei* by Pulsed Field Gel Electrophoresis (PFGE)". Detailed instructions for making the PFGE plugs can be found in the PulseNet QA/QC Manual PNL05. This document is available in the QA/QC conference on CDC Team or at www.cdc.gov/pulsenet/. If you can not access the protocol, please request it by sending an E-mail to PFGE@CDC.GOV.

1. Make 2-3 (disposable plug molds) or two (reusable plug molds) plugs of each test strain so they can be retested several times, if necessary.
2. Restrict one plug slice from the four test cultures (CDC16-98, CDC20-98, CDC24-98, and CDC68-98) and 3 plug slices of *Salmonella* ser. Braenderup H9812 (the PulseNet Universal Standard Strain) with *Xba*I (40-50 Units/plug slice) for 2 hours at 37°C..
3. Restrict one plug slice from three of the test cultures (CDC16-98, CDC20-98, and CDC24-98) with *Avr*II (*Bln*I; 25-30 Units/plug slice) for 2 hours at 37°C.
4. Load H9812 in Lanes 1, 5, 10 and the test strains as follows:

Lane	Certification Strain	Enzyme
1	H9812	XbaI
2	16-98	XbaI
3	20-98	XbaI
4	24-98	XbaI
5	H9812	XbaI
6	16-98	BlnI
7	20-98	BlnI
8	24-98	BlnI
9	68-98	XbaI
10	H9812	XbaI

Note: If you use combs with 15 teeth, load the plug slices in lanes 2-11 and leave the other lanes empty. Although the use of the ~0.5-mm wide 15-teeth combs [Bio-Rad, 170-4326] in the standard casting stand [14 x 13-cm] is not recommended for routine PFGE analysis of test isolates, the use of the smaller comb teeth will be allowed for certification.

5. Run the gel using the *E. coli* electrophoresis conditions. These run times are based on the equipment and reagents used at CDC. If the gels generated in your lab do not have the lowest band in strain H9812 approximately 1-1.5 cm from the bottom of the gel, the run time may have to be changed.

6. Staining, de-staining, and gel documentation (imaging):

   a. The gel image should fill the entire window of the imaging equipment (computer) screen (without cutting off wells or lower bands). Individuals submitting certification TIFFs that do not contain the wells or clearly show the bottom bands of the patterns that will automatically be asked to rerun the gel and submit a new certification TIFF. Images that have a file size that is not within the range of ~300-400 kb will be deemed unsatisfactory and certification file evaluators will ask for a resubmission.

   b. TIFFs with one lane that contains many shadow bands or multiple lanes with one or more shadow bands (indicating incomplete restriction) should not be submitted for certification. To eliminate incomplete restriction wash the plugs at least two more times with TE Buffer before restriction is repeated. If the problem persists repeat the restriction with more units of enzyme, for a longer amount of time and/or with a different lot of enzyme.

7. Bundle file creation

   a. In the bundle file, do not include lanes from more than 2 gels. Enter the corresponding CDC strain number in the BioNumerics "Key" field. The bundle file should contain 4 database entries as follows: 3 test strains restricted with *Xba*I and *Bln*I and one test strain restricted only with *Xba*I. If there are more than 4 entries in the bundle file, one or more lanes have not been linked properly.

   b. Create the bundle file using the lightning bolt icon.(refer to the Gel Analysis Guidelines PND04 for instructions on how to create a PulseNet Bundle File). Bundles created using this icon are "PulseNet bundles" and will contain only standardized PulseNet experiments and fields. "PulseNet bundles" can be recognized by the "PN" that is automatically added to the file name. If a non-PulseNet bundle is submitted, you will automatically be asked to resubmit.

8. Send the TIFF file and/or bundle (*.bdl) file of your gel images to CDC Pulse Net at PFGE@CDC.GOV within four weeks after receiving the strains.

a. In the email to CDC, include ***E. coli* Certification Gel** in the Subject line, so it can be forwarded to the correct person. Include the isolate number and restriction enzyme used for each lane on the gel in the body of the email. If you are sending certification TIFFs and bundle files for more than one organism, please send one email per organism.

b. Name the TIFF and bundle files of your gel images as follows:

TIFF: Use the unique identifier code that was assigned by CDC PulseNet for the first two to four letters of the file. The next 2 spaces will indicate the year the file was created. The next 3 spaces indicate the sequential number of the file submitted from your laboratory during a calendar year. For example: **GA09012.tif** is the twelfth file submitted from the state of Georgia during 2009.

Bundle: The bundle files are named in a similar manner as the TIFF files with the first two to four letters of the file name indicating the unique identifier code for your laboratory. The next 2 spaces indicate the year the bundle file was created, and the next 3 spaces indicate the sequential bundle file number from each laboratory. For example, the eighth bundle file submitted from the state of Georgia during 2009 would be named **GA09008PN.bdl**.

For each PulseNet pathogen, an individual may be certified for:

a. Gels only (i.e., laboratory methods for PFGE and image acquisition)
b. Analysis only (i.e., BioNumerics analysis and on-line access to the database), or
c. Both gels and analysis.

After the gel images are submitted, the PulseNet certification file evaluator will analyze the gels and inform your laboratory of your results ("Satisfactory" or "Needs Improvement") within four weeks of receiving the files. If the TIFF images are satisfactory, the person who submitted the TIFF will be eligible to send gel images to PulseNet for analysis; if the bundle files are satisfactory, the person who submitted the bundle files will be issued a SecurID device (also called a fob) and will be granted on-line access to the appropriate PulseNet national database. SecurID devices are issued to individuals within a laboratory. The devices cannot be shared and must be returned to CDC if the certified individual leaves his or her position in the laboratory. If the submitted certification files are not satisfactory, the individual will need to review the troubleshooting comments received from the evaluator and resubmit once results have improved. If the submitter fails certification three times, the individual will not be allowed to submit again for six months. Before resubmitting, the individual will be expected to work with CDC and/or their PulseNet Area Laboratory until satisfactory results are achieved. This includes, but is not limited to, troubleshooting and training in the PulseNet PFGE protocols, BioNumerics and the PulseNet masterscripts.

Appendix PNQ02-2

Laboratory Protocol to Reconstitute Lyophilized (Freeze-Dried) *Salmonella* Cultures

Biological Safety Warning: *Salmonella* strains are considered Level 2 biological agents by the U.S. Department of Health and Human Services. Use appropriate precautions when handling the vial or culture. Carry out laboratory work in a biological safety cabinet when applicable to ensure aseptic conditions and personal safety.

Note: Store the lyophilized cultures at 4^{o}C in the dark until they are reconstituted.

Materials Needed:

Sturdy sterile forceps
1 ml pipetman
1 ml sterile pipet tips
1 ul sterile inoculating loop

Reagents Needed:

Trypticase Soy + 5% Sheep Blood Agar plates (BAP) or equivalent media
Sterile grade reagent water
70% isopropyl alcohol

Procedure for Reviving Cultures:

Day 1

1. Document the isolate number(s) and the date(s) lyophilized for your records. Wipe the aluminum cover and outside of the vial with isopropyl alcohol. Using sturdy forceps, aseptically remove the aluminum cover and rubber stopper from the vial containing the lyophilized culture. Wipe the outside of the rubber stopper and neck of the vial with isopropyl alcohol before removing the stopper.

2. Re-suspend the lyophilized cells with 1ml of sterile grade reagent water. Allow to stand for a few minutes and/or mix gently to produce a uniform suspension. With an inoculating loop, streak a small amount of this suspension onto a blood agar plate (BAP) and incubate at 37ºC overnight.

Days 2 and 3

1. Check the BAP; if the culture appears pure, pick an isolated colony, and inoculate a fresh BAP for heavy growth; incubate at 37°C overnight. Use the growth from this plate to make PFGE plugs of the standard strains. Before making the plugs, transfer culture to fresh medium and incubate at 37°C overnight; this will ensure that the same culture can be retested, if necessary.

After the strains have been reconstituted according to the above directions, streak each culture to agar plates, pick an isolated colony, and subculture to another plate. Use the growth from the second plate to make the PFGE plugs. Please let me know if the package does not arrive in satisfactory condition, or if the cultures are not viable. **Freeze (-70°C) or stock these strains according to your laboratory's policy within 1 week of receiving them. Then, your laboratory will have stock cultures of this PulseNet certification set for future use, including the PulseNet certification of additional personnel.**

The strain numbers of the *Salmonella* cultures are as follows:

CDC61-99 CDC78-99 CDC87-03 CDC98-03

Please follow these supplemental instructions for making the PFGE plugs and running the gels for these strains. Refer to the "One-Day (24-28 h) Standardized Laboratory Protocol for Molecular Subtyping of *Escherichia coli*, non-typhoidal *Salmonella* serotypes and *Shigella sonnei* by Pulsed Field Gel Electrophoresis (PFGE)." Detailed instructions for making the PFGE plugs can be found in the PulseNet QA/QC Manual PNL05. This document is available in the QA/QC conference on CDC Team or at www.cdc.gov/pulsenet/. If you can not access the protocol, please request it by sending an E-mail to PFGE@CDC.GOV.

1. Make 2-3 (disposable plug molds) or two (reusable plug molds) plugs of each test strain so they can be retested several times, if necessary.

2. Restrict one plug slice from the four test cultures (CDC61-99, CDC78-99, CDC87-03, and CDC98-03) and 3 plug slices of *Salmonella* ser. Braenderup H9812 (the PulseNet Universal Standard Strain) with *Xba*I (40-50 Units/plug slice) for 2 hours at 37°C.
3. Restrict one plug slice from three of the test cultures (CDC61-99, CDC78-99, and CDC87-03) with *Avr*II (*Bln*I; 25-30 Units/plug slice) for 2 hours at 37°C.
4. Load H9812 in Lanes 1, 5, 10 and the test strains as follows:

Lane	Certification Strain	Enzyme
1	H9812	XbaI
2	61-99	XbaI
3	78-99	XbaI
4	87-03	XbaI
5	H9812	XbaI
6	61-99	BlnI
7	78-99	BlnI
8	87-03	BlnI
9	98-03	XbaI
10	H9812	XbaI

Note: If you use combs with 15 teeth, load the plug slices in lanes 2-11 and leave the other lanes empty. (The use of the ~0.5-mm wide 15-teeth combs [Bio-Rad, 170-4326] in the standard casting stand [14 x 13-cm] is not recommended for routine PFGE analysis of test isolates, but will be allowed for certification).

5. Run the gel using the *Salmonella* electrophoresis conditions. The run times listed in the protocol are based on the equipment and reagents used at CDC. If the gels generated in your lab do not have the lowest band in strain H9812 approximately 1-1.5 cm from the bottom of the gel, the run time may have to be changed.

6. Staining, de-staining, and gel documentation (imaging):

   a. The gel image should fill the entire window of the imaging equipment (computer) screen (without cutting off wells or lower bands). Individuals submitting a certification TIFFs that do not contain the wells or clearly show the bottom bands of the patterns will automatically be asked to rerun the gel and submit a new certification TIFF. Images that have a file size that is not within the range of ~300-400 kb will be deemed unsatisfactory and certification file evaluators will ask for a resubmission.

   b. TIFFs with one lane that contains many shadow bands or multiple lanes with one or more shadow bands (indicating incomplete restriction) should not be submitted for certification. To eliminate incomplete restriction wash the plugs at least two more times with TE Buffer before restriction is repeated. If the problem persists repeat the restriction with more units of enzyme, for a longer amount of time and/or with a different lot of enzyme.

7. Bundle file creation

   a. In the bundle file, do not include lanes from more than 2 gels. Enter the corresponding CDC strain number in the BioNumerics "Key" field. The bundle file should contain 4 database entries as follows: 3 test strains restricted with *Xba*I and *Bln*I and one test strains restricted only with *Xba*I. If there are more than 4 entries in the bundle file, one or more lanes have not been linked properly.

b. Create the bundle file using the lightning bolt icon.(refer to the Gel Analysis Guidelines PND04 for instructions on how to create a PulseNet Bundle File). Bundles created using this icon are "PulseNet bundles" and will contain only standardized PulseNet experiments and fields. "PulseNet bundles" can be recognized by the "PN" that is automatically added to the file name. If a non-PulseNet bundle is submitted, you will automatically be asked to resubmit.

8. Send the TIFF file and/or bundle (*.bdl) file of your gel images to CDC Pulse Net at PFGE@CDC.GOV within four weeks after receiving the strains.

a. In the email to CDC, include ***Salmonella*** **Certification Gel** in the Subject line, so it can be forwarded to the correct person. Include the isolate number and restriction enzyme used for each lane on the gel in the body of the email. If you are sending certification TIFFs and bundle files for more than one organism, please send one email per organism.

b. Name the TIFF and bundle files of your gel images as follows:

TIFF: Use the unique identifier code that was assigned by CDC PulseNet for the first two to four letters of the file. The next 2 spaces will indicate the year the file was created. The next 3 spaces indicate the sequential number of the file submitted from your laboratory during a calendar year. For example: **GA09012.tif** is the twelfth file submitted from the state of Georgia during 2009.

Bundle: The bundle files are named in a similar manner as the TIFF files with the first two to four letters of the file name indicating the unique identifier code for your laboratory. The next 2 spaces indicate the year the bundle file was created, and the next 3 spaces indicate the sequential bundle file number from each laboratory. For example, the eighth bundle file submitted from the state of Georgia during 2009 would be named **GA09008PN.bdl**.

For each PulseNet pathogen, an individual may be certified for:
a. Gels only (i.e., laboratory methods for PFGE and image acquisition)
b. Analysis only (i.e., BioNumerics analysis and on-line access to the database), or
c. Both gels and analysis.

After the gel images are submitted, the PulseNet certification file evaluator will analyze the gels and inform your laboratory of your results ("Satisfactory" or "Needs Improvement") within four weeks of receiving the files. If the TIFF images are satisfactory, the person who submitted the TIFF will be eligible to send gel images to PulseNet for analysis; if the bundle files are satisfactory, the person who submitted the bundle files will be issued a SecurID device (also called a fob) and will be granted on-line access to the appropriate PulseNet national database. SecurID devices are issued to individuals within a laboratory. The devices cannot be shared and must be returned to CDC if the certified individual leaves his or her position in the laboratory. If the submitted certification files are not satisfactory, the individual will need to review the troubleshooting comments received from the evaluator and resubmit once results have improved. If the submitter fails certification three times, the individual will not be allowed to submit again for six months. Before resubmitting, the individual will be expected to work with CDC and/or their PulseNet Area Laboratory until satisfactory results are achieved. This includes, but is not limited to, troubleshooting and training in the PulseNet PFGE protocols, BioNumerics and the PulseNet masterscripts

Appendix PNQ02-3

Laboratory Protocol to Reconstitute Lyophilized (Freeze-Dried) *Shigella* Cultures

Biological Safety Warning: *Shigella* strains are considered Level 2 biological agents by the U.S. Department of Health and Human Services. Use appropriate precautions when handling the vial or culture. Carry out laboratory work in a biological safety cabinet when applicable to ensure aseptic conditions and personal safety.

Note: Store the lyophilized cultures at 4^{o}C in the dark until they are reconstituted.

Materials Needed:

Sturdy sterile forceps
1 ml pipetman
1 ml sterile pipet tips
1 ul sterile inoculating loop

Reagents Needed:

Trypticase Soy + 5% Sheep Blood Agar plates (BAP) or equivalent media
Sterile grade reagent water
70% isopropyl alcohol

Procedure for Reviving Cultures:

Day 1

1. Document the isolate number(s) and the date(s) lyophilized for your records. Wipe the aluminum cover and outside of the vial with isopropyl alcohol. Using sturdy forceps, aseptically remove the aluminum cover and rubber stopper from the vial containing the lyophilized culture. Wipe the outside of the rubber stopper and neck of the vial with isopropyl alcohol before removing the stopper.

2. Re-suspend the lyophilized cells with 1ml of sterile grade reagent water. Allow to stand for a few minutes and/or mix gently to produce a uniform suspension. With an inoculating loop, streak a small amount of this suspension onto a blood agar plate (BAP) and incubate at 37ºC overnight.

Days 2 and 3

Check the BAP; if the culture appears pure, pick an isolated colony, and inoculate a fresh BAP for heavy growth; incubate at 37^{o}C overnight. Use the growth from this plate to make PFGE plugs of the standard strains. Before making the plugs, transfer culture to fresh medium and incubate at 37^{o}C overnight; this will ensure that the same culture can be retested, if necessary.

After the strains have been reconstituted according to the above directions, streak each culture to agar plates, pick an isolated colony, and subculture to another plate. Use the growth from the second plate to make the PFGE plugs. Please let me know if the package does not arrive in satisfactory condition, or if the cultures are not viable. **Freeze (-70°C) or stock these strains according to your laboratory's policy within 1 week after receiving them. Then, your laboratory will have stock cultures of this PulseNet certification set for future use, including PulseNet certification of additional personnel**.

Biosafety Warning: *Shigella* species have a low infectious dose and are demonstrated hazards to laboratory personnel; please use extreme caution and Biosafety Level 2 practices (at a minimum) when transferring and handling *Shigella* strains. Work in a biological safety cabinet when handling large amounts of cells. Disinfect or dispose of all plasticware and glassware that comes in contact with the cultures in a safe and appropriate manner.

The strain numbers of the *Shigella sonnei* cultures are as follows:

CDC90-01 CDC91-01 CDC94-01 CDC95-01

Please follow these supplemental instructions for making the PFGE plugs and running the gels for these strains. Refer to the "One-Day (24-28 h) Standardized Laboratory Protocol for Molecular Subtyping of *Escherichia coli*, non-typhoidal *Salmonella* serotypes and *Shigella sonnei* by Pulsed Field Gel Electrophoresis (PFGE)" Detailed instructions for making the PFGE plugs can be found in the PulseNet QA/QC Manual PNL06. This document is available in the QA/QC conference of the CDC Team Support or at www.cdc.gov/pulsenet/. If you can not access the protocol, please request it by sending an E-mail to PFGE@CDC.GOV.

1. Make 2-3 (disposable plug molds) or two (reusable plug molds) plugs of each test strain so they can be retested several times, if necessary.
2. Restrict one plug slice from each of the 4 test cultures (CDC90-01, CDC91-01, CDC94-01, and CDC95-01) and 3 plug slices of H9812 (the PulseNet Universal Standard Strain) with *Xba*I (40-50 Units per plug slice) for 2 hours at 37ºC.
3. Restrict one plug slice from three of the test cultures (CDC90-01, CDC91-01, and CDC94-01) with *Avr*II (*Bln*I; 25-30 Units/plug slice) for 2 hours at 37°C.
4. Load H9812 in Lanes 1, 6, 10 and the test strains as follows:

Lane	Certification Strain	Enzyme
1	H9812	XbaI
2	CDC90-01	XbaI
3	CDC91-01	XbaI
4	CDC94-01	XbaI
5	CDC95-01	XbaI
6	H9812	XbaI
7	CDC90-01	BlnI
8	CDC91-01	BlnI
9	CDC94-01	BlnI
10	H9812	XbaI

Note: If you use combs with 15 teeth, load the plug slices in lanes 2-11 and leave the other lanes empty (Although the use of the ~0.5-mm wide 15-teeth combs [Bio-Rad, 170-4326] in the standard casting stand [14 x 13-cm] is not recommended for routine PFGE analysis of test isolates, the use of the smaller comb teeth will be allowed for certification.).

5. Run the gel using the *Shigella* electrophoresis conditions. The run times listed in the protocol are based on the equipment and reagents used at CDC. If the gels generated in your lab do not have the lowest band in strain H9812 approximately 1-1.5 cm from the bottom of the gel, the run time may have to be changed.

6. Staining, de-staining, and gel documentation (imaging):

   a. The gel image should fill the entire window of the imaging equipment (computer) screen (without cutting off wells or lower bands). Individuals submitting certification TIFFs that do not contain the wells or clearly show the bottom bands of the patterns that will automatically be asked to rerun the gel and submit a new certification TIFF. Images that have a file size that is not within the range of ~300-400 kb will be deemed unsatisfactory and certification file evaluators will ask for a resubmission.

   b. TIFFs with one lane that contains many shadow bands or multiple lanes with one or more shadow bands (indicating incomplete restriction) should not be submitted for certification. To

eliminate incomplete restriction wash the plugs at least two more times with TE Buffer before restriction is repeated. If the problem persists, repeat the restriction with more units of enzyme, for a longer amount of time and/or with a different lot of enzyme.

7. Bundle file creation

   a. In the bundle file, do not include lanes from more than 2 gels. Enter the corresponding CDC strain number in the BioNumerics "Key" field. The bundle file should contain 4 database entries as follows: 3 test strains restricted with *Xba*I and *Bln*I and one test strain restricted only with *Xba*I. If there are more than 4 entries in the bundle file, one or more lanes have not been linked properly.

   b. Create the bundle file using the lightning bolt icon (refer to the Gel Analysis Guidelines PND04 for instructions on how to create a PulseNet Bundle File). Bundles created using this icon are "PulseNet bundles" and will contain only standardized PulseNet experiments and fields. "PulseNet bundles" can be recognized by the "PN" that is automatically added to the file name. If a non-PulseNet bundle is submitted, you will automatically be asked to resubmit.

8. Send the TIFF file and/or bundle (*.bdl) file of your gel images to CDC PulseNet at PFGE@CDC.GOV.

   a. In the e-mail to CDC, include ***Shigella* Certification** in the subject line, so it can be forwarded to the correct person. Include the isolate number and restriction enzyme used for each lane on the gel in the body of the email. If you are sending certification TIFFs and bundle files for more than one organism, please send one email per organism.

   b. Name the TIFF and bundle files of your gel images as follows:

   TIFF: Use the unique identifier code that was assigned by CDC PulseNet for the first two to four letters of the file. The next 2 spaces will indicate the year the file was created. The next 3 spaces indicate the sequential number of the file submitted from your laboratory during a calendar year. For example: **GA09012.tif** is the twelfth file submitted from the state of Georgia during 2009.

   Bundle: The bundle files are named in a similar manner as the TIFF files with the first two to four letters of the file name indicating the unique identifier code for your laboratory. The next 2 spaces indicate the year the bundle file was created, and the next 3 spaces indicate the sequential bundle file number from each laboratory. For example, the eighth bundle file submitted from the state of Georgia during 2009 would be named **GA09008PN.bdl**.

For each PulseNet pathogen, an individual may be certified for:

a. Gels only (i.e., laboratory methods for PFGE and image acquisition)
b. Analysis only (i.e., BioNumerics analysis and on-line access to the database), or
c. Both gels and analysis.

After the gel images are submitted, the PulseNet certification file evaluator will analyze the gels and inform your laboratory of your results ("Satisfactory" or "Needs Improvement") within four weeks of receiving the files. If the TIFF images are satisfactory, the person who submitted the TIFF will be eligible to send gel images to PulseNet for analysis; if the bundle files are satisfactory, the person who submitted the bundle files will be issued a SecurID device (also called a fob) and will be granted on-line access to the appropriate PulseNet national database. SecurID devices are issued to individuals within a laboratory. The devices cannot be shared and must be returned to CDC if

the certified individual leaves his or her position in the laboratory. If the submitted certification files are not satisfactory, the individual will need to review the troubleshooting comments received from the evaluator and resubmit once results have improved. If the submitter fails certification three times, the individual will not be allowed to submit again for six months. Before resubmitting, the individual will be expected to work with CDC and/or their PulseNet Area Laboratory until satisfactory results are achieved. This includes, but is not limited to, troubleshooting and training in the PulseNet PFGE protocols, BioNumerics and the PulseNet masterscripts.

Appendix PNQ02-4
Laboratory Protocol to Reconstitute Lyophilized (Freeze-Dried) *Listeria monocytogenes* Cultures

Biological Safety Warning: *Listeria monocytogenes* is considered a Level 2 biological agent by the U.S. Department of Health and Human Services. Use appropriate precautions when handling the vial or culture. Carry out laboratory work in a biological safety cabinet when applicable to ensure aseptic conditions and personal safety.

Note: Store the lyophilized culture at 4˚C in the dark until they are reconstituted.

Materials Needed:

Sturdy sterile forceps
1 ml pipetman
1 ml sterile pipet tips
1 µl sterile inoculating loop

Reagents Needed:

Blood agar plate or equivalent media
Brain heart infusion agar plates
Sterile grade reagent water
70% isopropyl alcohol

Procedure for Reviving Cultures:

1. Wipe the aluminum cover and outside of the vial with isopropyl alcohol. Using forceps, aseptically remove the aluminum cover and rubber stopper from the vial containing the lyophilized culture. Wipe the outside of the vial and stopper with alcohol after the metal cap is removed.

2. Re-suspend the lyophilized cells with 1.0 mL of sterile reagent grade water. Allow to stand for a few minutes and/or mix gently to produce a uniform suspension. Pipet 10µl of *Listeria* cell suspension onto a blood agar plate (BAP) and with a 10µl loop streak for growth. Incubate at 37ºC overnight.

3. Check the blood agar plate; if the culture appears pure, pick 2-3 representative colonies and inoculate a fresh brain heart infusion agar plate for heavy growth; incubate at 37˚C for 18 to 24 hours. Use the growth from the brain heart infusion agar plate to make agarose gel plugs as described in the Standardized Molecular Subtyping of Foodborne Bacterial Pathogens by Pulsed-Field Gel Electrophoresis.

Listeria monocytogenes certification isolate plug slice loading position:

Lane number	Isolate number	Restriction Enzyme
1	*S.* Braenderup H9812	*Xba*I

2	CDC-02-H8393	*Asc*I
3	CDC-03-H8394	*Asc*I
4	CDC-04-H8395	*Asc*I
5	*Lm* control H2446	*Asc*I
6	*S.* Braenderup H9812	*Xba*I
7	CDC-02-H8393	*Apa*I
8	CDC-03-H8394	*Apa*I
9	CDC-04-H8395	*Apa*I
10	*S.* Braenderup H9812	*Xba*I

Recommended gel casting bed and comb: Standard casting stand, 14 x 13 cm frame/platform and 10 well comb, 14 cm wide, 1.5-mm thick.

After the strains have been reconstituted according to the above instructions, streak each culture to a Trypticase Soy + 5% Sheep's blood agar plate (BAP) and allow them to incubate at 37°C for 24 hours to check for viability. Please let me know if the shipment does not arrive in satisfactory condition, or if the cultures are not viable. Freeze (-70˚C) or stock these strains immediately after reviving them according to your laboratory policy. The *L. monocytogenes* strains in the revised set are:

CDC#H8393 CDC#H8394 CDC#H8395 **CDC#H2446 control strain**

Please follow the *Listeria monocytogenes* standardized protocol (PNL04) for making the plugs and running the gel for these isolates. Refer to the Standardized Laboratory Protocol for Molecular Subtyping of Foodborne Bacterial Pathogens by PFGE (Section 5.3, Revised April, 2002).

Salmonella ser. Braenderup strain (H9812) is used as the reference standard. DNA of the H9812 strain **must be digested with *Xba*I** to give the appropriate band pattern. Follow instructions in the *E. coli* O157:H7 Standardized Laboratory protocol (PNL05) for making *S.* Braenderup plugs. If you do not have the *S.* Braenderup reference standard you may request it from PFGE@CDC.GOV. The chart shown above lists the positions where plug slices should be loaded on the gel.

1. Staining, de-staining, and gel documentation (imaging):

   a. The gel image should fill the entire window of the imaging equipment (computer) screen (without cutting off wells or lower bands). Individuals submitting certification TIFFs that do not contain the wells or clearly show the bottom bands of the patterns that will automatically be asked to rerun the gel and submit a new certification TIFF. Images that have a file size that is not within the range of ~300-400 kb will be deemed unsatisfactory and certification file evaluators will ask for a resubmission.

   b. TIFFs with one lane that contains many shadow bands or multiple lanes with one or more shadow bands (indicating incomplete restriction) should not be submitted for certification. To eliminate incomplete restriction wash the plugs at least two more times with TE Buffer before restriction is repeated. If the problem persists, repeat the restriction with more units of enzyme, for a longer amount of time and/or with a different lot of enzyme.

Follow the instructions in steps 1-4 below for analysis, creating the bundle (*.bdl) file, and sending the TIFF(s) and/or bundle file of your *Listeria monocytogenes* gel image(s) to CDC for certification.

1. In the bundle file, do not include lanes from more than two gels. Enter the corresponding CDC strain number in the BioNumerics "Key" field. The bundle file should contain 4 database entries as follows:

3 test strains restricted with *Asc*I and *Apa*I and 1 test strain restricted only with *Asc*I. If there are more than 4 entries in the bundle file, one or more lanes have not been linked properly.

2. Create the bundle file using the lightning bolt icon (see page 104 in the Appendix of the Dec. 2003 CDC BioNumerics manual). Bundles created using this icon are "PulseNet bundles" and will contain only standardized PulseNet experiments and fields. "PulseNet bundles" can be recognized by the "PN" that is automatically added to the file name. If a non-PulseNet bundle is submitted, you will automatically be asked to resubmit.

3. Send the TIFF file and/or bundle file of your gel images to CDC PulseNet at PFGE@CDC.GOV.

    a. In the e-mail to CDC, include the appropriate description of the gel (*Listeria* Certification) in the subject line, so it can be forwarded to the correct person. Include the isolate number and restriction enzyme used for <u>each</u> lane on the gel in the body of the e-mail or as an attachment. If you are sending certification TIFFs and bundle files for more than one organism, please send one e-mail per organism.

    b. Name the TIFF and bundle files of your gel images as follows:

    <u>TIFF</u>: Use he unique identifier code that was assigned by CDC PulseNet for the first two, three, or four letters of the file. The next 2 spaces will indicate the year the file was submitted. The next 3 spaces indicate the sequential number of the file submitted from your laboratory during a calendar year. For example: **GA09012.tif** is the twelfth file submitted from the state of Georgia during 2009.

    <u>Bundle</u>: The bundle files are named in a similar manner as the TIFF files with the first two, three, or four letters of the file name indicating the unique identifier code for your laboratory. The next 2 spaces indicate the year the bundle file was submitted, and the next 3 spaces indicate the sequential bundle file number from each laboratory. For example, the eighth bundle file submitted from the state of Georgia during 2009 would be named **GA09008PN.bdl**.

    c. Please refer to Section 15 of the PulseNet PFGE Manual for additional information on naming the TIFF and bundle files and submitting gel images to PulseNet.

4. For each PulseNet pathogen, an individual may be certified for:

    a. TIFF image only (i.e., laboratory methods for PFGE and image acquisition)
    b. Analysis only (i.e., BioNumerics analysis and on-line access to the database)
    c. Both TIFF and analysis

After the gel images are submitted, the certification file evaluator will analyze the gels and inform your laboratory of your results ("Satisfactory" or "Needs Improvement") within four weeks of receiving the files. If the TIFF images are satisfactory, the person who submitted the TIFF will be eligible to send gel images to PulseNet for analysis; if the bundle files are satisfactory, the person who submitted the bundle files will be issued a SecurID device (also called a fob) and will be granted on-line access to the appropriate PulseNet national database. (Up to two SecurID devices can be provided to a participating public health PulseNet laboratory. SecurID devices are issued to individuals within a laboratory. The devices cannot be shared and must be returned to CDC if the certified individual leaves his or her position in the laboratory.) If the submitted certification files are <u>not</u> satisfactory, the individual will need to review the troubleshooting comments received from the evaluator and resubmit once results have improved. If the submitter fails certification three times, the individual will not be allowed to submit again for six months. Before resubmitting, the individual will be expected to work with CDC and/or their PulseNet Area Laboratory until

satisfactory results are achieved. This includes, but is not limited to troubleshooting and training in the PulseNet PFGE protocols, BioNumerics and the PulseNet masterscripts.

Appendix PNQ02-5

Laboratory Protocol to Reconstitute Lyophilized (Freeze-Dried) *Campylobacter jejuni* Cultures

Biological Safety Warning: *Campylobacter jejuni* strains are considered Level 2 biological agents by the U.S. Department of Health and Human Services. Use appropriate precautions when handling the vial or culture. Carry out laboratory work in a biological safety cabinet when applicable to ensure aseptic conditions and personal safety.

Note: Store the lyophilized cultures at 4°C in the dark until they are reconstituted.

Materials Needed:

Sturdy sterile forceps
1 ml pipetman
1 ml sterile pipet tips
1 ul sterile inoculating loop

Reagents Needed:

BHI agar + 5% Rabbit Blood or equivalent *Campylobacter* plating media
Sterile grade reagent water
70% isopropyl alcohol

Procedure for Reviving Cultures:

Day 1

1. Document the isolate number(s) and the date(s) lyophilized for your records. Wipe the aluminum cover and outside of the vial with isopropyl alcohol. Using sturdy forceps, aseptically remove the aluminum cover and rubber stopper from the vial containing the lyophilized culture. Wipe the outside of the rubber stopper and neck of the vial with isopropyl alcohol before removing the stopper.

2. Re-suspend the lyophilized cells with 250 µl of sterile grade reagent water. Allow to stand for a few minutes and/or mix gently to produce a uniform suspension. Pipet 100µl of *Campylobacter* cell suspension onto a blood agar plate (BAP) and with a 10µl loop streak for growth. Incubate at 37ºC for 48 hours.

Days 2 and 3

1. Check the plate; if the culture appears pure, pick an isolated colony, and inoculate a fresh plate for heavy growth; incubate microaerobically for 24 hours at 37°C. Use the growth from this plate to make PFGE plugs of the *C. jejuni* strains. Before making the plugs, transfer culture to fresh medium and incubate microaerobically at 37°C for 24 hours; this will ensure that the same culture can be retested, if necessary.

After the strains have been reconstituted according to the above directions, streak each culture to agar plates, pick an isolated colony, and subculture to another plate. Use the growth from the second plate to make the PFGE plugs. Please let me know if the package does not arrive in satisfactory condition, or if the cultures are not viable. **Freeze (-70°C) or stock these strains according to your laboratory's policy within 1 week of receiving them. Then**

your laboratory will have stock cultures of this PulseNet certification set for future use, including the PulseNet certification of additional personnel.

The strain numbers of the *Campylobacter* cultures are as follows:

D424 D445 D2261 D2579

Please follow these supplemental instructions for making the PFGE plugs and running the gels for these strains. Refer to the "One-Day (24-28 h) Standardized Laboratory Protocol for Molecular Subtyping of *Campylobacter jejuni* by Pulsed Field Gel Electrophoresis (PFGE)" (PulseNet QA/QC Manual PNL03) for detailed instructions for making the *Campylobacter* PFGE plugs and the "One-Day (24-28 h) Standardized Laboratory Protocol for Molecular Subtyping of *Escherichia coli*, *Salmonella* serotypes and *Shigella sonnei* by Pulsed Field Gel Electrophoresis (PFGE)" (PulseNet QA/QC Manual PNL05) to make the H9812 standard plugs.
Both sections are available in the PulseNet QA/QC Manual. This document is available in the QA/QC conference of the CDC Team Support or at www.cdc.gov/pulsenet/. If you can not access the protocol, please request it by sending an E-mail to PFGE@CDC.GOV.

1. Make 2-3 (disposable plug molds) or two (reusable plug molds) plugs of each test strain so they can be retested several times, if necessary.
2. Restrict one plug slice from the four test cultures (D424, D445, D2261, and D2579) with *Sma*I for 2 - 4 hours at 25°C and 2 plug slices of *Salmonella* ser. Braenderup H9812 standard with *Xba*I for at least 2 hours at 37°C.
3. Load H9812 in Lanes 1 and 6 and the test strains as follows:

Lane	Certification Strain	Enzyme
1	H9812	XbaI
2	D424	SmaI
3	D445	SmaI
4	D2261	SmaI
5	D2579	SmaI
6	H9812	XbaI

Note: If you want to use combs with 15 teeth, load the plug slices in lanes 3-9 and leave the other lanes empty. (The use of the ~0.5-mm wide 15-teeth comb [Bio-Rad, 170-4326] in the standard casting stand [14 x 13-cm] is not recommended for routine PFGE analysis of test isolates, but its use will be allowed for certification.).

4. Run the gel using the *Campylobacter jejuni* electrophoresis conditions. The run times listed in the protocol are based on the equipment and reagents used at CDC. If the gels generated in your lab do not have the lowest band in strain H9812 approximately 1-1.5 cm from the bottom of the gel, the run time may have to be changed.

5. Staining, de-staining, and gel documentation (imaging):
   a. The gel image should fill the entire window of the imaging equipment (computer) screen (without cutting off wells or lower bands). Individuals submitting certification TIFFs that do not contain the wells or clearly show the bottom bands of the patterns that will automatically be asked to rerun the gel and submit a new certification TIFF. Images that have a file size that is not within the range of ~300-400 kb will be deemed unsatisfactory and certification file evaluators will ask for a resubmission.

   b. TIFFs with one lane that contains many shadow bands or multiple lanes with one or more shadow bands (indicating incomplete restriction) should not be submitted for certification. To eliminate incomplete restriction wash the plugs at least two more times with TE Buffer before

restriction is repeated. If the problem persist repeat the restriction with more units of enzyme, for a longer amount of time and/or with a different lot of enzyme.

7. Bundle file creation
   a. In the bundle file, do not include lanes from more than 2 gels. Enter the corresponding CDC strain number in the BioNumerics "Key" field. The bundle file should contain 4 database entries (the 4 test strains) linked with *Sma*I.
   b. Create the bundle file using the lightning bolt icon (refer to the Gel Analysis Guidelines PND04 for instructions on how to create a PulseNet Bundle File). Bundles created using this icon are "PulseNet bundles" and will contain only standardized PulseNet experiments and fields. "PulseNet bundles" can be recognized by the "PN" that is automatically added to the file name. If a non-PulseNet bundle is submitted, you will automatically be asked to resubmit.

8. Send the TIFF file and/or bundle (*.bdl) file of your gel images to the CDC Pulse Net Database Administrator at PFGE@CDC.GOV within four weeks after receiving the strains.
   a. In the email to CDC, include ***Campylobacter* Certification** in the Subject line, so it can be forwarded to the correct person. Include the isolate number and restriction enzyme used for each lane on the gel in the body of the email. If you are sending certification TIFFs and bundle files for more than one organism, please send one email per organism.
   b. Name the TIFF and bundle files of your gel images as follows:
      TIFF: Use the unique identifier code that was assigned by CDC PulseNet for the first two to four letters of the file. The next 2 spaces will indicate the year the file was created. The next 3 spaces indicate the sequential number of the file submitted from your laboratory during a calendar year. For example: **GA09012.tif** is the twelfth file submitted from the state of Georgia during 2009. Bundle: The bundle files are named in a similar manner as the TIFF files with the first two to four letters of the file name indicating the unique identifier code. The next 2 spaces indicate the year the bundle file was created, and the next 3 spaces indicate the sequential bundle file number from each laboratory. For example, the eighth bundle file submitted from the state of Georgia during 2009 would be named **GA09008PN.bdl**.

For each PulseNet pathogen, an individual may be certified for:

a. Gels only (i.e., laboratory methods for PFGE and image acquisition)
b. Analysis only (i.e., BioNumerics analysis and on-line access to the database), or
c. Both gels and analysis.

After the gel images are submitted, the PulseNet certification file evaluator will analyze the gels and inform your laboratory of your results ("Satisfactory" or "Needs Improvement") within four weeks of receiving the files. If the TIFF images are satisfactory, the person who submitted the TIFF will be eligible to send gel images to PulseNet for analysis; if the bundle files are satisfactory, the person who submitted the bundle files will be issued a SecurID device (also called a fob) and will be granted on-line access to the appropriate PulseNet national database. SecurID devices are issued to individuals within a laboratory. The devices cannot be shared and must be returned to CDC if the certified individual leaves his or her position in the laboratory. If the submitted certification files are not satisfactory, the individual will need to review the troubleshooting comments received from the evaluator and resubmit once results have improved. If the submitter fails certification three times, the individual will not be allowed to submit again for six months. Before resubmitting, the individual will be expected to work with CDC and/or their PulseNet Area Laboratory until satisfactory results are achieved. This includes, but is not limited to, troubleshooting and training in the PulseNet PFGE protocols, BioNumerics and the PulseNet masterscripts.

Appendix PNQ02-6

Laboratory Protocol to Reconstitute Lyophilized (Freeze-Dried) *Vibrio cholerae* Cultures

This package contains lyophilized cultures of the four *Vibrio cholerae* strains that are used for PulseNet Certification. After the strains have been reconstituted according to the enclosed directions, streak each culture to agar plates, pick an isolated colony, and subculture to another plate. Use the growth from the second plate to make the PFGE plugs. Please let us know if the package does not arrive in satisfactory condition, or if the cultures are not viable. **Freeze (-70°C) or stock these strains according to your laboratory's policy using 24 – 48 h growth within one week after reconstituting them. Then, your laboratory will have stock cultures of this PulseNet certification set for future use, including the PulseNet certification of additional personnel**.

The strain numbers of the *Vibrio* cultures are as follows:

CDC-100 CDC-104 CDC-105 CDC-106

Please follow these supplemental instructions for making the PFGE plugs and running the gels for these strains. Refer to the "One-Day (24-28 h) Standardized Laboratory Protocol for Molecular Subtyping of *Vibrio cholerae* by Pulsed Field Gel Electrophoresis (PFGE)" (PulseNet QA/QC Manual PNL06) for detailed instructions for making the *Vibrio* PFGE plugs and the "One-Day (24-28 h) Standardized Laboratory Protocol for Molecular Subtyping of *Escherichia coli*, *Salmonella* serotypes and *Shigella sonnei* by Pulsed Field Gel Electrophoresis (PFGE)" (PulseNet QA/QC Manual PNL05) to make the H9812 standard plugs. Both sections are available in the PulseNet QA/QC Manual. This document is available in the QA/QC conference on CDC Team or at www.cdc.gov/pulsenet/. If you can not access the protocol, please request it by sending an E-mail to PFGE@CDC.GOV.

1. Make 2-3 (disposable plug molds) or two (reusable plug molds) plugs of each test strain so they can be retested several times, if necessary.
2. Restrict one plug slice from the four test cultures (CDC-100, CDC-104, CDC-105, and CDC-106) with *Sfi*I for 4 hours at 50°C and 3 plug slices of *Salmonella* ser. Braenderup H9812 standard with *Xba*I for at least 2 hours at 37°C.
3. Restrict one plug slice from the three test cultures (CDC-104, CDC-105, and CDC-106) with *Not*I for 4 hours at 37°C.
4. Load H9812 in Lanes 1, 6, 10 and the test strains as follows:

Lane	Certification Strain	Enzyme
1	H9812	XbaI
2	CDC-100	SfiI
3	CDC-104	SfiI
4	CDC-105	SfiI
5	CDC-106	SfiI
6	H9812	XbaI
7	CDC-104	NotI
8	CDC-105	NotI
9	CDC-106	NotI
10	H9812	XbaI

Note: If you want to use combs with 15 teeth, load the plug slices in lanes 2-11 and leave the other lanes empty. (The use of the ~0.5-mm wide 15-teeth comb [Bio-Rad, 170-4326] in the standard casting stand [14 x 13-cm] is not recommended for routine PFGE analysis of test isolates, but its use will be allowed for certification.).

5. Run the gel using the *V. cholerae* electrophoresis conditions. The run times listed in the protocol are based on the equipment and reagents used at CDC. If the gels generated in your lab do not have the lowest band in strain H9812 approximately 1-1.5 cm from the bottom of the gel, the run time may have to be changed.

6. Staining, de-staining, and gel documentation (imaging):

   a. The gel image should fill the entire window of the imaging equipment (computer) screen (without cutting off wells or lower bands). Individuals submitting certification TIFFs that do not contain the wells or clearly show the bottom bands of the patterns that will automatically be asked to rerun the gel and submit a new certification TIFF. Images that have a file size that is not within the range of ~300-400 kb will be deemed unsatisfactory and certification file evaluators will ask for a resubmission.

   b. TIFFs with one lane that contains many shadow bands or multiple lanes with one or more shadow bands (indicating incomplete restriction) should not be submitted for certification. To eliminate incomplete restriction wash the plugs at least two more times with TE Buffer before restriction is repeated. If the problem persist repeat the restriction with more units of enzyme, for a longer amount of time and/or with a different lot of enzyme.

6. Bundle file creation

   a. In the bundle file, do not include lanes from more than 2 gels. Enter the corresponding CDC strain number in the BioNumerics "Key" field. The bundle file should contain 4 database entries as follows: 3 test strains restricted with *Sfi*I and *Not*I and one test strain restricted only with *Sfi*I. If there are more than 4 entries in the bundle file, one or more lanes have not been linked properly.

   b. Create the bundle file using the lightning bolt icon (refer to the Gel Analysis Guidelines PND04 for instructions on how to create a PulseNet Bundle File). Bundles created using this icon are "PulseNet bundles" and will contain only standardized PulseNet experiments and fields. "PulseNet bundles" can be recognized by the "PN" that is automatically added to the file name. If a non-PulseNet bundle is submitted, you will automatically be asked to resubmit.

7. Send the TIFF file and/or bundle (*.bdl) file of your gel image to CDC at PFGE@cdc.gov within four weeks after receiving the strains.

   a. In the email to CDC, include **Vcholerae Certification** in the Subject line, so it can be forwarded to the correct person. Include the isolate number and restriction enzyme used for each lane on the gel in the body of the email. If you are sending certification TIFFs and bundle files for more than one organism, please send one email per organism.

   b. Name the TIFF and bundle files of your gel images as follows:

      TIFF: Use the unique identifier code that was assigned by CDC PulseNet for the first two to four letters of the file. The next 2 spaces will indicate the year the file was created. The next 3 spaces indicate the sequential number of the file submitted from your laboratory during a calendar year. For example: **GA09012.tif** is the twelfth file submitted from the Georgia Public Health Laboratory during 2009.

      Bundle: The bundle files are named in a similar manner as the TIFF files with the first two to four letters of the file name indicating the unique identifier code. The next 2 spaces indicate the year the bundle file was created, and the next 3 spaces indicate the sequential

bundle file number from each laboratory. For example, the eighth bundle file submitted from the Georgia Public Health Laboratory during 2009 would be named **GA09008PN.bdl**. Remember, "PN" is automatically added to the file name.

For each PulseNet pathogen, an individual may be certified for:

a. Gels only (i.e., laboratory methods for PFGE and image acquisition)
b. Analysis only (i.e., BioNumerics analysis and on-line access to the database), or
c. Both gels and analysis.

After the gel images are submitted, the PulseNet certification file evaluator will analyze the gels and inform your laboratory of your results ("Satisfactory" or "Needs Improvement") within four weeks of receiving the files. If the TIFF images are satisfactory, the person who submitted the TIFF will be eligible to send gel images to PulseNet for analysis; if the bundle files are satisfactory, the person who submitted the bundle files will be issued a SecurID device (also called a fob) and will be granted on-line access to the appropriate PulseNet national database. SecurID devices are issued to individuals within a laboratory. The devices cannot be shared and must be returned to CDC if the certified individual leaves his or her position in the laboratory.

If the TIFF images or bundle files are not satisfactory, the submitter will need to review the troubleshooting comments received from the evaluator and resubmit once results have improved. If the submitter fails certification three times, the individual will not be allowed to submit again for six months. Before resubmitting, the individual will be expected to work with CDC and/or their PulseNet Area Laboratory until satisfactory results are achieved. This includes, but is not limited to, troubleshooting and training in the PulseNet PFGE protocols, BioNumerics and the PulseNet Master Scripts.

Appendix PNQ02-7

Laboratory Protocol to Reconstitute Lyophilized (Freeze-Dried) *Vibrio parahaemolyticus* Cultures

Biological Safety Warning: *Vibrio parahaemolyticus* strains are considered Level 2 biological agents by the U.S. Department of Health and Human Services. Use appropriate precautions when handling the vial or culture. Carry out laboratory work in a biological safety cabinet when applicable to ensure aseptic conditions and personal safety.

Note: Store the lyophilized cultures at 4°C in the dark until they are reconstituted.

Materials Needed:

Sterile sturdy forceps
1 ml pipetman
1 ml sterile pipet tips
1 ul sterile inoculating loop

Reagents Needed:

Trypticase Soy + 5% Sheep Blood Agar plates (BAP) or equivalent media
Sterile grade reagent water or Trypticase Soy Broth (TSB)
70% isopropyl alcohol

Procedure for Reviving Cultures:

Day 1

1. Document the isolate number (s) and the date(s) lyophilized for your records. Wipe the aluminum cover and outside of the vial with isopropyl alcohol. Using sturdy forceps, aseptically remove the aluminum cover and rubber stopper from the vial containing the lyophilized culture. Wipe the outside of the rubber stopper and neck of the vial with isopropyl alcohol before removing the stopper.

2. Re-suspend the lyophilized cells with 1.0 ml of sterile grade reagent water. Allow to stand for a few minutes and/or mix gently to produce a uniform suspension. Pipet 100µl of cell suspension onto a blood agar plate (BAP) and with a 10µl loop streak for growth. Incubate at 37ºC overnight.

Days 2 and 3

1. Check the BAP; if the culture appears pure, pick an isolated colony, and inoculate a fresh BAP for heavy growth; incubate at 37°C overnight. Use the growth from this plate to make PFGE plugs of the standard strains. Before making the plugs, transfer culture to fresh medium and incubate at 37°C overnight; this will ensure that the same culture can be retested, if necessary.

After the strains have been reconstituted according to the above directions, streak each culture to agar plates, pick an isolated colony, and subculture to another plate. Use the growth from the second plate to make the PFGE plugs. Please let us know if the package does not arrive in satisfactory condition, or if the cultures are not viable. **Freeze (-70°C) or stock these strains according to your laboratory's policy using 24 – 48 h growth within one week after reconstituting them. Then, your laboratory will have stock cultures of this PulseNet certification set for future use, including the PulseNet certification of additional personnel**.

The strain numbers of the *Vibrio* cultures are as follows:

CDC#200-07 CDC#201-07 CDC#202-07 CDC#203-07

Please follow these supplemental instructions for making the PFGE plugs and running the gels for these strains. Refer to the "One-Day (24-28 h) Standardized Laboratory Protocol for Molecular Subtyping of *Vibrio paraheamolyticus* by Pulsed Field Gel Electrophoresis (PFGE)" (PulseNet QA/QC Manual PNL22) for detailed instructions for making the *Vibrio* PFGE plugs and the "One-Day (24-28 h) Standardized Laboratory Protocol for Molecular Subtyping of *Escherichia coli*, *Salmonella* serotypes and *Shigella sonnei* by Pulsed Field Gel Electrophoresis (PFGE)" (PulseNet QA/QC Manual PNL05) to make the H9812 standard plugs. Both sections are available in the PulseNet QA/QC Manual. This document is available in the QA/QC conference on CDC Team or at www.cdc.gov/pulsenet/. If you can not access the protocol, please request it by sending an E-mail to PFGE@CDC.GOV.

1. Make 2-3 (disposable plug molds) or two (reusable plug molds) plugs of each test strain so they can be retested several times, if necessary.
2. Restrict one plug slice from the four test cultures (CDC#200-07, CDC#201-07, CDC#202-07, CDC#203-07) with *Sfi*I for 4 hours at 50°C and 3 plug slices of *Salmonella* ser. Braenderup H9812 standard with *Xba*I for at least 2 hours at 37°C.
3. Restrict one plug slice from three of the three test cultures (CDC#200-07, CDC#201-07, and CDC#202-07) with *Not*I for 4 hours at 37°C.
4. Load H9812 in Lanes 1, 6, 10 and the test strains as follows:

Lane	Certification Strain	Enzyme
1	H9812	XbaI

2	CDC#200-07	SfiI
3	CDC#201-07	SfiI
4	CDC#202-07	SfiI
5	CDC#203-07	SfiI
6	H9812	XbaI
7	CDC#200-07	NotI
8	CDC#201-07	NotI
9	CDC#202-07	NotI
10	H9812	XbaI

Note: If you want to use combs with 15 teeth, load the plug slices in lanes 2-11 and leave the other lanes empty. (The use of the ~0.5-mm wide 15-teeth comb [Bio-Rad, 170-4326] in the standard casting stand [14 x 13-cm] is not recommended for routine PFGE analysis of test isolates, but its use will be allowed for certification.).

5. Run the gel using the *V. paraheamolyticus* electrophoresis conditions. The run times listed in the protocol are based on the equipment and reagents used at CDC. If the gels generated in your lab do not have the lowest band in strain H9812 approximately 1-1.5 cm from the bottom of the gel, the run time may have to be changed.

6. Staining, de-staining, and gel documentation (imaging):
   a. The gel image should fill the entire window of the imaging equipment (computer) screen (without cutting off wells or lower bands). Individuals submitting certification TIFFs that do not contain the wells or clearly show the bottom bands of the patterns that will automatically be asked to rerun the gel and submit a new certification TIFF. Images that have a file size that is not within the range of ~300-400 kb will be deemed unsatisfactory and certification file evaluators will ask for a resubmission.

   b. TIFFs with one lane that contains many shadow bands or multiple lanes with one or more shadow bands (indicating incomplete restriction) should not be submitted for certification. To eliminate incomplete restriction wash the plugs at least two more times with TE Buffer before restriction is repeated. If the problem persists repeat the restriction with more units of enzyme, for a longer amount of time and/or with a different lot of enzyme.

7. Bundle file creation
   a. In the bundle file, do not include lanes from more than 2 gels. Enter the corresponding CDC strain number in the BioNumerics "Key" field. The bundle file should contain 4 database entries as follows: 3 test strains restricted with *Sfi*I and *Not*I and one test strain restricted only with *Sfi*I. If there are more than 4 entries in the bundle file, one or more lanes have not been linked properly.
   b. Create the bundle file using the lightning bolt icon (refer to the Gel Analysis Guidelines PND04 for instructions on how to create a PulseNet Bundle File.). Bundles created using this icon are "PulseNet bundles" and will contain only standardized PulseNet experiments and fields. "PulseNet bundles" can be recognized by the "PN" that is automatically added to the file name. If a non-PulseNet bundle is submitted, you will automatically be asked to resubmit.

8. Send the TIFF file and/or bundle (*.bdl) file of your gel image to CDC at PFGE@cdc.gov within four weeks after receiving the strains.

   a. In the email to CDC, include **Vparahaem Certification** in the Subject line, so it can be forwarded to the correct person. Include the isolate number and restriction enzyme used for each lane on the gel in the body of the email. If you are sending certification TIFFs and bundle files for more than one organism, please send one email per organism.
   b. Name the TIFF and bundle files of your gel images as follows:

TIFF: Use the unique laboratory identifier code that was assigned by CDC PulseNet for the first two to four letters of the file. The next 2 spaces will indicate the year the file was created. The next 3 spaces indicate the sequential number of the file submitted from your laboratory during a calendar year. For example: **GA09012.tif** is the twelfth file submitted from the Georgia Public Health Laboratory during 2009.

Bundle: The bundle files are named in a similar manner as the TIFF files with the first two to four letters of the file name indicating the unique identifier code. The next 2 spaces indicate the year the bundle file was created, and the next 3 spaces indicate the sequential bundle file number from each laboratory. For example, the eighth bundle file submitted from the Georgia Public Health Laboratory during 2009 would be named **GA09008PN.bdl**. Remember, "PN" is automatically added to the file name.

For each PulseNet pathogen, an individual may be certified for:

a. Gels only (i.e., laboratory methods for PFGE and image acquisition)
b. Analysis only (i.e., BioNumerics analysis and on-line access to the database), or
c. Both gels and analysis.

After the gel images are submitted, the PulseNet certification file evaluator will analyze the gels and inform your laboratory of your results ("Satisfactory" or "Needs Improvement") within four weeks of receiving the files. If the TIFF images are satisfactory, the person who submitted the TIFF will be eligible to send gel images to PulseNet for analysis; if the bundle files are satisfactory, the person who submitted the bundle files will be issued a SecurID device (also called a fob) and will be granted on-line access to the appropriate PulseNet national database. SecurID devices are issued to individuals within a laboratory. The devices cannot be shared and must be returned to CDC if the certified individual leaves his or her position in the laboratory. If the TIFF images or bundle files are not satisfactory, the submitter will need to review the troubleshooting comments received from the evaluator and resubmit once results have improved. If the submitter fails certification three times, the individual will not be allowed to submit again for six months. Before resubmitting, the individual will be expected to work with CDC and/or their PulseNet Area Laboratory until satisfactory results are achieved. This includes, but is not limited to, troubleshooting and training in the PulseNet PFGE protocols, BioNumerics and the PulseNet Master Scripts.

Appendix PNQ02-8

Analysis Certification Protocol for *E. coli* Non O157 (STEC)

PulseNet participants may become analysis-certified for *E. coli* Non O157 (STEC) by analyzing a TIFF sent by CDC (KC09104.tif) and creating a PulseNet bundle file. There must be at least one person in your laboratory that is currently TIFF-certified in *E. coli* O157:H7 before anyone else from your laboratory becomes analysis-certified for *E. coli* Non O157.

1. Lane Information for KC09104.tif.

Note 1: If more than one person in your laboratory will be submitting this certification set, you may wish to rename the TIFF using your initials (i.e. JKA09104.tif or KC09104JKA.tif) before you import it into your local database (otherwise the TIFF will be overwritten each time it is imported by a different person).

Note 2: When assigning reference markers in step 3 (normalization) of analysis place the marker at the top of the band at 668.9 Kb and the top band of the doublet at 167.1 Kb as the reference standard in the database suggests.

Lane	Certification Strain	Enzyme
1	H9812	XbaI

2	3074-01	XbaI
3	3508-03	XbaI
4	K5159	XbaI
5	K4947	XbaI
6	H9812	XbaI
7	3074-01	BlnI
8	3508-03	BlnI
9	K5159	BlnI
10	H9812	XbaI

2. Bundle File Creation
   a. In the bundle file enter the corresponding CDC strain number in the BioNumerics "Key" field. If there are more than 4 entries in the bundle file, one or more lanes have not been linked properly. The standard lanes should never be linked.

   b. Create the bundle file using the lightning bolt icon (refer to the Gel Analysis Guidelines PND04 for instructions on how to create a PulseNet Bundle File). Bundles created using this icon are "PulseNet bundles" and will contain only standardized PulseNet experiments and fields. "PulseNet bundles" can be recognized by the "PN" that is automatically added to the file name. If a non-PulseNet bundle is submitted, you will automatically be asked to resubmit.

3. Send the bundle (*.bdl) file of your gel image to CDC at PFGE@cdc.gov.

   a. In the email to CDC, include ***E. coli* Non O157 Certification** in the Subject line, so it will be directed to the correct person. If you are submitting certification TIFFs and bundle files for more than one organism, please send one email per organism.

   b. Name the bundle file containing your analysis as follows:
   Bundle files are named in a similar manner as TIFF files with the first two to four letters of the file name indicating your unique laboratory identifier code. The next 2 spaces indicate the year the bundle file was created, and the next 3 spaces indicate the sequential bundle file number from each laboratory. For example, the eighth bundle file submitted from the Georgia Public Health Laboratory during 2009 would be named **GA09008PN.bdl**. Remember, "PN" is automatically added to the file name.

After your analysis bundle file is submitted, the PulseNet certification evaluator will analyze your submission and inform your laboratory of your results ("Satisfactory" or "Needs Improvement") within four weeks of receiving the files. If the bundle file is satisfactory, the person who submitted the bundle file will be issued a SecurID device (also called a fob) and will be granted on-line access to the appropriate PulseNet national database. SecurID devices are issued to individuals within a laboratory. The devices cannot be shared and must be returned to CDC if the certified individual leaves his or her position in the laboratory. If the bundle file is not satisfactory, the submitter will need to review the troubleshooting comments received from the evaluator and resubmit once results have improved. If the submitter fails certification three times, the individual will not be allowed to submit again for six months. Before resubmitting, the individual will be expected to work with CDC and/or their PulseNet Area Laboratory until satisfactory results are achieved. This includes, but is not limited to, troubleshooting and training in the PulseNet protocols, BioNumerics and the PulseNet Master Scripts.

Appendix PNQ02-9

Analysis Certification Protocol for *C. botulinum*

This package contains cultures of four *Clostridium botulinum* strains used for **PulseNet Certification**. Since this is a Select Agent transfer, your Responsible Official (RO) must complete Section 3 of APHIS/CDC Form 2 and send a copy to APHIS/CDC within two business days of receipt. If the package has been damaged to the extent that a release of select agent may have occurred, your laboratory's RO must immediately report to APHIS/CDC. Please refer to National Select Agent Registry website (http://www.selectagents.gov/TransferForm.html) for additional information.

Stock these four *C. botulinum* strains according to your laboratory's policy within one week after receiving them. Then, your laboratory will have stock cultures of this PulseNet certification set for future use, including the PulseNet certification of additional personnel.

The strain numbers of the *C. botulinum* cultures are as follows:

CDC5328 CDC44575 CDC37456 CDC55707

Please follow these supplemental instructions for making the PFGE plugs and running the gels for these strains. Refer to the "**Five Day Standardized Laboratory Protocol for Molecular Subtyping of *Clostridium botulinum* by Pulsed Field Gel Electrophoresis (PFGE**). This document is available as PNL25 in the QA/QC Manual conference on CDC Team or at www.cdc.gov/pulsenet/. If you cannot access the protocol, please request it by sending an E-mail to PFGE@CDC.GOV.

1. Streak each test culture for colony isolation onto EYA plates. Record isolate number and date on each plate. Incubate under anaerobic conditions at 37°C (± 2) for two days.

2. If the culture appears pure, pick an isolated colony and streak for colony isolation onto ANA-BAP plates. Incubate under anaerobic conditions overnight at 37°C ± 2°C.

3. Make two or three disposable plug molds plugs, or two reusable plug molds plugs of each test strain so they can be retested several times, if necessary.

4. Restrict three plug slices of *Salmonella* ser. Braenderup H9812 (the PulseNet Universal Standard Strain) with *Xba*I (60 Units/plug slice) for 2 hours at 37°C.

5. Restrict one plug slice from the four test cultures (CDC5328, CDC44575, CDC37456, and CDC55707) with *Sma*I (50 Units/plug slice) for 4 hours at 25°C.

6. Restrict one plug slice from three of the test cultures (CDC5328, CDC44575, and CDC37456) with *Xho*I (100 Units/plug slice) for 3 hours at 37°C.

7. Load H9812 and the test strains as follows:

Lane	Certification Strain	Enzyme
1	H9812	*Xba*I
2	CDC5328	*Sma*I
3	CDC44575	*Sma*I
4	CDC37456	*Sma*I
5	H9812	*Xba*I

6	CDC5328	*Xho*I
7	CDC44575	*Xho*I
8	CDC37456	*Xho*I
9	CDC55707	*Sma*I
10	H9812	*Xba*I

Note: If you use combs with 15 teeth, load the plug slices in lanes 2-11 and leave the other lanes empty (Although the use of the ~0.5-mm wide 15-teeth combs [Bio-Rad, 170-4326] in the standard casting stand [14 x 13-cm] is not recommended for routine PFGE analysis of test isolates, the use of the smaller comb teeth will be allowed for certification.).

8. Run the gel using the *C. botulinum* electrophoresis conditions. These run times are based on the equipment and reagents used at CDC. If the gels generated in your lab do not have the lowest band in strain H9812 approximately 1-1.5 cm from the bottom of the gel, the run time may have to be changed.

9. Staining, de-staining, and gel documentation (imaging):

    a. The gel image should fill the entire window of the imaging equipment (computer) screen (without cutting off wells or the bottom of the gel). An individual submitting a certification TIFF with wells cut off or bottom bands that are not visible will automatically be asked to rerun the gel and submit a new certification TIFF. Images that have a file size that is not within the range of ~300-400 kb will be deemed unsatisfactory and certification file evaluators will ask for a resubmission.

    b. TIFFs with one lane that contains many shadow bands or multiple lanes with one or more shadow bands (indicating incomplete restriction) should not be submitted for certification. To eliminate incomplete restriction wash the plugs at least two more times with TE Buffer before restriction is repeated. If the problem persists, repeat the restriction with more units of enzyme, for a longer amount of time and/or with a different lot of enzyme.

10. Bundle file creation
    a. In the bundle file, do not include lanes from more than 2 gels. Enter the corresponding CDC strain number in the BioNumerics "Key" field. The bundle file should contain 4 database entries as follows: 3 test strains restricted with *Sma*I and *Xho*I and one test strain restricted only with *SmaI*. If there are more than 4 entries in the bundle file, one or more lanes have not been linked properly.

    b Create the bundle file using the lightning bolt icon (refer to the Gel Analysis Guidelines PND04 for instructions on how to create a PulseNet Bundle File). Bundles created using this icon are "PulseNet bundles" and will contain only standardized PulseNet experiments and fields. "PulseNet bundles" can be recognized by the "PN" that is automatically added to the file name. If a non-PulseNet bundle is submitted, you will automatically be asked to resubmit.

11. Send the TIFF file and/or bundle (*.bdl) file of your gel images to CDC PulseNet at PFGE@CDC.GOV within four weeks after receiving the strains.

    a. In the email to CDC, include ***C. botulinum*** **Certification** in the Subject line, so it can be forwarded to the correct person. Include the isolate number and restriction enzyme used for each lane on the gel in the body of the email.

    b. Name the TIFF and bundle files of your gel images as follows:

TIFF: Use the unique identifier code that was assigned by CDC PulseNet for the first two or three letters of the file. The next 2 spaces will indicate the year the file was submitted. The next 3 spaces indicate the sequential number of the file submitted from your laboratory during a calendar year. For example: **GA10012.tif** is the twelfth file submitted from the state of Georgia during 2010.

Bundle: The bundle files are named in a similar manner as the TIFF files with the first 2 or three letters of the file name indicating the unique identifier code for your laboratory. The next 2 spaces indicate the year the bundle file was submitted, and the next 3 spaces indicate the sequential bundle file number from each laboratory. For example, the eighth bundle file submitted from the state of Georgia during 2010 would be named **GA10008PN.bdl**.

For each PulseNet pathogen, an individual may be certified for:

a. Gels only (i.e., laboratory methods for PFGE and image acquisition)
b. Analysis only (i.e., BioNumerics analysis and on-line access to the database), or
c. Both gels and analysis.

After the gel images are submitted, the PulseNet certification file evaluator will analyze the gels and inform your laboratory of your results ("Satisfactory" or "Needs Improvement") within four weeks of receiving the files. If the TIFF images are satisfactory, the person who submitted the TIFF will be eligible to send gel images to PulseNet for analysis; if the bundle files are satisfactory, the person who submitted the bundle files will be issued a SecurID device (also called a fob) and will be granted on-line access to the appropriate PulseNet national database. SecurID devices are issued to individuals within a laboratory. The devices cannot be shared and must be returned to CDC if the certified individual leaves his or her position in the laboratory. If the submitted certification files are not satisfactory, the individual will need to review the troubleshooting comments received from the evaluator and resubmit once results have improved. If the submitter fails certification three times, the individual will not be allowed to submit again for six months. Before resubmitting, the individual will be expected to work with CDC and/or their PulseNet Area Laboratory until satisfactory results are achieved. This includes, but is not limited to, troubleshooting and training in the PulseNet PFGE protocols, BioNumerics and the PulseNet masterscripts.

Appendix PNQ02-10

Shigella flexneri Analysis Certification

PulseNet participants may become analysis-certified for *S. flexneri* by analyzing a TIFF sent by CDC (MC14025.tif) and creating a PulseNet bundle file. There must be at least one person in your laboratory that is currently gel and analysis-certified in *Shigella sp*. before anyone else from your laboratory becomes analysis-certified for *Shigella flexneri*.

1. Lane Information for MC14025.tif.

Note 1: If more than one person in your laboratory will be submitting this certification set, you may wish to rename the TIFF using your initials (i.e. DS14025I.tif or MC14025DS.tif) before you import it into your local database (otherwise the TIFF will be overwritten each time it is imported by a different person).

Lane	Certification Strain	Enzyme
1	H9812	XbaI
2	AM46721	NotI
3	AM48331	NotI
4	2013c-3571	NotI
5	2012AM-0435	NotI
6	H9812	XbaI
7	AM46721	XbaI
8	AM48331	XbaI
9	2013c-3571	XbaI
10	H9812	XbaI

2. Bundle File Creation

   a. In the bundle file enter the corresponding CDC strain number in the BioNumerics "Key" field. If there are more than 4 entries in the bundle file, one or more lanes have not been linked properly. The standard lanes should never be linked.

   b. Create the bundle file using the lightning bolt icon (refer to the Gel Analysis Guidelines PND04 for instructions on how to create a PulseNet Bundle File). Bundles created using this icon are "PulseNet bundles" and will contain only standardized PulseNet experiments and fields. "PulseNet bundles" can be recognized by the "PN" that is automatically added to the file name. If a non-PulseNet bundle is submitted, you will automatically be asked to resubmit.

3. Send the bundle (*.bdl) file of your gel image to CDC at PFGE@cdc.gov.

   a. In the email to CDC, include ***Shigella Flexneri*** **Certification** in the Subject line, so it will be directed to the correct person. If you are submitting certification TIFFs and bundle files for more than one organism, please send one email per organism.

   b. Name the bundle file containing your analysis as follows:
   Bundle files are named in a similar manner as TIFF files with the first two to four letters of the file name indicating your unique laboratory identifier code. The next 2 spaces indicate the year the bundle file was created, and the next 3 spaces indicate the sequential bundle file number from each laboratory. For example, the eighth bundle file submitted from the Georgia Public Health Laboratory during 2009 would be named **GA09008PN.bdl**. Remember, "PN" is automatically added to the file name. If you find yourself attempting to add this extension, you have not created the bundle file correctly.

After your analysis bundle file is submitted, the PulseNet certification evaluator will analyze your submission and inform your laboratory of your results ("Satisfactory" or "Needs Improvement") within four weeks of receiving the files. If the bundle file is satisfactory, the person who submitted the bundle file will be issued a SecurID device (also called a fob) and will be granted on-line access to the appropriate PulseNet national database. SecurID devices are issued to individuals within a laboratory.

The devices cannot be shared and must be returned to CDC if the certified individual leaves his or her position in the laboratory. If the bundle file is not satisfactory, the submitter will need to review the troubleshooting comments received from the evaluator and resubmit once results have improved. If the submitter fails certification three times, the individual will not be allowed to submit again for six months. Before resubmitting, the individual will be expected to work with CDC and/or their PulseNet Area Laboratory until satisfactory results are achieved. This includes, but is not limited to, troubleshooting and training in the PulseNet protocols, BioNumerics and the PulseNet Master Scripts.

Please let us know if you have questions or further clarification is needed.

1. PURPOSE: To describe the procedure for evaluating certification files sent by PulseNet participating laboratories. This is part of the PulseNet QA/QC program.

2. SCOPE: This procedure applies to PulseNet personnel who evaluate certification files and certify individuals for gels (laboratory methods for PFGE and image acquisition) and/or BioNumerics gel analysis. It also applies to individuals who review certification reports.

3. DEFINITIONS/TERMS:

3.1 Certification files: TIFF and/or bundle files submitted by PulseNet participating laboratories for certification evaluation.
3.2 QA/QC: Quality Assurance/Quality Control
3.3 PFGE: Pulsed-field Gel Electrophoresis
3.4 BioNumerics: Gel analysis software used by PulseNet, developed by Applied Maths, Belgium
3.5 SOP: Standard Operating Procedure
3.6 TIFF: Tagged Image File Format, a file of a gel image that can be analyzed in BioNumerics
3.7 Certification file evaluator: An individual who evaluates certification files
3.8 TIFF quality: The grading of the appearance and ease of analysis of a TIFF according to the PulseNet TIFF Grading Guidelines. This is a main component of the evaluation of a TIFF submitted for certification
3.9 Gel analysis assessment: The grading of the whole analysis of a TIFF, including gel and lane definition, normalization, and band marking, according to the PulseNet Gel Analysis Guidelines. This is a main component of the evaluation of a bundle file submitted for certification
3.10 Bundle file: A file with a .bdl extension that is produced in BioNumerics and contains the analysis of at least one lane of a gel image
3.11 CDC: Centers for Disease Control and Prevention
3.12 Certification file reviewer: An individual who reviews and signs off on the certification reports submitted by the certification file evaluator
3.13 Comparison TIFFs: One or more TIFFs run by CDC for a specific pathogen for use in comparing PFGE patterns and band resolution against submitted certification TIFFs. Comparison TIFFs can also be a group of certification TIFFs submitted by several laboratories to monitor PFGE patterns and band resolution over several submitting laboratories. The latter is most easily accomplished through a saved list in BioNumerics
3.14 Comparison list: A list of analyzed lanes from comparison TIFFs that is saved in BioNumerics and used to compare to the analysis of the submitted TIFF by the certification file evaluator and the analysis of the submitter in the certification bundle file
3.15 Gel certified: Formerly "TIFF certified," an individual or laboratory that is certified in laboratory methods for PFGE and image acquisition
3.16 Analysis certified: An individual who is certified in BioNumerics gel analysis

4. RESPONSIBILITIES

4.1 Individuals performing PulseNet-related work (i.e., preparing PFGE gels and/or analyzing TIFF images) must submit certification file(s) and have them evaluated before being allowed to submit TIFF images and BioNumerics analyses directly to the PulseNet national databases. See PNQ02 for information on how to request certification sets.

4.2 Individuals evaluating certification files (evaluators) must:
4.2.1 Assess the TIFF quality and the ease of analysis of the TIFFs submitted for certification and assess the gel analysis, including band marking, of bundle files submitted for certification. All files submitted should be evaluated based on the current PulseNet standards of TIFF quality and gel analysis.
4.2.2 Evaluate submitted files and submit a written report, using the existing templates, to CDC reviewers within the time frame written in the certification instructions sent to the participating laboratories with the certification strain sets.

4.2.3 Modify reports based on CDC reviewers' comments, if necessary. Resubmit reports to CDC as soon as possible.

4.2.4 Keep electronic files in an organized folder system and keep hard copies of TIFFs, worksheets, reports, and any analyses (e.g., dendrograms, lane-to-lane comparisons) generated in files organized by laboratory.

4.3 Individuals reviewing certification reports submitted by the evaluator (reviewers) must:

4.3.1 Review submitted reports and their associated cover letters and certificates within the time frame written in the certification instructions sent to the participating laboratories with the certification strain sets.

4.3.2 Submit signed reports to the next reviewer or to the Database Team Leader (Kelley Hise).

5. PROCEDURE

5.1 TIFF and/or bundle files are received via email to the PFGE inbox are moved into the *organism* pending subfolder within the QA/QC folder. The person assigned to evaluate that organisms' certification file will retrieve the submissions from this location.

5.2 The email is printed and the attached files are saved to a folder on the hard drive (or network drive) that indicates which pathogen, which laboratory submitted the files, and which person in the laboratory submitted the files (P:\QAQC\Certification\Certification-Final Reports*organism**lab ID-initials of submitter*).

5.3 The file names and the date received are recorded in the appropriate Excel sheet (e.g., Salmonella Certification Status_Steward.xls). The Excel sheets are saved to P:\QAQC\Certification\Certification Tables and updated regularly.

5.4 When ready to analyze, open the TIFF in an image program. Invert to black bands on a white background, if not submitted this way. Print out the TIFF picture, filling the whole 8 ½ x 11" sheet with the image, if possible.

5.5 Grade the TIFF visually; using the TIFF Grading Guidelines and also compare PFGE patterns to hard copies of comparison TIFF(s).

5.5.1 If the TIFF is not passable (e.g., too much debris, bands not resolved well enough for analysis, one or more PFGE patterns are not correct), email the sender to repeat the TIFF. Give specific instructions about how to improve the next gel. Write on the printed email that you requested a repeat submission and file in your working folder. Record repeat submission request on the appropriate Excel sheet.

5.5.2 If the TIFF is passable, perform analysis as outlined in 5.6 below.

5.6 Analysis of certification TIFFs

5.6.1 Fill out the top third of a log-in and analysis sheet (login & analysis_worksheet.doc), recording information about the current certification files and about any previous certification files submitted but repeated.

5.6.2 In the middle third of the log-in and analysis sheet, record information about the gel quality. Use the PulseNet TIFF Grading Guidelines, circling a rating for each category listed (4 being the highest rating and 1 being the lowest). To the right of each category, record specific information pertaining to that rating (e.g., Bands: clear & distinct? 2 "Bands very fuzzy and difficult to analyze in some places".)

5.6.3 In the bottom third of the log-in and analysis sheet, record information about the following:

5.6.3.1 Standards – Are the standards in the correct lanes? Are the patterns correct and distinguishable according to the current PulseNet standards and comparison TIFF(s)?

5.6.3.2 TIFF: "By eye"-match comparison gel? – Are the PFGE patterns of the test lanes correct and distinguishable according to the current PulseNet standards and comparison TIFF(s)?

5.6.4 Analyze the TIFF in BioNumerics. Analyze all 10 lanes of the TIFF (standard and test lanes). Code each "key" entry with the Laboratory ID, the submitter's initials, and the strain number (e.g., CDC-KH-H9812).

5.6.4.1 If no bundle file was submitted, perform a dendrogram match with the comparison

list (the *Salmonella* list is named "2003_cert"; the *E. coli* list is named "2003comp" and the *Listeria* list is named "Compcert") using PulseNet standard settings. Proceed to step 5.6.6 of this document.

5.6.4.2 If a bundle file was submitted, go to step 5.6.5 below.

5.6.5 Bundle file analysis

5.6.5.1 Highlight the existing bundle file in BioNumerics and check the information before opening. Note whether there are non-PulseNet standardized fields and/or experiments in the bundle file. Make sure all entries are in the bundle file and linked correctly. If the lanes are linked incorrectly or if there are no standards in the bundle file, send an email to request a repeat submission, noting any other changes that need to be made in the repeat submission.

5.6.5.2 Open the bundle file and select the lanes (noting if the lanes are linked incorrectly).

5.6.5.3 Using PulseNet standard settings, perform a dendrogram match of the comparison list, the bundle file analysis, and the analysis of the TIFF by the evaluator. Proceed to step 5.6.6 of this document.

5.6.6 Analysis of TIFF and/or bundle file once dendrogram is generated in BioNumerics.

5.6.6.1 Print out a hard copy of the dendrogram and go through each isolate match, circling where the discrepancies are and looking at the submitted TIFF to see if that TIFF has a different band resolution than the comparison.

5.6.6.2 Underneath the dendrogram, record information for each isolate with a discrepancy. For example:

5.6.6.2.1 "A single band is resolved at ~235 kb. Some labs are resolving a doublet at this position." If some laboratories are resolving a particular area as a single band and some as a doublet, this is passable. If all labs are resolving a particular area as a doublet and the certification TIFF shows a thick single band, the TIFF should be repeated (do not pass the TIFF or bundle file for certification), especially if this occurs in more than one place on the TIFF.

5.6.6.2.2 "A doublet is marked on the bundle file at ~400 kb that appears as a single band on the TIFF." – If a laboratory "over-marks" several areas of the TIFF, request a repeat bundle file.

5.6.6.2.3 "A doublet is marked on the bundle file at ~170 kb. It is difficult to distinguish a doublet from a single band at ~170 kb on the TIFF." – Use this comment when bands are fuzzy and difficult to distinguish in a certain area and the submitter has marked the area on the bundle differently than the certification evaluator has marked it. Ask for a repeat submission if the fuzzy bands occur over more than several places on the TIFF and the evaluator feels that better band resolution would dramatically improve analysis.

5.6.6.2.4 "A band is marked in error at ~55 kb where no band appears on the TIFF". – If a laboratory marks stray bands in more than one place, request a repeat bundle file.

5.6.6.2.5 Standard lanes with bands not marked – ask for a repeat bundle file if standard bands are not marked at all. If band(s) at ~170 kb (the bands not used for normalization) are not marked, record standard comment about marking appropriate bands for normalization but mark all bands during band finding.

5.6.6.3 Note any bands marked on the bundle file that consistently appear above or below the bands marked by the evaluator. This could be due to incorrect normalization by the submitter. Check to make sure the standard the submitter used is correct. Ask for a repeat bundle submission if the normalization is incorrect. The submitter can be gel certified, but not analysis certified until a new bundle file is evaluated and passed.

5.6.6.4 In the bottom third of the log-in and analysis worksheet, record information about the dendrogram analyses:

5.6.6.4.1 TIFF: Analyzed TIFF matches comparisons? - How well does the analysis of the submitted TIFF by the certification file evaluator match the comparison list of TIFF(s) analysis? This should include information about the band resolution and ease of analysis of the submitted TIFF. A note of "See dendrogram notes" in this box on the worksheet may be sufficient.

5.6.6.4.2 Bundle: Analyzed TIFF matches bundle? – How well does the submitter's bundle file analysis match the analysis of the submitted TIFF by the evaluator? This should include information about inaccurate normalization or band marking. A note of "See dendrogram notes" in this box on the worksheet may be sufficient. If a bundle file was not submitted, no information should be recorded in this box.

5.6.6.4.3 Bundle: Bundle patterns match comparison patterns? – How well does the submitter's bundle file analysis match the patterns generated by CDC and other laboratories. The CDC patterns are those within the list used to compare to the bundle file in the dendrogram generated. If necessary, the certification file evaluator may perform a dendrogram match using submissions from other laboratories to see if doublets or single bands are being submitted in a certain area of a particular lane. If a bundle file was not submitted, no information should be recorded in this box.

5.6.6.4.4 Report Comments – Use this box for recording information such as if the band resolution is much better or much worse than other submissions.

5.7 After analysis, save the report template for that pathogen to the folder where the certification files were saved (P:\QAQC\Certification\Certification-Final Reports*organism**lab ID-initials of submitter*). Save the report template using the submitter's name (e.g., Salm 2003 cert report_KH.doc).

5.8 Complete the report

5.8.1 Type in the comments recorded on the dendrogram for each isolate with a discrepancy or other comment.

5.8.2 Type in suggestions for improvement and additional comments at the bottom of the report. Use the Standard Comments document (Appendix PNQ04-1) for wording and troubleshooting tips.

5.8.3 Type in information about previous submissions and the use of non-PulseNet standard fields and experiments in the bundle file at the bottom of the comments section. See Standard Comments document for wording.

5.8.4 Make sure the circles around the "TIFF satisfactory" and "bundle satisfactory" are correct for each report.

5.8.5 Print out two copies of the report – one to send to CDC and one for the evaluator's files.

5.9 After completing the report, save the cover letter template for that pathogen to the folder where the certification files and report were saved (P:\QAQC\Certification\Certification-Final Reports*organism**lab ID-initials of submitter*). Save the cover letter template using the submitter's name (e.g., Certification letter_DHHS letterhead_Salm_KH.doc). Add the current date and the name of the submitter to the cover letter. Change the check mark as appropriate. Print out one copy to send to CDC.

5.10 Open the Certification Certificates.ppt file and personalize the appropriate certificate. Print out one copy to send to CDC.

5.11 Record the information about the completed certification on the Excel sheet (e.g., "date certified," "date delivered to CDC").

5.12 Add the completed certification to a running list of all certifications completed by the evaluator. Record the name of the submitter and his/her laboratory, whether they were certified for gel and/or analysis, the pathogen, the date of the report (date analyzed), the date delivered to CDC, and any comments about the analysis or the TIFF.

5.13 Paperclip the cover letter, report, and certificate together. Send to the Database Team Leader (Kelley Hise) at CDC. The Team Leader will distribute the certifications among the Database Team for review. Send an email to the Database Team Leader (Kelley Hise). In the email, include a list of the certifications being sent to CDC. List the pathogen, the certified individual's name, the laboratory ID, and what the individual was certified for (i.e., gels and analysis, gels only, or analysis only).

5.14 Paperclip all the analyses, emails, TIFFs, etc. together with the log-in and analysis sheet on top, and file in evaluator's files in the appropriate laboratory folder.

5.15 Review certification files.

5.15.1 When received by CDC, the Database Team member and Methods Validation Laboratory Team member will review the reports in a timely manner in accordance with the certification instructions sent out with the strains to the participating laboratories.

5.15.2 The review will consist of checking the TIFF quality assessment and the comments associated with each analysis discrepancy. The reviewer will only sign his/her name if he/she agrees with the evaluator's assessment of the certification files. If the reviewer does not agree with the evaluator's assessment, the reviewer must contact the evaluator and discuss modifying the certification report.

5.15.3 Once the Database Team reviewer approves a certification, he or she must send an email to the PulseNet Technical Steward listing who was certified, their lab ID, organism for which they were certified, and what they were certified for (gels, analysis, or both).

5.16 TIFF images that pass certification and review indicate that the submitter is gel certified. Bundle files that pass certification and review indicate that the submitter is analysis certified. The submitter is considered certified as long as the laboratory successfully completes annual proficiency testing. If the submitted certification files do not pass the certification evaluation, the individual will need to review the troubleshooting comments received from the evaluator and resubmit once results have improved. If the submitter fails certification three times, the individual will not be allowed to submit again for six months. Before resubmitting, the individual will be expected to work with CDC and/or their PulseNet Area Laboratory until satisfactory results are achieved. This includes, but is not limited to troubleshooting and training in the PulseNet PFGE protocols, BioNumerics and the PulseNet masterscripts.

6. FLOW CHART:

7. BIBLIOGRAPHY:

8. CONTACTS:

8.1 Database Team Leader
Kelley Hise
KHise@cdc.gov
(404) 639-0704

8.2 PulseNet Technical Steward
Susan Hunter
SHunter@cdc.gov
(404) 639-1749

9. AMENDMENTS:

Appendix PNQ04-1.

Standard Comments for Certification and Proficiency Testing Reports

"General" Comments:

- A gel image that fills the entire window on the imaging equipment screen (without cutting off wells or the bottom of the gel) may improve analysis.
- A gel image that fills the entire window on the imaging equipment screen (without cutting off wells or the bottom of the gel) may make analysis easier.
- Lanes X-Y are skewed slightly but the skew does not interfere with the analysis.
- The TIFF file was renamed <labID>051602.tif by evaluator for analysis. In the future, please name all files sent to CDC according to the standardized PulseNet naming system. The first two digits should be the laboratory ID (e.g., GA), the second two digits should be the year (e.g., 04), and the last three digits should be the unique number of the TIFF or bundle file submitted that year from your laboratory (e.g., 001).

Band Marking:

- Consistency in band marking could be improved by marking thick bands as singlets unless two bands (a doublet) can be visualized on the TIFF.
- Bands down to the last band of the standard (~20 kb) should be marked. If test bands are close to the last band of the standard by a visual check of the TIFF, mark the band. Most labs are marking the bands at ~20 kb in lanes 7 and 8. Sometimes these bands run a little above the standard and sometimes they run a little below the standard.
- *Listeria* 2003 (03-H8394): A singlet is marked at ~150 kb on bundle file. Some laboratories are resolving and marking 2 bands in this area. [The light band directly under the bright band at ~150 kb should be marked on the bundle file.] [A singlet is marked at ~150 kb on bundle file. Some laboratories are resolving and marking the lighter area directly underneath the dark band at ~150 kb as a second band.]
- *Listeria* 2003 (04-H8395): A doublet is marked at ~50 kb on bundle file. Some laboratories are resolving and marking a third band at ~40 kb.

Standard Lanes:

- Please include standard lanes in bundle file for certification.
- Please place standards in the assigned lanes described in the certification or proficiency testing protocols.
- The outer two standard lanes skewed inward, but the skew did not interfere with the analysis.
- Bands of some of the standard lanes (especially lanes 1 and 15) are lighter than bands of the test strains. This did not affect analysis. However, more consistent standardization of cell concentrations among all test and standard strains would improve gel quality.
- The band distortion in the first and last lanes could cause inaccurate analysis if the band placement was not adequately checked during normalization.
- Although for normalization with standard strain H9812 the ninth band at ~180 kb is not marked, for band finding, it should be marked as it appears on the TIFF. In this case, it should be marked as a single band.

- *E. coli* 2003 H9812: The area at ~170 is marked as a single band on the bundle file, but appears as a doublet on the TIFF.

- *Listeria* 2003: The top two bands in each standard lane are closer together than on other certification gels. Make sure you are following the *Listeria* standardized protocol, especially the comb placement, for optimum band separation.

- *E. coli* 2003: In the H9812 standard lanes, a few laboratories are resolving doublets at ~170 kb, ~76 kb, and ~33 kb such as appears in lane 5 of this TIFF. Lane 5 has very good band resolution. When doublets are resolved in the noted areas, make sure the area of the doublet marked during normalization is consistent with the area of any single bands at the same position on other lanes. For areas resolved as doublets but normalized as one band, you may wish to normalize using the midpoint between the two doublet bands. However, in band finding, the bands should be marked as they appear on the TIFF.

Gel Not Clean/Shadow Bands Present:

- Bands are difficult to see on the TIFF and mark on the bundle file because the gel background is not clean. Spots and other debris may be reduced by cleaning all staining and destaining containers and preparing fresh reagents with clean glassware. Make sure that all agarose goes into solution when it is heated with 0.5X TBE.

- Background from DNA degradation (i.e., smearing in the lanes) may be reduced by carefully preparing new plugs to prevent shearing. Background may also occur because the agarose was too hot when the plugs were made or when the gel was poured and/or because of poor quality reagents. Washing the plugs at least two more times with TE Buffer may also reduce background.

- "Shadow" or "ghost" bands are present. These probably indicate incomplete restriction and should not be present on a gel. If shadow bands appear on gels in the future, repeat restriction with more units of enzyme, for a longer amount of time and/or with a different lot of enzyme. Wash the plugs at least two more times with TE Buffer before restriction is repeated.

- The *Bln*I pattern of 24-98 contains an extra faint band at ~140 kb. This band has been present on certification TIFFs received from other laboratories. This faint band may be the result of incomplete restriction or it could be due to a mutation in the strain itself. If this certification set must be used again, repeat restriction with more units of enzyme, for a longer amount of time and/or with a different lot of enzyme. Wash the plugs at least two more times with TE Buffer before restriction is repeated. If that does not eliminate the faint band, you may wish to consider requesting a new 24-98 strain from CDC by emailing PFGE@cdc.gov.

Top Bands:

- *Salmonella* 2003: The distance between the first and second bands of the patterns on the TIFF is less than on the comparison TIFFs. This difference may make comparison of your gels to patterns in the national database difficult, especially when the first bands of test strain patterns are near the top of the gel. For example, the distance difference appears to have affected the pattern of CDC 61-99 (*Bln*I). On the TIFF, a thick band appears at ~1070 kb for CDC 61-99 (*Bln*I) where a doublet appears on the comparison TIFFs. Make sure you are running the gels according to the standardized protocol on 1% SeaKem Gold agarose (BioWhittacker) with CHEF settings of 30 Kb – 700 Kb [2.2 s – 63.8 s] for *Salmonella* species. You may also try running the gel for a longer period of time (without cutting off bottom bands). Contact Mary Ann Fair (mal3@cdc.gov) for help if this does not solve the problem.

Bottom Bands:

- Running the gel so that the last band of the standard is approximately 1.0-1.5 cm from the bottom of the gel (per PulseNet protocol) may improve separation of bands.

- The certification was marked unsatisfactory because the bottom bands of the standard are not visible on XX.tif and are too low on XX.tif. Please run the gel so that the last band of the standard is approximately 1.0-1.5 cm from the bottom as per the PulseNet standard PFGE protocol.

- The bottom bands of the standard are too low. The gel should be run so that the last band of the standard is approximately 1.0-1.5 cm from the bottom per the PulseNet standard PFGE protocol.

Resolution:

- This is a good TIFF but could be improved by increasing band sharpness.
- Improvements in band resolution and gel background clarity will aid analysis and improve comparisons with the national database.
- Fuzzy bands may occur because of an imaging problem or because the agarose was too hot when the plugs were made or when the gel was poured, because of incomplete plug washing, and/or because of poor quality reagents. On future gels, you may want to check your TIFF imaging by determining if a fluorescent ruler placed beside the gel is in focus. If the ruler is not in focus, the bands on the gel may not be either. You may want to check the filter on your GelDoc system to make sure it is clean.
- On this gel, it appears that the wells are out of focus. This may indicate that the bands are not focused. On future gels, you may want to check your TIFF imaging by viewing the gel directly on the UV box or by determining if a fluorescent ruler placed beside the gel is in focus. If the ruler is not in focus, the bands on the gel may not be either.
- Bands seem to disappear at the bottom of the gel. This affected the bundle file analysis.
- Lanes with bands missing at the bottom of the gel and/or where the pattern is light may require repeating restriction with a larger plug slice, more units of enzyme, for a longer amount of time, and/or with a different lot of enzyme. Wash the plugs at least two more times with TE Buffer to remove excess lysis reagents or other impurities. Preparing new plugs with more concentrated cell suspensions may also improve band appearance. Light bands could also be the result of prolonged exposure to UV light before capture of the image.
- The bands are distinct, but, in most lanes, very light. Better standardization of cell concentrations among all test and standard strains would improve gel quality and TIFF imaging. Lanes where the pattern is light may require repeating restriction with a larger plug slice or preparing new plugs with more concentrated cell suspensions. Light bands could also be the result of prolonged exposure to UV light before capture of the image. [Can add: Make sure that the UV filter is in place while photographing the gel and that fresh ethidium bromide solution in the proper concentration (stock of 10 mg/ml diluted to 1:10,000 or 10 microliters in 100 ml reagent grade/deionized water) is used for staining gels.]
- The bands tend to be very light at the bottom of the gel. Correcting this may require repeating restriction with a larger plug slice, more units of enzyme, for a longer amount of time, and/or with a different lot of enzyme. You could also try washing the plugs at least two more times with TE Buffer to remove excess lysis reagents or other impurities. Light bands could also be the result of prolonged exposure to UV light before capture of the image.
- This gel appears to have a different band resolution than the comparison gels; some thick single bands on the comparison patterns appear as doublets on this gel.
- Improving the resolution of the lower molecular weight bands would improve analysis.
- Bands are fused, fuzzy, and difficult to distinguish. Use of a comb with 10 mm-wide teeth (instead of 5.5 mm-wide teeth) and running the gel for a longer time may improve the separation of bands.
- Better standardization of cell concentrations among all test and standard strains would improve gel quality.
- In some test lanes, thick bands (possibly due to a slight DNA overload) make doublets difficult to distinguish.
- There appears to be too much DNA in the lanes. Use smaller plug slices or prepare new plugs with less concentrated cell suspensions to improve separation of bands.

…to improve band appearance and separation for ease of analysis.

- The bands appear thick. This could be the result of too much DNA in the lanes or an over-integration issue in image capture. Use smaller plug slices or prepare new plugs with less concentrated cell suspensions to improve separation of bands. Compare your gel as it appears on the UV box to the TIFF image. If thick bands do not appear on the gel but do appear on the TIFF image, refer to the GelDoc manual for ways to improve your TIFF imaging (e.g., saturated pixels function).

- *Listeria*: Turning the comb around and running the gel so that the last band of the standard is approximately 1.0-1.5 cm from the bottom of the gel may improve separation of bands.

- Several doublets and triplets are difficult to distinguish from single bands and from doublets, respectively. For *Listeria* gels, the comb is turned around so that the teeth face the top of the gel; run the gel long enough so that the last band of the standard is approximately 1.0-1.5 cm from the bottom of the gel to help improve resolution and separation of bands.

- Bands appear wavy. This could be due to one or more of the following: plug damage from the pipet tip or spatula during removal from buffer or loading onto the gel; the comb is not clean; there are bubbles on the plug slices during loading on a gel; the plugs are not firm or are too thin, both of which may cause increased likelihood of damage from a pipet tip. Fragile plugs may be caused by the agarose concentration not being high enough or by overheated agarose.

- The bands appear wavy. Careful handling of the plugs during plug preparation and loading may reduce distortions. Make sure that the plug slices are firmly attached to the comb before slowly pouring the agarose into the gel mold so as not to dislodge the plug slices.

- Frowning bands could occur because of buffer flow or temperature fluctuations during the running of the gel, because the gels sat too long before they were run, because the plugs were too thick, or for another unknown reason. Bands with more curve than those on this TIFF would be difficult to analyze accurately.

- Several lanes contain bands that are distorted. Careful handling of the plugs during plug preparation and loading may reduce distortions.

Analysis:

- Comparing your band markings in the software to a hard copy of your original TIFF may improve analysis.

- Improvements in band resolution and gel background clarity will aid analysis and improve comparisons with the national database.

- During the first step of analysis in BioNumerics (1. Strips), place the top of the green box frame directly under the wells and the bottom of the green box frame at the bottom edge of the gel. This will standardize lane sizes, which may produce more accurate normalizations and improve comparisons with the national database.

- Also during the first analysis step, using the linear adjustment under “Edit tone curve” may provide better band clarity for analysis.

- The bands in the bundle appear dark compared to the bands in the comparison lanes. This may be the result of using the “enhance weak band” feature in the “edit tone curve” option under Step 1 of the BioNumerics analysis. Too much weak band enhancement may make analysis more difficult. Remember to compare your band markings in the software to a hard copy of your original TIFF.

- The gel strips in XX.bdl are too wide. After the lanes are defined in BioNumerics, adjust the “thickness” under “edit settings” to increase or decrease the thickness of the defined gel strips so that the left and right edges of the strips are just inside the outer edge of the bands. This will ensure that the bands will appear in a size for proper

analysis. If the gel strips are defined incorrectly (too wide), the resulting narrow bands with large white patches on either side of the bands are difficult to analyze.

- Do not overmark bands during analysis. If you think an area may be a doublet but no white space is apparent in the middle of the area or no indentations appear on the sides of the area separating two bands, mark the area as a single band or rerun a gel that has better band resolution to check the area.

- To decide if an area is a doublet or single band, look on the TIFF for white space in the middle of the area or indentations on the sides of the area separating two bands. If white space and/or indentations are present, mark the area as a doublet.

- Make sure the normalization markers are placed consistently from standard lane to standard lane at the bottom of the TIFF where doublets are resolved (~30 kb). The normalization markers should be placed on the doublet in the same way for each standard lane: either on the top band, the bottom band or in between the 2 bands of the doublet.

- The band marking in the bundle was good but could be improved by the presence of sharper bands and more consistency in band marking from lane to lane (e.g., marking thick bands as singlets unless two bands [a doublet] can be visualized on the TIFF).

- One extra band is marked at >2000 kb in the following lanes: Lane 5 (H2446), Lane 4 (H8395), and Lane 9 (H8395). To prevent this from happening in the future, you could do one or both of the following:
  a. When first going to Step 4 Bands in BioNumerics, look at the whole gel picture (without a zoom) and delete obviously extraneous bands. Make sure you have taken off the normalization so that all extraneous bands have been deleted. Note - It looks like you've drawn the top of your green box outlining the gel area (in Step 1 BioNumerics) low. Try putting the top of the green box directly under the wells next time.
  b. After band finding, run a dendrogram of all the analyzed lanes. Extraneous high bands (and low bands) will show up easily on the dendrogram.

- In the bundle file, a doublet was marked at ~160 kb. This area is difficult to distinguish from a singlet on the TIFF – there is little (if any) white area in the middle and no defined indentations on the side separating 2 bands. Most laboratories are resolving and marking a singlet at this position.

- Compare the doublet marked at ~270 kb in lane 8 (CDC 24-98, *Bln*I) on the bundle file to the singlet marked at ~360 kb in lane 2 (CDC 16-98, *Xba*I). Because the bands in the two areas look similar on this TIFF, they should be marked consistently. When determining whether a band is a singlet or a doublet, look for white space in between the bands and/or indentations (i.e., shoulders) on the sides of the area separating two bands.

- Before linking a lane to the local database, make sure the fingerprint type (e.g., PFGE-XbaI, PFGE-BlnI, etc.) is correct. Changing fingerprints is necessary when more than one enzyme is used for restriction on a particular gel. If the lanes are linked with incorrect fingerprint types, possible duplicate entries in the database could occur. To change the fingerprint type, right-click on the desired lane and select "Change fingerprint type of lane…" After the fingerprint type is changed, you can link the lane to an entry in the database. Both fingerprint types should be indicated with a green dot next to the one entry in the BioNumerics database.

- Previous TIFF files submitted on m/d/yy (AA.tif) and on m/d/yy (BB.tif) were unsatisfactory due to a large amount of specks present on the TIFF (AA.tif) and due to curving, distorted bands (BB.tif). The previous bundle file submitted (BB.bdl) contained too many bands at the bottom of CDC 24-98 and did not include the standard lanes.

- This report analyzes the bundle submitted mm/dd/yy. Previous bundles submitted mm/dd/yy and mm/dd/yy were edited to delete extra bands. The auto band finding feature in BioNumerics marked many extra bands on this TIFF. In addition, some single bands on the TIFF were marked as doublets in previous bundles.

- This report analyzes the bundle submitted mm/dd/yy. A previous bundle submitted mm/dd/yy (AA.bdl) was edited to mark bands at the bottom of the TIFF and to change a doublet to a single band, as it appeared on the TIFF.

- AA.tif and AA.bdl were analyzed for this report. Previous TIFFs submitted mm/dd/yy (XX.tif) and mm/dd/yy (YY.tif) were unsatisfactory due to poor band resolution and an incorrect pattern for 61-99 (*Bln*I), respectively.

- AA.bdl (submitted mm/dd/yy) was analyzed for this report. A previous bundle submitted mm/dd/yy (XX.bdl) was unsatisfactory due to inaccurate band finding in the H9812 standard lanes. Lane 3 of AA.tif contains a repeat of CDC 68-98.

- AA.bdl contained fields and experiments that are not in the standardized PulseNet scripts. After installing the December 2003 Master Scripts, create PulseNet bundle files using the bundle file lightning bolt icon on the left side of the BioNumerics screen (PulseNet BioNumerics Version 2.5 Training Manual, December 2003, Page 104). Bundle files created using this icon will contain only PulseNet fields.

- When you have edited your analysis, please create a PulseNet bundle (use the lightning bolt icon, see page 104 in the Appendix of the Dec. 2003 CDC BioNumerics manual) and submit your new bundle (you may call it XXb.bdl, if you wish) with your XX.tif file to PFGE@cdc.gov, including the words “E. coli certification” in the subject line.

1. PURPOSE: To describe the procedure for the performance and evaluation of proficiency testing within PulseNet. This is part of the PulseNet PFGE QA/QC program.

2. SCOPE: This procedure applies to PulseNet personnel at participating laboratories who perform proficiency testing and to the personnel who organize the proficiency testing rounds, ship proficiency testing strains, evaluate and review the proficiency testing submissions, and send out proficiency testing results.

3. DEFINITIONS/TERMS:

3.1 Proficiency Testing (PT): An annual assessment of the quality of the work being performed in PulseNet participating laboratories. For each pathogen, PFGE proficiency testing includes two parts – a "TIFF sent by CDC" and a "TIFF generated by the participating laboratory (i.e., in-house TIFF)."
3.2 QA/QC: Quality Assurance/Quality Control
3.3 PFGE: Pulsed-field Gel Electrophoresis
3.4 TIFF: Tagged Image File Format. A file of a gel image that can be analyzed in BioNumerics.
3.5 Certification files: TIFF and/or bundle files submitted by PulseNet participating laboratories for certification evaluation
3.6 SOP: Standard Operating Procedure
3.7 CDC: Centers for Disease Control and Prevention
3.8 Gel certified (or TIFF certified): An individual or laboratory that is certified in laboratory methods for PFGE and image acquisition
3.9 "In-house TIFF": TIFF generated by the participating laboratory. This is a part of the proficiency testing program where laboratories run a gel and produce a TIFF of the gel that contains the *Salmonella* Braenderup H9812 standards and the proficiency testing strain. The TIFF is analyzed and submitted to the organism-specific online PT database. If no one in the laboratory is analysis certified, they should refer to section 4.2.1.2 for submission instructions.
3.10 Analysis certified: An individual who is certified in BioNumerics gel analysis
3.11 BioNumerics: Gel analysis software used by PulseNet, developed by Applied Maths, Belgium
3.13 "TIFF sent by CDC": This is a part of the proficiency testing program where all laboratories analyze the same TIFF sent to them by CDC
3.14 TIFF quality: The grading of the appearance and ease of analysis of a TIFF according to the PulseNet TIFF Grading Guidelines (PNQ01). This is a main component of the evaluation of a TIFF submitted for certification.
3.16 Proficiency testing evaluation: A report that contains the evaluation and results of the participant's proficiency test. See template in appendix PNQ04-3.
3.17 Proficiency testing packet: An electronic PDF file including a cover letter and the proficiency testing evaluation that is emailed to the participant(s) who performed the PT, the primary PulseNet contact for the laboratory and the participant's laboratory director.
3.18 Certification file evaluator (evaluator): An individual who evaluates and signs off on the certification reports submitted by PulseNet participants.
3.19 Certification file reviewer (reviewer): An individual who reviews and signs off on the certification reports submitted by the certification file evaluator. There is a reviewer from the PulseNet Database Team and one from the PulseNet Methods and Development Laboratory for each organism.
3.20 EDLB: Enteric Diseases Laboratory Branch
3.21 ITSO ticket: An internal CDC request submitted to CDC's Information Technology Services Office via http://intranet.cdc.gov/itso/ServiceDesk/default2.htm

4. RESPONSIBILITIES

4.1 Individuals performing PulseNet-related work (i.e., preparing PFGE gels and/or analyzing TIFF images) must be certified before being able to participate in the proficiency testing program. See PNQ02 (SOP for Certification of PulseNet Personnel) for information on becoming certified.
4.2 Certified individuals at PulseNet participating laboratories must:

4.2.1 Perform proficiency testing annually for each pathogen in which they are certified, as part of continuing in-house QA/QC. All certified individuals in a laboratory perform proficiency testing in-house, and one set of results per pathogen per laboratory is selected and submitted to the organism specific proficiency testing database at CDC.

4.2.1.1 Gel certified individuals run and image a gel containing the proficiency testing strain for each pathogen in which they are certified. The TIFF generated by the participating laboratory (in-house TIFF) is produced.

4.2.1.2 If the laboratory does not have an analysis certified individual on staff, the TIFF file generated from the proficiency testing is sent via email to pfge@cdc.gov with the subject line of "*organism* Proficiency Testing." In addition, the lab must provide lane information (indicate what isolate is in what lane), including the standards.

4.2.1.3 Analysis certified individuals analyze the in-house TIFF and the TIFF sent by CDC in BioNumerics for each pathogen in which they are certified. They upload the analyses and TIFFs to the organism specific proficiency testing database at CDC.

4.2.1.4 An individual certified in gels and analysis may produce the TIFF generated by the participating laboratory, analyze the proficiency testing TIFFs, and upload the results. For some labs this may be a combined effort of multiple staff members, one certified to run gels and one certified to perform analysis and upload results to CDC.

4.3 Individual(s) preparing isolates for shipment must:

4.3.1 Exchange emails with the evaluator(s) in order to decide which strains will be sent out.

4.3.2 Order and pick up shipment supplies and contact the CDC shipping department two months in advance to schedule a shipping date and provide an estimate of what organisms and how many shipments will be going out (Domestic and International). Note: You will need to request UN3733 shipping containers for each shipment of *E. coli* O157:H7 and category B shipping bags for all other organisms at this time.

4.3.3 Prepare isolates for shipment as stated in the procedure section 5.2.

4.4 Individuals evaluating proficiency testing results (evaluators) must:

4.4.1 Send the individual(s) preparing isolates for shipment a list of certified laboratories scheduled to participate, edit documentation for the proficiency testing round, select the TIFFs sent by CDC, assist in selecting the proficiency testing strains, and contact the participating laboratories as stated under the procedure section 5.1.

4.4.2 Confirm and update participant's mailing addresses to create shipping labels

4.4.3 Submit an ITSO ticket to the attention of PulseNet's IT Support (see contacts section 8) to notify them of impending PT round. Give at least two weeks advance notice and make sure to note the expected shipment week for cultures. Work with PulseNet IT Support to confirm participant's access to the databases (go through the list to make sure the contacts are still PulseNet participants and discuss any discrepancies in records).

4.4.4 Assess the TIFF quality and ease of analysis of the TIFFs submitted for proficiency testing and assess the gel analysis of proficiency testing submissions. All TIFFs and analyses submitted should be evaluated based on the current PulseNet standards of TIFF quality (PNQ01) and gel analysis (PND04).

4.4.5 Use the "PT_Development" Microsoft Access database located in \\Cdc\project\CCID_NCZVED_DFBMD_PulseNet\QAQC\Proficiency Testing to evaluate the proficiency testing submissions. A short cut to access the database may be saved on your desktop for easy access. Instructions for using this database are listed under appendix PNQ04-6. Once all the reports are completed, email the CDC reviewers to let them know to review the reports and insert electronic signature and date.

4.4.6 Modify reports based on CDC reviewers' comments, if necessary. Resubmit reports to CDC reviewers as soon as possible.

4.4.7 Create the cover letters using the template saved here \\Cdc\project\CCID_NCZVED_DFBMD_PulseNet\QAQC\Proficiency Testing\Templates

4.4.8 Convert final PT packets (cover letters and evaluations) into one PDF. Email the laboratory's PT packet to the participant(s), primary PulseNet contact and laboratory director.

4.4.9 Using the existing templates \\cdc\project\CCID_NCZVED_DFBMD_PulseNet\QAQC\Proficiency Testing\Templates create summary documents once all results are complete and post within the PulseNet Announcements forum on the PulseNet/OutbreakNet SharePoint site.

4.5 The Individuals reviewing proficiency testing evaluations submitted by the evaluator (reviewers) must:

4.5.1 Review the submitted evaluations and send any feedback to the evaluator (i.e. if you disagree with any of the evaluator's decisions or if you would like to edit any portion of the report).

4.5.2 The database team reviewer enters their scanned signature and date on each report in the PT Access Database. Instructions are listed in appendix PNQ04-6. Once all the reports are signed, the database reviewer should notify the laboratory reviewer that the reports are ready for approval.

4.5.3 The laboratory reviewer enters their scanned signature and date on each report in the PT Access Database. Instructions are listed in appendix PNQ04-6. Once all the reports are signed, the laboratory reviewer should notify the evaluator that the reports are ready for distribution.

5. PROCEDURE

5.1 Evaluators organizing an upcoming proficiency testing round:

5.1.1 Contact the individual(s) preparing isolates for shipment of the proficiency testing strains to select strains for the round. Do this at least 6-8 weeks prior to desired shipment date.

5.1.2 Determine desired shipment date. Consult the individual(s) preparing isolates for shipment to make sure the proposed date is acceptable. Fall and Spring rounds are typically ~6 months apart (i.e. Fall ships *E. coli* O157:H7, *Salmonella and Shigella* in October, Spring ships *Listeria*, *Campylobacter*, *Vibrio parahaemolyticus and cholerae* in April).

5.1.3 Edit the participants list, proficiency testing instructions, cover letters, and address labels for use in the upcoming round of testing. Templates are located \\cdc\project\CCID_NCZVED_DFBMD_PulseNet\QAQC\Proficiency Testing\Templates

5.1.3.1 Email the participants list and address labels to the individual(s) preparing isolates for shipment at least two weeks prior to shipment date

5.1.4 Email international participants to request any necessary import permits. Participants may email or fax their permits to PFGE@cdc.gov or (404) 639-3333.

5.1.5 Determine which images (one per organism) to use for the "TIFF sent by CDC" portion of the test. If a suitable TIFF cannot be found and exported from the national database then request one from the CDC Methods and Development lab at least three weeks prior to shipment of PT strains. The TIFF needs to have both enzymes present (with the exception of *Campylobacter*) and be of good to excellent quality.

5.1.6 Once the PT strains have shipped, email all the participating laboratories. Include the cover letter, instructions, TIFFs sent by CDC and any other helpful information. Let them know when to expect the strains to arrive. See appendix PNQ04-1 for a template.

5.1.6.1 Record which strains were used during this round in the "PT Strains_Tracking.doc" saved \\Cdc\project\CCID_NCZVED_DFBMD_PulseNet\QAQC\Proficiency Testing

5.1.6.2 Make sure that PulseNet IT Support emails the Proficiency Testing Database logins to all of the participants the same week strains are shipped

5.1.7 Post the instructions and TIFFs sent by CDC on SharePoint within the PulseNet Announcements forum. Title the posting "*yyyy round* Proficiency Testing."

5.1.8 Email the participating laboratories approximately one to two weeks before the proficiency testing results are due, giving notice of the impending deadline.

5.1.8.1 Promptly respond to any participants who have requested deadline extensions.

5.2 Individuals preparing and shipping strains for proficiency testing:

5.2.1 At 4-6 weeks before strains are shipped:

5.2.1.1 Decide which strains will be sent to certified labs for proficiency testing by selecting two strains for each organism for preliminary testing.

5.2.1.2 Reconstitute at least two lyophilized vials of each strain, pick 2 different colonies from each isolation plate and make PFGE plugs from the four cultures for each strain.

5.2.2 Run a gel according to the appropriate organism specific PulseNet protocol with the new PFGE plugs. Confirm the patterns by sending the TIFF to the evaluators to compare to the previous patterns obtained for the strain.

5.2.3 At 3-4 weeks before strains are shipped:

5.2.3.1 Coordinate with the individual(s) organizing the proficiency testing round about the "TIFFs sent by CDC." If a suitable TIFF (excellent or good quality with H9812 standards in appropriate lanes and an appropriate strain cut with the primary and secondary enzymes) cannot be found by the evaluator, run a suitable TIFF that can be used for "the TIFF sent by CDC" portion of the PT.

5.2.3.2 Send the TIFF to the individuals organizing the proficiency testing to be sure that the gel image is satisfactory and select strain to be used for each organism so there will be time to repeat it or find another one, if necessary.

5.2.4 At 2-3 weeks before strains are shipped:

5.2.4.1 Receive list of participating laboratories organized by category of shipment. For example, *E. coli* only, *Salmonella* only, *Listeria* only, *E. coli* and *Salmonella*, *E. coli* and *Shigella*, *Salmonella* and *Shigella*, and *E. coli*, *Salmonella*, and *Shigella*.

5.2.4.2 Decide on the coding of the proficiency testing strains:

5.2.4.2.1 Strains are typically named using a two character descriptive of the organism (i.e. Salmonella: SA), P for Proficiency, a two digit year (i.e. 14 for 2014), a dash (-), then the CDC ID (i.e. 8099 for 80-99). For example the Salmonella PT strain with CDC ID 80-99 sent in 2013 was SAP13-8099. Consult with the evaluator for final strain designations.

5.2.4.2.2 Print out labels for:

5.2.4.2.2.1 Lyophilized vials – Include PulseNet Proficiency Test, Genus and species, Lyophilization Date, PT strain ID (SAP13-8099), and instructions for reconstituting (Reconstitute in 1ml H20).

5.2.4.2.2.2 Address/Return Labels for Category A shipments containing *E. coli* O157:H7- Instructions should read (If not claimed after 5 days return to: (Insert Shipper's Name). U. S. Department of Health and Human Services, Centers for Disease Control and Prevention, Phone#:(XXX) XXX-XXXX, 1600 Clifton Rd, Mailstop X, Atlanta GA, 30022" .

5.2.4.2.2.3 Labels defining contents of package- Ex. 1x1.0 ml Escherichia coli O157:H7, 1 x 1.0 ml Salmonella enterica serotype Typhimurium.

5.2.4.2.3 Replace the original label on the vials with the proficiency testing lyophilized vial label (this can be done the week before cultures are shipped).

5.2.4.2.4 Order and/or pick up enough of the following supplies for the shipments:

5.2.4.2.4.1 Shipping form CDC57.7, Rev. 7/2004 or submit electronic request (Scerison).

5.2.4.2.4.2 Peel-off mailing labels, CDC 0.689 Rev. 12/96 (Check with someone in the shipping department because they can supply labels with sender, mailstop and phone number already printed.)

5.2.4.2.4.3 Styrofoam sleeves- supplied by the Shipping Dept.

5.2.4.2.4.4 Pick up UN3733 shipping containers for each shipment of *E. coli* O157:H7 and Category B shipping bags supplied by the shipping department for all other organisms.

5.2.5 At 1-2 weeks before strains are shipped:

5.2.5.1 Coordinate with the shipping department to determine which days certain groups of shipments will go out. For example, the week of PT shipping all shipments containing only *E. coli* O157 will go out Tuesday, all shipments containing *E. coli* O157, *Salmonella* and *Shigella* will go out Wednesday in addition to the *Salmonella* only shipments. Adjust shipment date if necessary.

5.2.5.2 Send shipping department separate lists of addresses organized by category of shipment and number each address. For Example, *E. coli* O157 only (and number 1-10), *Salmonella* and *Shigella* (number 11-20), etc.

5.2.6 On the Thursday or Friday of the week before the strains are shipped:

5.2.6.1 Separate lyophilized proficiency testing strains according to shipment type (1 culture, 2 cultures, etc.) and put in labeled styrofoam sleeves (E and S; E, L and S, etc.). Refrigerate in shipment specific containers according to category (UN3733 shipping containers for each shipment containing *E. coli* O157:H7 and Category B shipping bags for all others)

5.2.6.2 Place a sticker indicating contents of shipment (refer to Section 5.2.4.2.2.3) on the outside of the container (for *E. coli* O157:H7 place it on the canister, for all others place on Category B bag).

5.2.6.3 For domestic shipments, fill out information on Shipping Form (CDC57.7, Rev. 7/2004) or submit electronic request through Scerison and address and phone number of recipient on mailing labels. Separate into appropriate categories.

5.2.6.3.1 If the same items are being shipped to 3 or more domestic labs, only one shipping form has to be filled out per order; in the "Ship To:" section, fill in the number of laboratories and attach a separate sheet with the numbered list of names, addresses and phone numbers of the labs receiving the cultures. The individual mailing labels still have to be completed for each lab.

5.2.6.3.2 International shipments require a separate shipping form for each laboratory and "Declaration for Export of Biologicals, Chemicals, Equipment, or Technical Data" Form (CDC 50.117 Rev. 3/2000) in triplicate. Include a copy of an Import Permit for each country, if required. Spell out both the genus and species name (i.e., *Escherichia coli*, and not *E. coli*) on the forms. If shipping *E. coli, Salmonella spp., Shigella spp., Vibrio spp*., fill out an "Enteric Checklist Form".

5.2.7 On the day of shipment

5.2.7.1 Bring necessary paperwork described above in section 5.2.6.3 and containers to the shipping department by 10:00 am so they will have time to pack and ship the same day. Ask them to notify you if shipments will be delayed.

5.2.7.2 Notify the evaluator once strains have shipped so they may notify laboratories and request that the labs let you know if the package does **not** arrive by Friday, so it can be tracked. The labs do not send the empty shipping boxes back to CDC.

5.3 Individuals at participating laboratories:

5.3.1 Must perform the proficiency testing according to the current proficiency testing instructions that are emailed and posted on SharePoint within the PulseNet Announcements forum.

5.3.2 All certified individuals in a laboratory should perform proficiency testing annually as part of in-house QA/QC. However, only one set of results per pathogen per laboratory should be chosen for upload/submission to the organism specific PT database at CDC.

5.3.3 Laboratories must submit proficiency testing TIFFs and analyses performed by certified individuals. Results submitted by non-certified individuals will automatically fail the proficiency testing round.

5.3.4 All results must be submitted correctly by the submission deadline to avoid penalty. Laboratories that cannot meet the deadline may submit a request for extension to PFGE@cdc.gov before the submission deadline.

5.3.5 Laboratories must submit a "submission email" as described in the PT instructions to the PFGE inbox after their results have been submitted.

5.3.6 Laboratories must pass proficiency testing annually to maintain certification.

5.3.6.1 Laboratories that fail one proficiency testing round must resubmit the proficiency testing results for that pathogen again using the same strains. The evaluator will email a failure notification letter (see appendix PNQ04-5) to the participant, primary PulseNet contact, laboratory director and the appropriate PulseNet Area Lab. Resubmission due dates will be determined by the evaluator.

5.3.6.2 Laboratories that fail for a particular organism two times in a row will lose their certification and must submit routine gels to PFGE@cdc.gov . Individuals at those laboratories must submit new certification files and pass certification again before reinstatement of their certification. Please see PNQ02 for PulseNet Certification information.

5.3.6.3 CDC has the right to revoke certifications at any time

5.4 Individuals evaluating proficiency testing results (evaluators):

5.4.1 In the last week of the deadline for submissions, send an email reminder to all the participating laboratories and check the submissions.
5.4.2 Open the PT_Development Access Database to begin evaluations
5.4.3 Evaluate one pathogen at a time
5.4.3.1 Click the green plus sign next to "New Analysis (PFGE)" to open the evaluation template. Fill in the appropriate information in the "Create New Analysis" box that opens (Year, Season, LabID, Organism and Resubmission). Click OK.
5.4.3.2 There are four tabs across the top: Gel Prep, Gel Analysis, Submission of Results and Other Info
5.4.3.2.1 Type in any comments or notes in the "Comments" column next to the appropriate section (e.g., normalization, band marking, etc.). The comments should explain any point deductions and/or any PFGE patterns that did not match the majority of submissions received.
5.4.3.2.2 In the "PT Comments" section, type in any TIFF grading comments that would explain the reason for the grade and add any more suggestions for TIFF improvement, any more detailed notes about the band marking or band resolution, etc.
5.4.3.2.3 Use the submission email sent by each laboratory to fill in the Other Info tab fields (e.g., person who prepared gel, equipment used, etc.).
5.4.3.2.4 Enter your scanned signature and date the reports as instructed in appendix PNQ04-6
5.4.3.2.5 Enter the images into each report as instructed in appendix PNQ04-6
5.4.3.2.6 Save the reports under \\Cdc\project\CCID_NCZVED_DFBMD_PulseNet\QAQC\Proficiency Testing*yyyy round*\Reports*organism* and notify the reviewer once they are ready for their review
5.4.4 TIFF evaluation
5.4.4.1 In the organism specific PT Admin database objectively grade the in-house TIFFs according to the PulseNet TIFF Grading Guidelines. TIFFs should receive a grade of excellent, good, fair, or poor. Record the corresponding points and any comments for improvement in the proficiency testing report (Gel Prep tab in Access).Refer to the Standard Comments for Troubleshooting for example statements \\cdc\project\CCID_NCZVED_DFBMD_PulseNet\QAQC\Troubleshooting.
5.4.5 Gel analysis evaluation
5.4.5.1 In the organism specific PT Admin database pull up all the proficiency testing submissions for that round in BioNumerics. Check to make sure all submissions are there, that they are linked properly, and that the strain numbers are entered correctly into the "Key field."
5.4.5.2 Divide all submissions for each pathogen into the two proficiency testing parts, the "TIFF sent by CDC" and the "TIFF generated in-house." Create a dendrogram for each enzyme of all the submissions for each part of the proficiency testing. Printing the dendrograms to write comments and note discrepancies as a whole may be helpful while evaluating and when you begin to compile the summary documents to post on SharePoint. Saving the submissions as comparisons may also be helpful.
5.4.5.3 Record the corresponding points and any comments for improvement in the proficiency testing report (Gel Analysis tab in Access).
5.4.5.4 "TIFF sent by CDC" evaluation
5.4.5.4.1 Compare band markings to the CDC submission, which should be analyzed according to the PulseNet Gel Analysis Guidelines.
5.4.5.4.2 On the dendrogram, pay attention to any areas with discrepancies as compared to the CDC submission and the majority of submissions. Record any comments about the discrepancy and at what molecular weight the discrepancy took place on the appropriate laboratory's evaluations. Only deduct points for areas that are marked differently than they are resolved.
5.4.5.4.3 Open the TIFFs and check for any normalization discrepancies. This could be indicated by bands on a submission that are consistently lower or higher than other submissions received. For the "TIFF sent by CDC," all laboratories are analyzing the same TIFF, so all bands should be the same down the dendrogram. Record any comments for normalization or if the submission should be failed because of a normalization error.

5.4.5.4.4 Record any submissions that are to have band marking points deducted, and record the number of deducted points.

5.4.5.4.4.1 Three points are deducted for each discrepancy ≥300 kb (eyeball the top part of the lane)

5.4.5.4.4.2 Two points are deducted for each discrepancy from 299-100 kb (eyeball the middle of the lane)

5.4.5.4.4.3 One point is deducted for each discrepancy <100 kb (eyeball the bottom part of the lane)

5.4.5.5 "TIFF generated in-house" evaluation

5.4.5.5.1 Look at the band markings compared to the CDC submission which should be analyzed according to the PulseNet Gel Analysis Guidelines (PND04). This will help determine the quality of the band resolution of the submissions.

5.4.5.5.2 Compare each submission (i.e., each lane) to the actual TIFF submitted by the participating laboratory.

5.4.5.5.2.1 Note whether the band marking matches the TIFF, according to the PulseNet Gel Analysis Guidelines (PND04). If the band marking does not visually match the TIFF, make a comment on the appropriate laboratory's evaluation about the discrepancies and at what molecular weight the discrepancies took place. If the band marking matches the TIFF but is different from the majority of submissions received, note that on the dendrogram as well. If necessary, deduct appropriate number of points as described in section 5.4.5.3.4.

5.4.5.5.2.2 Check for any normalization discrepancies on the TIFF. Normalization issues could also be seen by looking at the dendrogram for bands on a submission that are consistently lower or higher than other submissions received. Record any comments for normalization or if the submission should be failed because of a normalization error.

5.4.5.5.2.3 Toggle between the normalization step and analysis step to be sure that there are no bands marked above or below the bands of the reference system (when automatic band marking is used sometimes bands are marked outside of the reference system)

5.4.6 Cover Letter

5.4.6.1 Use the cover letter template in appendix PNQ04-4 and edit the document as necessary. Save on the network drive as "LabID_Cover Letter.doc" \\cdc\project\CCID_NCZVED_DFBMD_PulseNet\QAQC\Proficiency Testing*yyyy Round*\Reports\Cover Letters. Do this for each participating laboratory.

5.4.7 When all reports are finalized, create a PT summary posting showing summary statistics for the proficiency testing round (e.g., number of laboratories participating, number that failed, number of excellent TIFFs, etc.). Templates can be found \\cdc\project\CCID_NCZVED_DFBMD_PulseNet\QAQC\Proficiency Testing\Templates.

5.4.8 Create summary documents for posting on SharePoint. These documents should summarize the equipment and enzyme manufacturers used for the proficiency testing survey and also show pertinent information about the proficiency testing results. For examples please see the following: "TIFF examples.ppt" and "Summary of Enzyme and Equipment.xls" Templates can be found \\cdc\project\CCID_NCZVED_DFBMD_PulseNet\QAQC\Proficiency Testing\Templates.

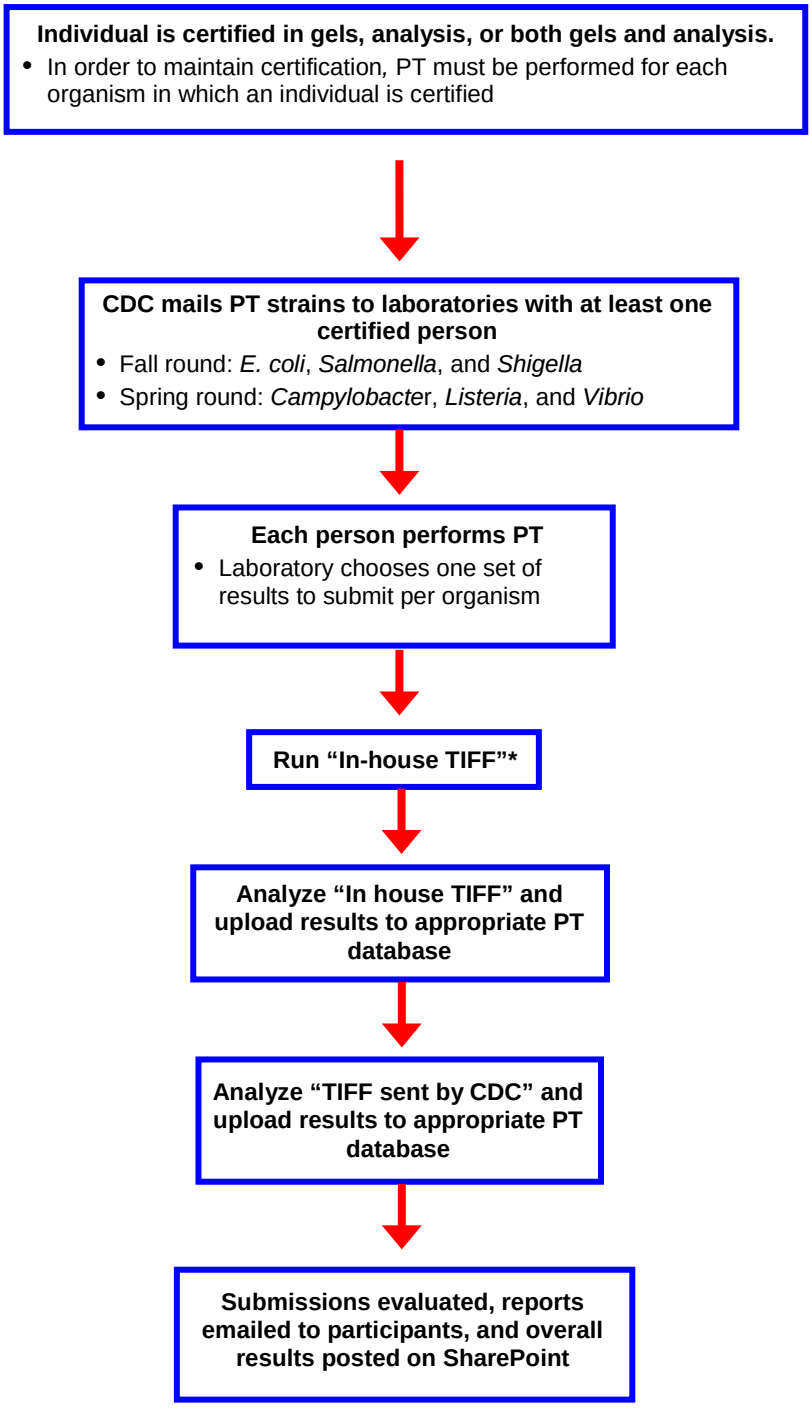
5.5 Individuals reviewing proficiency testing packets (reviewers).

5.5.1 The review will consist of checking the TIFF quality assessment and the comments associated with each analysis discrepancy. The reviewer will enter their electronic signature if he/she agrees with the evaluator's assessment of the proficiency testing files. If the reviewer does not agree with the evaluator's assessment, the reviewer must contact the evaluator and discuss modifying the proficiency testing report.

5.6 Evaluators sending out proficiency testing packets:

5.6.1 Convert final PT packets (cover letters and evaluations) into one PDF. Save the final PDFs on the network drive \\cdc\project\CCID_NCZVED_DFBMD_PulseNet\QAQC\Proficiency Testing*yyyy round*\Final Reports. Email the laboratory's PT packet (final PDF) to the participant(s), main PulseNet contact and laboratory director. Refer back to section 5.3.6 if a laboratory should fail the proficiency test.

6. FLOW CHART:


Individual is certified in gels, analysis, or both gels and analysis.
• In order to maintain certification, PT must be performed for each organism in which an individual is certified
CDC mails PT strains to laboratories with at least one certified person
• Fall round: E. coli, Salmonella, and Shigella
• Spring round: Campylobacter, Listeria, and Vibrio
Each person performs PT
• Laboratory chooses one set of results to submit per organism
Run "In-house TIFF"*
Analyze "In house TIFF" and upload results to appropriate PT database
Analyze "TIFF sent by CDC" and upload results to appropriate PT database
Submissions evaluated, reports emailed to participants, and overall results posted on SharePoint

7. BIBLIOGRAPHY:

8. CONTACTS:

8.1 CDC PulseNet Database Unit
(404) 639-4558
PFGE@cdc.gov

8.2 CDC PulseNet Methods and Development Laboratory Unit
(404) 639-4558
PFGE@cdc.gov

8.3 CDC Shipping: Yvonne Stifel
(404) 639-3355
YStivel@cdc.gov

8.4 PulseNet Information Technology
Brenda Brown
(404) 639-3942
bsb6@cdc.gov

9. AMENDMENTS:

9.1 July 2014: Entire document was updated to reflect a paperless process for sending proficiency testing instructions and final evaluations. PT instructions and results will no longer be mailed; all instructional documents will be emailed and posted on SharePoint within the PulseNet Announcements forum. PT database logins will be emailed separately. All final evaluations will be emailed to the participant(s), the primary PulseNet contact and the laboratory director.

Appendix PNQ04-1

Template email announcing PT shipment of strains and TIFFs sent by CDC

Greetings everyone,

It is time again for PulseNet Proficiency Testing (PT). Persons who were certified for [*Insert the organisms included in the round of PT*] on or before [*month date, year*] are expected to complete PT this round. Each participating laboratory receives a PT strain and a "TIFF sent by CDC" for the organism(s) for which the laboratory is currently certified. Please make sure to share this information with other certified personnel within your laboratory. The strains and emails are only being sent to one person per laboratory, however all instructions and TIFFs have also been posted on SharePoint within the PulseNet Announcements forum.

The PT strains for [*Insert the organisms included in the round of PT*] were shipped on [*date*]. PT database logins are being sent in a separate email this week. If you do not receive the PT strains and/or PT database logins by [*date*], please let us know.

The attached TIFFs are to be analyzed and submitted for the "TIFFs sent by CDC" portion of PT. Lane information may be found in the attached instructions.

If you have any questions, email PFGE@cdc.gov with "Proficiency Testing" in the subject line.

Thank you,

[Name of contact(s)]
PulseNet QA/QC Program
[Phone number(s)]

Appendix PNQ04-3

Proficiency Testing Evaluation Template

NOTE: When evaluating a PT resubmission, the title of the report should be **(*Round #, i.e. 10th*) PulseNet Proficiency Testing Survey (*round yyyy*) Resubmission Report Form**

(*Round #, i.e. 10th*) PulseNet Proficiency Testing Survey (*round yyyy*) Report Form

Organism:	Date lyophilized:	Date results received TIFF by CDC:
Laboratory:	Date strains shipped:	TIFF by lab:

	Possible Points	No. of Points Received	Comments
I. Gel Preparation (50 pts)			
A. Overall gel and TIFF quality	Excellent (Pass 20 pts) Good (Pass 15 pts) Fair (Pass 10 pts) Poor (Fail)		
B. Correct pattern (visual) attained in:			
1. Lane containing *[primary enzyme]* pattern	Pass (13 pts) or Fail		
2. Lane containing *[secondary enzyme]* pattern	Pass (13 pts) or Fail		
C. Were directions followed?			
1. Correct file name	1 pt		
2. Run in requested lanes	1 pt		
3. Run on routine gel	1 pt		
4. Correct standard and pattern	Pass (1 pt) or Fail		
II. Gel Analysis - TIFF generated by participating lab (22 pts)			
A. Correct normalization for:			
1. Lane containing *[primary enzyme]* pattern	6 pts or Fail		
2. Lane containing *[secondary enzyme]* pattern	6 pts or Fail		
B. Band marking compared to Guidelines for:			
1. Lane containing *[primary enzyme]* pattern	5 pts		
2. Lane containing *[secondary enzyme]* pattern	5 pts		
III. Gel Analysis - TIFF sent by CDC (22 pts)			
A. Correct normalization for:			
1. Lane containing *[primary enzyme]* pattern	6 pts or Fail		
2. Lane containing *[secondary enzyme]* pattern	6 pts or Fail		

B. Band marking compared to Guidelines for:	
1. Lane containing *[primary enzyme]* pattern	5 pts
2. Lane containing *[secondary enzyme]* pattern	5 pts

IV. <u>Submission of Results</u> (6 pts)

A. All results sent/uploaded for:	
1. TIFF generated by participating laboratory	Pass (1 pt) or Fail
2. TIFF sent by CDC	Pass (1 pt) or Fail
B. TIFF file generated in-house was uploaded	2 pts
C. Correct strain numbers used in BioNumerics	1 pt
D. Lanes linked correctly in BioNumerics	1 pt

Total points received	
Overall proficiency testing result	Pass or Fail

Person who prepared the gel:

Person who performed the analysis:

Person who uploaded the analysis:

Equipment used:

Enzymes ordered from:

Proficiency Testing Comments:

Some or all items in I-A, I-B, I-C, II-A, III-A, and IV-A on the report form are in Pass / Fail format. If you fail any one of the questions, you fail the proficiency testing round. Laboratories that pass will accumulate the specified points. For band marking, all isolates were compared to the PulseNet Gel Analysis Guidelines. If band marking differed from the guidelines, up to 5 points were deducted for each band difference according to the position of the difference in three organism-dependent zones as follows: -3 points in the top 1/3 of the gel; -2 points in the middle 1/3 of the gel; -1 point each (up to 4 points) in the bottom 1/3 of the gel. A passing score is >=85% (>=85/100).

Proficiency Testing Analysis:

Performed By: _____ **Date of Report:**

name, email@cdc.gov, phone number

Reviewed By: _____ **Date of Review:**
name, email@cdc.gov, phone number

Approved By: _____ **Date of Approval:**
name, email@cdc.gov, phone number

[A COPY OF THE SUBMITTED IMAGE SHOULD BE INSERTED HERE]

Appendix PNQ04-4

Proficiency Testing Cover Letter Template

Month yyyy

Dear *Participant(s), Primary PulseNet Contact,* and *Lab Director*,

The *Fall/Spring yyyy* round of PulseNet Proficiency Testing for *Organisms* has been completed. The results for your laboratory are enclosed.

Below is the breakdown of the number of laboratories who have passed the *Fall/Spring yyyy* round:

Salmonella: 81/84 laboratories passed 6 are currently pending*
E. coli: 78/80 laboratories passed 4 are currently pending*
Shigella: 61/62 laboratories passed 5 are currently pending*

*Laboratories that did not pass were notified in *month* along with a request for resubmission.

A score of 85 points or higher was needed to pass this proficiency testing round. A thorough description of the scoring system is included in each report, as well as specific comments and suggestions for your laboratory, when applicable. Detailed summary reports and tips for improvement are posted in PulseNet Announcements on SharePoint. PulseNet laboratory staff should have access to this forum.

Successful completion of the Proficiency Testing for the specified organisms maintains your standing as a certified PulseNet laboratory. If you have any questions please contact me or send an email to PFGE@cdc.gov. We appreciate your participation and continued support of PulseNet.

Sincerely,

name
contact info

Cc: Food Safety Team, Association of Public Health Laboratories
PulseNet Unit, Centers for Disease Control and Prevention

Appendix PNQ04-5

Proficiency Testing Notification of Failure Template

[Save on CDC Letterhead]

[Date]

[Name and info of lab director],

The [*laboratory*] PulseNet laboratory did not pass the [*round yyyy*] round of the PulseNet Proficiency Testing for [*organism]*. [Insert reason for fail]. The *[laboratory*] laboratory must resubmit the [*error example: analysis of the "TIFF sent by CDC" with proper normalization*] by [*due date*.]

[*Person*] was notified on [*date*] and a resubmission was requested by the above mentioned deadline. Comments and suggestions for improvement were provided. PulseNet participants are always reminded to contact their designated PulseNet Area Laboratory or CDC with any questions or for troubleshooting assistance.

If no resubmission is received, or if the resubmission is not satisfactory, this round of proficiency testing is considered failed. If the resubmitted results are satisfactory, the round is recorded as a pass. Resubmission evaluations will be emailed to participants and their laboratory directors upon completion.

Please let us know if you have any questions or concerns.

Thank you,

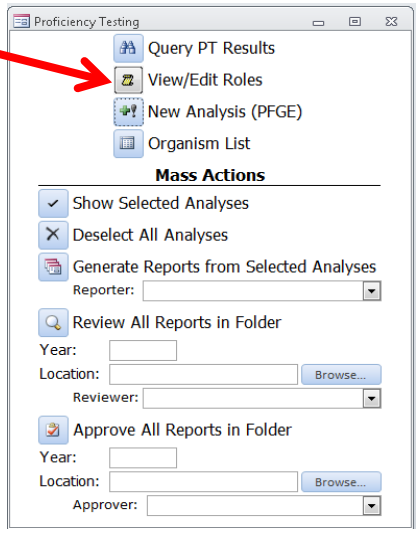
Name
Contact info

Appendix PNQ04-6

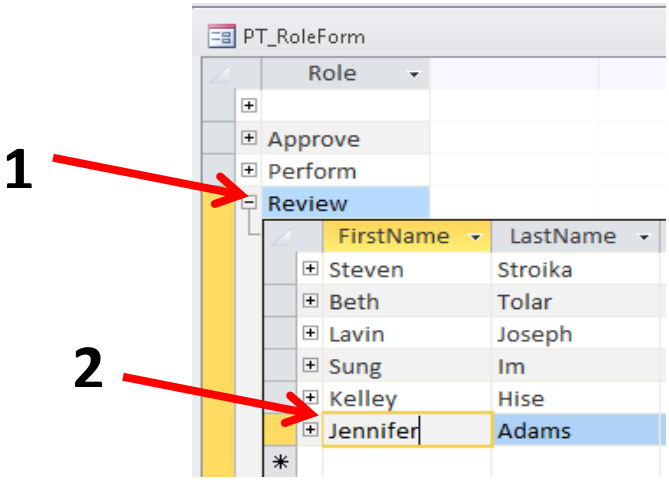
Instructions for Setup and Use of the PT_Development Access Database

The location of the database is \\Cdc\project\CCID_NCZVED_DFBMD_PulseNet\QAQC\Proficiency Testing
For those who evaluate, review or approve PT results you may wish to create a desktop shortcut for easy access.

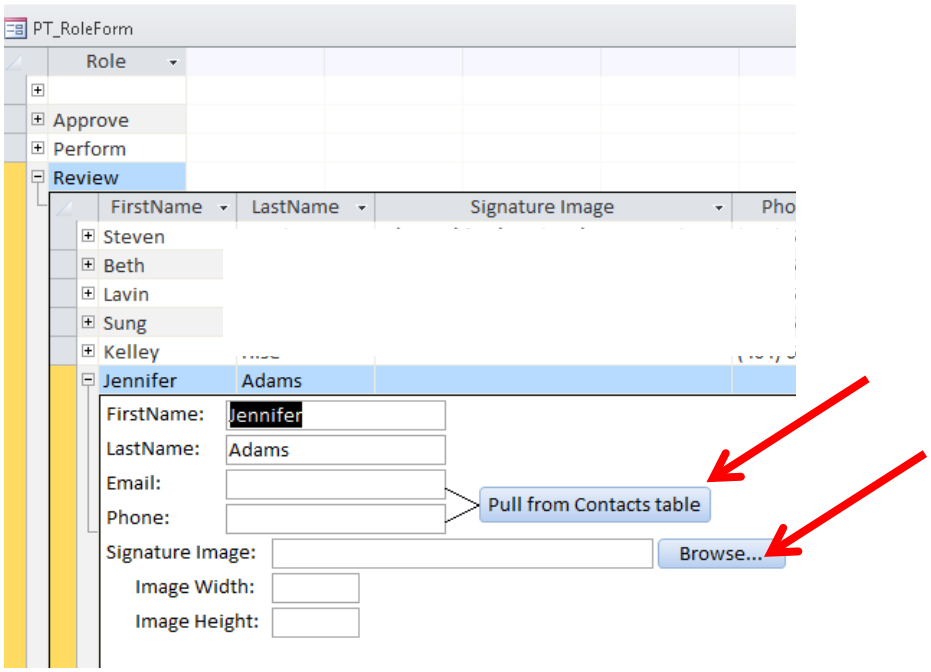
1. To add your personal information (name, phone, email and signature), open the PT_Development Access Database and click the View/Edit Roles button



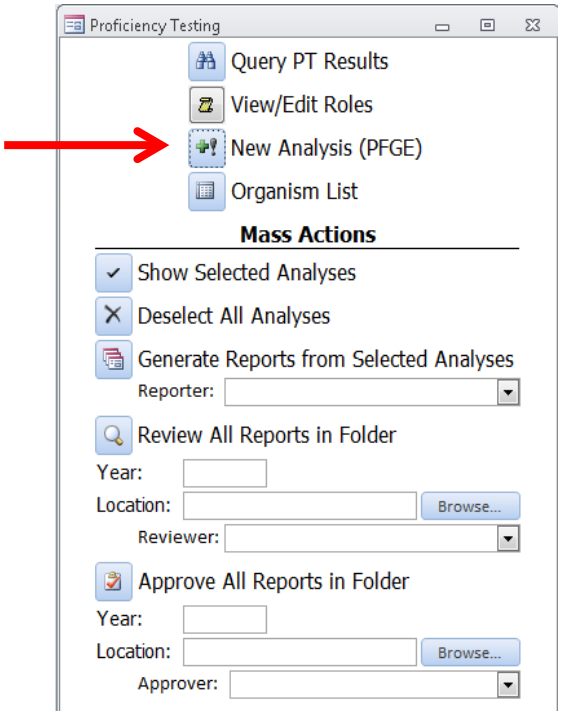
2. Click the plus sign next to your role (evaluators click “perform”, database reviewers click “review”, laboratory reviewers click “approve”). Then Type your first and last name into the next available box.



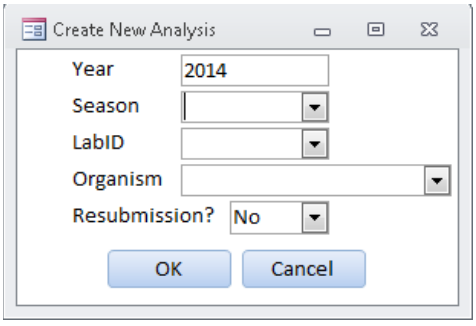
3. Click the plus sign next to your name to enter the remaining information. Click "Pull from Contacts table" to auto populate your email and phone number (the information is pulled from the PulseNet Contacts Database). Then click "Browse" to import your electronic signature.
   Note 1: You must have already saved a scanned image of your signature on a private drive (such as your desktop or C drive). The image must be cropped so that your signature does not appear shrunken (zoom in to crop). The default image width will be 120 and height will be 30.
   Note 2: It is highly recommended that you test using your signature in a single report before you use it to mass review.



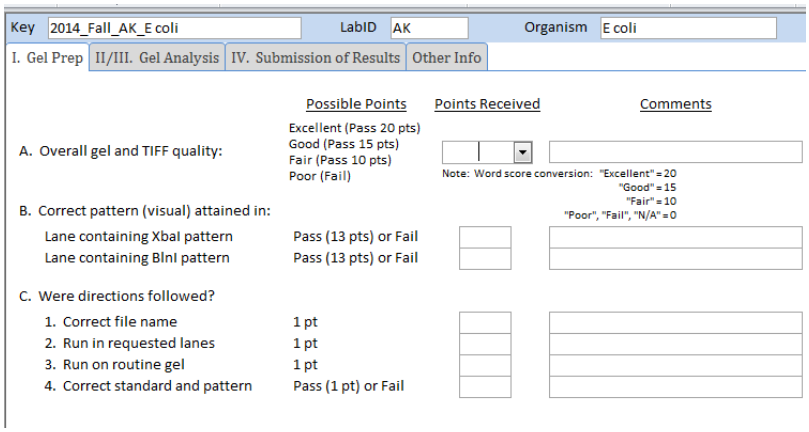
4. **Instructions for Evaluator:**
To begin the evaluation, click the green plus sign next to "New Analysis"



The year will auto populate (make sure it is for the correct round of PT). Use the pull-down menus to populate the rest of the data fields.

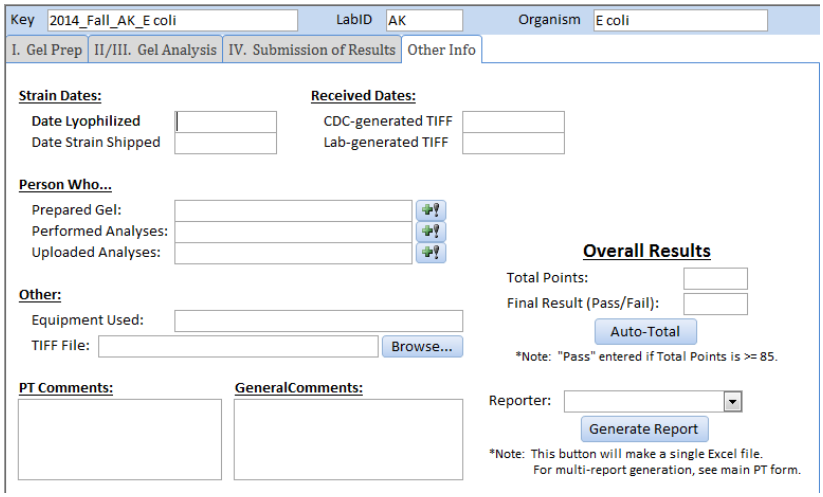


There are four tabs across the top of the page: Gel Prep, Gel Analysis, Submission of Results and Other Info. Click the tab to enter information into the report form as you are evaluating the PT submission. Enter comments and points under the appropriate headings.

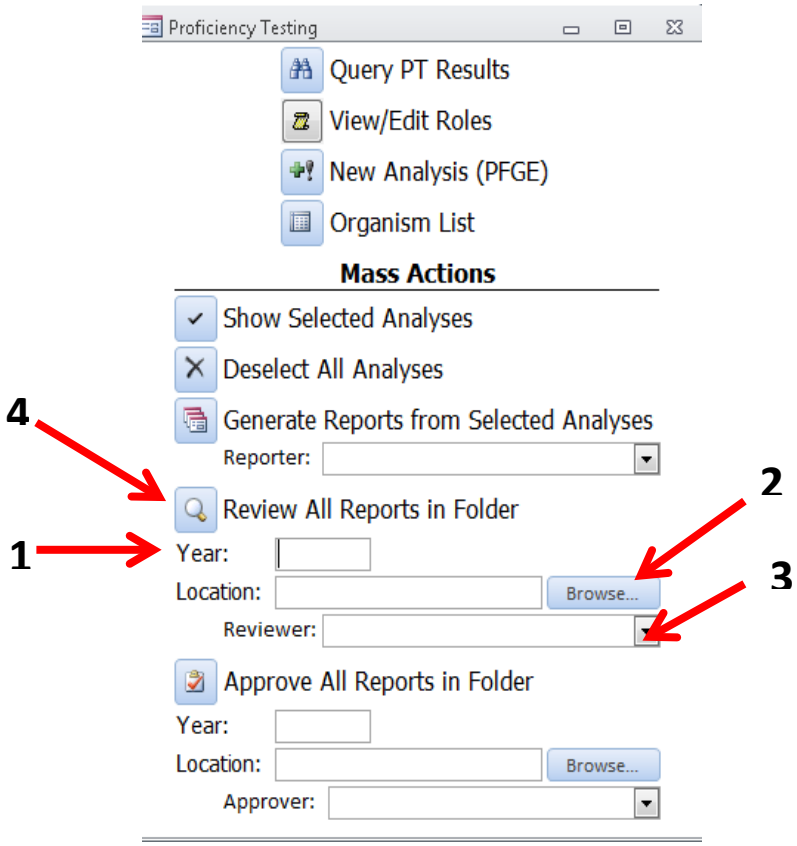


When you get to the Other Info tab, use the information provided in the PT submission emails sent from the participating laboratories to complete this section (strain and received dates, person who…, and other). For the Comments, enter any general comments you have for improving gel quality, analysis or submission.

When you are ready to score the overall results, click the auto-total button to auto populate the total points and final result (pass/fail) section. If you want to go ahead and generate the final report then, use the pull-down to select your name and click the "generate report" button. This will generate a single excel file. If you wish to wait until you are finished with all the reports to sign off, then use the tool in the main screen to mass sign.



5. **Instructions for Mass Review/Approve:** First enter the year (i.e. 2014), then click "Browse" to navigate to the location where all of the reports are saved (the evaluator will notify the reviewers of the location).Click the pull-down arrow next to Reviewer to select your name from the list. Then click the magnifying glass button next to "Review All Reports in Folder". This should automatically insert your signature and the current date into all of the reports. Please check a few to make sure this worked properly. **Note:** The arrows point to the fields used by the Database Reviewers. The laboratory reviewer should use the section of tools under "Approve All Reports in Folder" in the same order as described above and pictured below to complete the review/approval process.



6. If any files weren't able to be reviewed or approved, you'll get a pop-up message telling you which ones had an error. Otherwise, you'll receive a confirmation message that says "All files successfully reviewed/approved".

1. **PURPOSE:** To describe the procedure for certifying PulseNet personnel to enable full participation in PulseNet MLVA activities.

2. **SCOPE:** This procedure applies to all PulseNet personnel performing MLVA and creating peak files.

3. **DEFINITIONS/TERMS:**
   MLVA: Multiple-locus variable-number tandem repeat analysis
   DNA: Deoxyribonucleic acid
   PCR: Polymerase chain reaction
   CDC: Centers for Disease Control and Prevention
   SOP: Standard Operating Procedure

4. **RESPONSIBILITIES:**

4.1 Individuals performing PulseNet MLVA-related work must submit certification file(s) and have them reviewed before being able to submit peak files to the PulseNet MLVA Database.
  4.1.1 Submitted certification files must document the submitter's highest level of competence in producing peak files.
  4.1.2 Individuals can be certified for peak files only at this time.

5. **PROCEDURE:**

5.1 PulseNet participants request the *E. coli* O157 and *S. enterica* serotypes Typhimurium and Enteritidis MLVA certification sets from CDC (pfge@cdc.gov) if they do not already have them.
5.2 CDC sends the requested certification set and detailed instructions (see Appendices PNQ05-1 through PNQ05-5) to reconstitute the cultures and primers, make DNA templates, perform PCR and fragment analysis and export the peak file from the sequencer in the ".CSV" format according to the standardized laboratory protocol (PNL19, PNL21, and PNL27).
5.3 Peak file(s) are submitted to CDC for review. See Appendices PNQ05-2, PNQ05-3 and PNQ05-4 for submission instructions.
5.4 Submitters are notified in writing of the results of their certification file evaluation.
  5.4.1 If the submitted certification file passes the certification evaluation, the submitter is considered certified as long as they remain in their current laboratory and that laboratory successfully completes annual proficiency testing. If a person relocates to a different PulseNet laboratory, they must be recertified.
  5.4.2 If the submitted certification files do not pass the certification evaluation
    5.4.2.1 The individual will need to review the troubleshooting comments received from the evaluator and resubmit once results have improved.
    5.4.2.2 If the submitter fails certification three times, the individual will not be allowed to submit again for six months. Before resubmitting, the individual will be expected to work with CDC until satisfactory results are achieved. This includes, but is not limited to troubleshooting and training in the PulseNet MLVA protocol.

6. FLOW CHART:

7. BIBLIOGRAPHY:

8. CONTACTS:

8.1 Eija Hyytia-Trees, D.V.M., Ph.D.
PulseNet Next Generation Subtyping Methods Unit, EDLB, DFWED, CDC
(404) 639-3672
EHyytia-Trees@cdc.gov

8.2 Patti Lafon, M.S.
PulseNet Next Generation Subtyping Methods Unit, EDLB, DFWED, CDC
(404) 639-2828
PLafon@cdc.gov

9. AMENDMENTS:

9.1 Appendix PNQ05-3 Instructions: PulseNet Certification for MLVA Peak Files of *S.* Typhimurium - Beckman Coulter CEQ 8000 Platform added 2/5/2008.

9.2 Appendix PNQ05-3: The internal ladder isolates were assigned CDC identification numbers 10/6/2009.

9.3 Appendix PNQ05-4: Primer reconstitution instructions were added 3/2/2010. A list of certification package contents was added to the top of each PNQ05-2 and PNQ05-3.

9.4 Appendix PNQ05-4 was added 3/26/2010. This document details the certification procedure for the *S. enterica* serotype Enteritidis MLVA. Former Appendix PNQ05-4 (primer reconstitution instructions) was renamed PNQ05-5 and was amended to reflect the addition of *S.* Enteritidis MLVA certification.

9.5 Appendix PNQ05-1: Removed section regarding TSA Stabs: Day 1. All certification strains and controls are now lyophilized 4/19/2013.

9.6 Document title now includes the 8800 and GeXP Genetic Analyzers 4/19/2013..

9.7Appendix PNQ05-2: Revised contents to include 11 lyophilized vials. Updated Eija Trees' job title and affiliation. Included the GeXP and 8800 Genetic Analyzers in the text 4/19/13 .

9.8Appendix PNQ05-3: Revised contents to include 11 lyophilized vials. Updated Eija Trees' job title and affiliation. Included the GeXP and 8800 Genetic Analyzers in the text 4/19/13 .

9.7 Appendix PNQ05-4: Revised contents to include 11 lyophilized vials. Updated Eija Trees' job title and affiliation. Included the GeXP and 8800 Genetic Analyzers in the text 4/19/13.

Appendix PNQ05-1

Laboratory Protocol to Reconstitute Lyophilized (Freeze-Dried) *E. coli* O157:H7,*S.* Typhimurium, and *S. enterica* serotype Enteritidis

Biological Safety Warning: *E. coli* 0157:H7 and *Salmonella* strains are considered Level 2 biological agents by the U.S. Department of Health and Human Services. Use appropriate precautions when handling the vial or culture. Carry out laboratory work in a biological safety cabinet when applicable to ensure aseptic conditions and personal safety.

Note: Store the lyophilized cultures and TSA stabs at 4°C in the dark until they are reconstituted and subcultured.

Materials Needed:

Sterile sturdy forceps
1 ml pipetman
1 ml sterile pipet tips
1 µl sterile inoculating loop

Reagents Needed:

Trypticase Soy + 5% Sheep Blood Agar plates (BAP) or equivalent media
Sterile grade reagent water or Trypticase Soy Broth (TSB)
70% isopropyl alcohol

Procedure for Reviving Cultures:

Lyophilized cultures: Day 1

1. Document the isolate number (s) and the date(s) lyophilized for your records. Wipe the aluminum cover and outside of the vial with isopropyl alcohol. Using sturdy forceps, aseptically remove the aluminum cover and rubber stopper from the vial containing the lyophilized culture. Wipe the outside of the rubber stopper and neck of the vial with isopropyl alcohol before removing the stopper.

2. Re-suspend the lyophilized cells with 1.0 ml of sterile grade reagent water. Allow to stand for a few minutes and/or mix gently to produce a uniform suspension. With an inoculating loop, streak a small amount of this suspension onto a blood agar plate (BAP) and incubate at 37°C overnight.

Days 2 and 3

1. Check the BAP; if the culture appears pure, pick an isolated colony, and streak it on a fresh BAP; incubate at 37°C overnight. Use the growth from this plate to make DNA templates of the certification strains. Transfer culture to fresh medium and incubate at 37°C overnight; this will ensure that the same culture can be retested, if necessary.

Appendix PNQ05-2

Instructions: PulseNet Certification for MLVA Peak Files of *E. coli* O157:H7-Beckman Coulter CEQ 8000/8800/GeXP Platform

Dear PulseNet Participant,

This package should contain:

- 16 vials of primers (please see Appendix PNQ05-5 for primer reconstitution instructions)
- 11 vials of lyophilized cultures of *E. coli* O157:H7:
  - 8 Certification Strains
  - 1 Positive Control Strain (EDL933)
  - 2 Internal Ladder Strains (EC04PN0139 and EC04PN0570)

After the certification strains have been reconstituted according to the directions in Appendix PNQ05-1, streak each culture onto agar plates (overnight incubation at 37^{o}C), pick an isolated colony, and subculture to another plate. Use the growth from the second plate to make the DNA templates. Please let me know if this package does not arrive in a satisfactory condition, or if the cultures are not viable. **Please, make a stock culture (freeze at -70°C) of each of the strains according to your laboratory's policy within 1 week from receiving them. Long-term storage of these cultures will ensure the availability of the PulseNet certification set for future use, including MLVA certification of additional personnel**.

The strain numbers of the *E. coli* cultures are as follows:

CDC# 01-98 CDC# 05-98 CDC# 07-98 CDC# 08-98 CDC# 12-98 CDC# 24-98

CD# 11 (G5286) CDC# 48 (G7602)

Please follow these supplemental instructions for testing the certification isolates by MLVA. Refer to the "Laboratory standard operating procedure for PulseNet MLVA of Shiga toxin-producing *Escherichia coli* O157 (STEC O157)-Beckman Coulter CEQ 8000/8800/GeXP Platform (PNL19)" for detailed instructions.

1. Make DNA templates from each test isolate, positive control EDL933, and internal ladder isolates EC04PN0139 and EC04PN0570.

2. Perform PCR and fragment analysis following the instructions of the standard protocol with the possible exception of the primer concentration modifications your laboratory may have had to make to optimize the PCR assays.

   a. Make sure to include your PulseNet laboratory ID (the unique identifier code that was assigned to your laboratory by CDC PulseNet) in front of the CDC strain ID number

and your initials after the strain ID number, i.e. follow the strain ID format *labID*_CDC01-98xx.

3. Export the peak file from the sequencer in the .CSV format. Please notice that in addition to the certification set isolates, the peak file must also contain the positive control strain EDL933 in duplicate for each reaction, one negative control for each reaction, the internal ladder in duplicate, and the molecular size standard peaks (D1 peaks).

a. Name the peak files according to the standardized PulseNet naming system:

Use the laboratory ID that was assigned to your laboratory by CDC PulseNet for the first two to four letters of the file. The next 2 spaces will indicate the year the file was generated. The next 4 spaces indicate the month and the date the run was performed. For example: **GA090219.csv** is a peak file run on Feb 19th, 2009 at the GA Public Health Laboratory. If multiple runs are performed on a same day, differentiate the peak files using sequential numbers, for example GA090219-1, GA090219-2.

4. Send the peak file to CDC PulseNet at pfge@cdc.gov within four weeks after receiving the strains.

a. In the email to CDC, include ***E. coli* O157 MLVA Certification** in the subject line.

Currently, for *E. coli* O157, an individual may be certified for peak file submission only. Once the *E. coli* O157 national MLVA database is available on-line, individuals may also be certified for analysis.

After the peak files are submitted, the PulseNet certification file evaluator will analyze the files and inform your laboratory of your results ("Satisfactory" or "Needs Improvement") within four weeks of receiving the files. If the peak file is satisfactory, the person who submitted the file will be eligible to send peak files to PulseNet for analysis. If the submitted certification files are not satisfactory, the individual will need to review the troubleshooting comments received from the evaluator and resubmit once results have improved. If the submitter fails certification three times, the individual will not be allowed to submit again for six months. Before resubmitting, the individual will be expected to work with CDC until satisfactory results are achieved.

Please let me know if you have questions or further clarification is needed.

Good luck,

Eija Trees, D.V.M., Ph.D.
Unit Chief
PulseNet Next Generation Subtyping Methods Unit
EDLB, DFWED, CDC
Tel: 404-639-3672
E-mail: EHyytia-Trees@cdc.gov

Appendix PNQ05-3

Instructions: PulseNet Certification for MLVA Peak Files of *S. enterica* serotype Typhimurium - Beckman Coulter CEQ 8000/8800/GeXP Platform

Dear PulseNet Participant,

This package should contain:

- 14 vials of primers (please see Appendix PNQ05-5 for primer reconstitution instructions)
- 11 vials of lyophilized cultures of *Salmonella* Typhimurium
    - 8 Certification strains
    - 1 Positive control (LT2)
    - 2 Internal Ladder isolates (2009K0825 and 2009K0826)

After the strains have been reconstituted according to the directions in Appendix PNQ05-1, streak each culture onto agar plates (overnight incubation at 37^{o}C), pick an isolated colony, and subculture to another plate. Use the growth from the second plate to make the DNA templates. Please let me know if this package does not arrive in a satisfactory condition, or if the cultures are not viable. **Please, make a stock culture (freeze at -70°C) of each of the strains according to your laboratory's policy within 1 week from receiving them. Long-term storage of these cultures will ensure the availability of the PulseNet certification set for future use, including MLVA certification of additional personnel**.

The strain numbers of the *S.enterica* serotype Typhimurium cultures are as follows:

CDC# 61-99 CDC# 63-99 CDC# 76-99 CDC# 78-99 CDC# 80-99 CDC# 81-99

CD# 83-99 CDC# H8290

Please follow these supplemental instructions for testing the certification isolates by MLVA. Refer to the "Laboratory standard operating procedure for PulseNet MLVA of *Salmonella enterica* serotype Typhimurium (*S*. Typhimurium) - Beckman Coulter CEQ 8000 Platform (PNL21)" for detailed instructions.

1. Make DNA templates from each test isolate, positive control LT2, and internal ladder isolates 2009K0825 and 2009K0826.

2. Perform PCR and fragment analysis following the instructions of the standard protocol with the possible exception of the primer concentration modifications your laboratory may have had to make to optimize the PCR assays.

    a. Make sure to include your PulseNet laboratory ID (the unique identifier code that was assigned to your laboratory by CDC PulseNet) in front of the CDC strain ID number

and your initials after the strain ID number, i.e. follow the strain ID format *labID*_CDC61-99xx.

3. Export the peak file from the sequencer in the .CSV format. Please notice that in addition to the certification set isolates, the peak file must also contain the positive control strain LT2 in duplicate for each reaction, one negative control for each reaction, the internal ladder in duplicate, and the molecular size standard peaks (D1 peaks).

a. Name the peak files according to the standardized PulseNet naming system:

Use the laboratory ID that was assigned to your laboratory by CDC PulseNet for the first two to four letters of the file. The next 2 spaces will indicate the year the file was generated. The next 4 spaces indicate the month and the date the run was performed. For example: **GA090219.csv** is a peak file run on Feb 19th, 2009 at the GA Public Health Laboratory. If multiple runs are performed on a same day, differentiate the peak files using sequential numbers, for example GA090219-1, GA090219-2.

4. Send the peak file to CDC PulseNet at pfge@cdc.gov within four weeks after receiving the strains.

a. In the email to CDC, include ***S.* Typhimurium MLVA Certification** in the subject line.

Currently, for *S.* Typhimurium, an individual may be certified for peak file submission only. Once the *S.* Typhimurium national MLVA database is available on-line, individuals may also be certified for analysis.

After the peak files are submitted, the PulseNet certification file evaluator will analyze the files and inform your laboratory of your results ("Satisfactory" or "Needs Improvement") within four weeks of receiving the files. If the peak file is satisfactory, the person who submitted the file will be eligible to send peak files to PulseNet for analysis. If the submitted certification files are not satisfactory, the individual will need to review the troubleshooting comments received from the evaluator and resubmit once results have improved. If the submitter fails certification three times, the individual will not be allowed to submit again for six months. Before resubmitting, the individual will be expected to work with CDC until satisfactory results are achieved.

Please let me know if you have questions or further clarification is needed.

Good luck,

Eija Trees, D.V.M., Ph.D.
Unit Chief PulseNet Next Generation Subtyping Methods Unit
EDLB, DFWED, CDC
Tel: 404-639-3672
E-mail: EHyytia-Trees@cdc.gov

Appendix PNQ05-4

Instructions: PulseNet Certification for MLVA Peak Files of *S. enterica* serotype Enteritidis - Beckman Coulter CEQ 8000/8800/GeXP Platform

Dear PulseNet Participant,

This package should contain:

- 14 vials of primers (please see Appendix PNQ05-5 for primer reconstitution instructions)
- 11 vials of lyophilized cultures of *Salmonella* Enteritidis
    - 8 Certification Strains
    - 1 Positive control (K1891)
    - 2 Internal Ladder isolates (H9560 and 2010K0017)

According to the directions in Appendix PNQ06-1, streak each culture onto agar plates (overnight incubation at 37^{o}C), pick an isolated colony, and subculture to another plate. Use the growth from the second plate to make the DNA templates. Please let me know if this package does not arrive in satisfactory condition, or if the cultures are not viable. **Please, make a stock culture (freeze at -70°C) of each of the strains according to your laboratory's policy within 1 week from receiving them. Long-term storage of these cultures will ensure the availability of the PulseNet certification set for future use, including MLVA certification of additional personnel**.

The strain numbers of the *S. enterica* serotype Enteritidis cultures are as follows:

CDC# K2148 CDC# H9654 CDC# 2009K0432 CDC# K3307 CDC# J0932

CDC# K4417 CDC# K2158 CDC# K0746

Please follow these supplemental instructions for testing the certification isolates by MLVA. Refer to the "Laboratory standard operating procedure for PulseNet MLVA of *Salmonella enterica* serotype Enteritidis- Beckman Coulter CEQ 8000/8800/GeXP Platform (PNL27)" for detailed instructions.

1. Make DNA templates from each test isolate, positive control K1891, and internal ladder isolates H9560 and 2010K0017.

2. Perform PCR and fragment analysis following the instructions of the standard protocol with the possible exception of the primer concentration modifications your laboratory may have had to make to optimize the PCR assays.

    a. Make sure to include your PulseNet laboratory ID (the unique identifier code that was assigned to your laboratory by CDC PulseNet) in front of the CDC strain ID

number and your initials after the strain ID number, i.e. follow the strain ID format *labID*_CDCK2148xx.

3. Export the peak file from the sequencer in the .CSV format. Please notice that in addition to the certification set isolates, the peak file must also contain the positive control strain K1891 in duplicate for each reaction, one negative control for each reaction, the internal ladder in duplicate, and the molecular size standard peaks (D1 peaks).

a. Name the peak files according to the standardized PulseNet naming system:

Use the laboratory ID that was assigned to your laboratory by CDC PulseNet for the first two to four letters of the file. The next 2 spaces will indicate the year the file was generated. The next 4 spaces indicate the month and the date the run was performed. For example: **GA090219.csv** is a peak file run on Feb 19th, 2009 at the GA Public Health Laboratory. If you perform multiple runs on a same day, differentiate the peak files using sequential numbers, for example GA090219-1, GA090219-2.

4. Send the peak file to CDC PulseNet at pfge@cdc.gov within four weeks after receiving the strains.

a. In the email to CDC, include ***S.* Enteritidis MLVA Certification** in the subject line.

Currently, for *S. enterica* serotype Enteritidis, an individual may be certified for peak file submission only. Once the national *S.* Enteritidis MLVA database is available on-line, individuals may also be certified for analysis.

After the peak files are submitted, the PulseNet certification file evaluator will analyze the files and inform your laboratory of your results ("Satisfactory" or "Needs Improvement") within four weeks of receiving the files. If the peak file is satisfactory, the person who submitted the file will be eligible to send peak files to PulseNet for analysis. If the submitted certification files are not satisfactory, the individual will need to review the troubleshooting comments received from the evaluator and resubmit once results have improved. If the submitter fails certification three times, the individual will not be allowed to submit again for six months. Before resubmitting, the individual will be expected to work with CDC until satisfactory results are achieved.

Please let me know if you have questions or further clarification is needed.

Good luck,

Eija Trees, D.V.M., Ph.D.
Unit Chief PulseNet Next Generation Subtyping Methods Unit
EDLB, DFWED, CDC
Tel: 404-639-3672
E-mail: EHyytia-Trees@cdc.gov

Appendix PNQ05-5

Instructions: Reconstitution of Primers for *E. coli* O157:H7 and *Salmonella enterica serotypes* Typhimurium and Enteritidis

E. coli O157:H7:

Reconstitution of the primers:

The amount of primer in each vial is indicated on the label. In order to prepare a **100 µM** stock, please reconstitute the primers by adding following amounts of distilled water:

Amount of primer in the vial	**Water needed for a 100 µM stock**
10 nM	100 µl
40 nM	400 µl

Preparation of the working concentrations from the 100 µM stock:

Working concentration:	**Water (µl) + primer (µl)**
25 µM	30.0 + 10.0
5 µM	47.5 + 2.5
2.5 µM	48.75 + 1.25
1 µM	99.0 + 1.0

Salmonella Typhimurium:

Reconstitution of the primers:

The amount of primer in each vial is indicated on the label. In order to prepare a **100 µM** stock, please reconstitute the primers by adding following amounts of distilled water:

Amount of primer in the vial	**Water needed for a 100 µM stock**
10 nM	100 µl
20 nM	200 µl
40 nM	400 µl

Preparation of the working concentrations from the 100 µM stock:

Working concentration:	**Water (µl) + primer (µl)**
5 µM	47.5 + 2.5
2.5 µM	48.75 + 1.25

***Salmonella* Enteritidis:**

Reconstitution of the primers:

The amount of primer in each vial is indicated on the label. In order to prepare a **100 µM** stock, please reconstitute the primers by adding following amounts of distilled water:

Amount of primer in the vial	**Water needed for a 100 µM stock**
10 nM	100 µl
20 nM	200 µl

Preparation of the working concentrations from the 100 µM stock:

Working concentration:	**Water (µl) + primer (µl)**
12.5 µM	43.75 + 6.25
2.5 µM	48.75 + 1.25
1 µM	99.0 + 1.0

1. PURPOSE: To describe the procedure for certifying PulseNet personnel to enable full participation in PulseNet MLVA activities.

2. SCOPE: This procedure applies to all PulseNet personnel performing MLVA and creating peak files.

3. DEFINITIONS/TERMS:

3.1 MLVA: Multiple-locus variable-number tandem repeat analysis
3.2 PCR: Polymerase chain reaction
3.3 DNA: Deoxyribonucleic acid
3.4 CDC: Centers for Disease Control and Prevention
3.5 SOP: Standard Operating Procedure

4. RESPONSIBILITIES:

4.1 Individuals performing PulseNet MLVA-related work must submit certification file(s) and have them reviewed before being able to submit peak files to the PulseNet MLVA Database.
  4.1.1 Submitted certification files must document the submitter's highest level of competence in producing peak files.
  4.1.2 Individuals can be certified for peak files only at this time.

5. PROCEDURE:

5.1 PulseNet participants request the *E. coli* O157 and *Salmonella enterica* serotypes Typhimurium and Enteritidis MLVA certification sets from CDC (pfge@cdc.gov) if they do not already have them.
5.2 CDC sends the requested certification set and detailed instructions (see Appendices PNQ06-1 through PNQ06-5) to reconstitute the cultures and primers, make DNA templates, perform PCR and fragment analysis and export the peak file from the sequencer in the ".txt" format according to the standardized laboratory protocols (PNL23, PNL24, PNL26, PNL28, PNL29, PNL30).
5.3 Peak file(s) are submitted to CDC for review. See Appendices PNQ06-2, PNQ06-3 and PNQ06-4 for submission instructions.
5.4 Submitters are notified in writing of the results of their certification file evaluation.
  5.4.1 If the submitted certification file passes the certification evaluation, the submitter is considered certified as long as they remain in their current laboratory and that laboratory successfully completes annual proficiency testing. If a person relocates to a different PulseNet laboratory, they must be recertified.
  5.4.2 If the submitted certification files do not pass the certification evaluation
    5.4.2.1 The individual will need to review the troubleshooting comments received from the evaluator and resubmit once results have improved.
    5.4.2.2 If the submitter fails certification three times, the individual will not be allowed to submit again for six months. Before resubmitting, the individual will be expected to

work with CDC until satisfactory results are achieved. This includes, but is not limited to troubleshooting and training in the PulseNet MLVA protocol.

6. FLOW CHART:

7. BIBLIOGRAPHY:

8. CONTACTS:

8.1 Eija Hyytia-Trees, D.V.M., Ph.D.
PulseNet Next Generation Subtyping Methods Unit, EDLB, DFWED, CDC
(404) 639-3672
EHyytia-Trees@cdc.gov

8.2 Patti Lafon, M.S.
PulseNet Next Generation Subtyping Methods Unit, EDLB, DFWED, CDC
(404) 639-2828
PLafon@cdc.gov

9. AMENDMENTS:

9.1 Appendix PNQ06-3 was added 10/1/2009. This document details the certification procedure for the *S. enterica* serotype Typhimurium MLVA.

9.2 Appendix PNQ06-4: Primer reconstitution instructions were added 3/2/2010. A list of certification package contents was added to the top of each PNQ06-2 and PNQ06-3.

9.3 Appendix PNQ06-4 was added 3/17/2010. This document details the certification procedure for the *S. enterica* serotype Enteritidis MLVA. Former Appendix PNQ06-4 (primer reconstitution instructions) was renamed PNQ06-5 and was amended to reflect the addition of *S.* Enteritidis MLVA certification.

9.4 Document Title now includes the 3500 Genetic Analyzer 4/19/2013.

9.5 Appendix PNQ06-1: Removed section regarding TSA Stabs: Day 1. All certification strains and controls are now lyophilized 4/19/2013.

9.6 Appendix PNQ06-2: Revised contents to include 11 lyophilized vials. Updated Eija Trees' job title and affiliation. Included the reference to PNL28 (3500) in the text 4/19/13 .

9.7 Appendix PNQ06-3: Revised contents to include 11 lyophilized vials. Updated Eija Trees' job title and affiliation. Included the reference to PNL29 (3500) in the text 4/19/13 .

9.8 Appendix PNQ06-4: Revised contents to include 11 lyophilized vials. Updated Eija Trees' job title and affiliation. Included the reference to PNL30 (3500) in the text 4/19/13 .

Appendix PNQ06-1

Laboratory Protocol to Reconstitute Lyophilized (Freeze-Dried) *E. coli* O157:H7, *S. enterica* serotype Typhimurium and *S. enterica* serotype Enteritidis

Biological Safety Warning: *E. coli* 0157:H7, *S. enterica* serotypes Typhimurium and Enteritidis strains are considered Level 2 biological agents by the U.S. Department of Health and Human Services. Use appropriate precautions when handling the vial or culture. Carry out laboratory work in a biological safety cabinet when applicable to ensure aseptic conditions and personal safety.

Note: Store the lyophilized cultures and TSA stabs at 4°C in the dark until they are reconstituted and subcultured.

Materials Needed:

Sterile sturdy forceps
1 ml pipetman
1 ml sterile pipet tips
1 µl sterile inoculating loop

Reagents Needed:

Trypticase Soy + 5% Sheep Blood Agar plates (BAP) or equivalent media
Sterile grade reagent water or Trypticase Soy Broth (TSB)
70% isopropyl alcohol

Procedure for Reviving Cultures:

Lyophilized cultures: Day 1

1. Document the isolate number (s) and the date(s) lyophilized for your records. Wipe the aluminum cover and outside of the vial with isopropyl alcohol. Using sturdy forceps, aseptically remove the aluminum cover and rubber stopper from the vial containing the lyophilized culture. Wipe the outside of the rubber stopper and neck of the vial with isopropyl alcohol before removing the stopper.

2. Re-suspend the lyophilized cells with 1.0 ml of sterile grade reagent water. Allow to stand for a few minutes and/or mix gently to produce a uniform suspension. With an inoculating loop, streak a small amount of this suspension onto a blood agar plate (BAP) and incubate at 37°C overnight.

Days 2 and 3

1. Check the BAP; if the culture appears pure, pick an isolated colony, and streak it on a fresh BAP; incubate at 37°C overnight. Use the growth from this plate to make DNA templates of the certification strains. Transfer culture to fresh medium and incubate at 37°C overnight; this will ensure that the same culture can be retested, if necessary.

Appendix PNQ06-2

Instructions: PulseNet Certification for MLVA Peak Files of *E. coli* O157:H7-Applied Biosystems Genetic Analyzer 3130/3500 Platform

Dear PulseNet Participant,

This package should contain:

- 16 vials of primers (please see Appendix PNQ06-5 for primer reconstitution instructions)
- 11 vials of lyophilized cultures of *E. coli* O157:H7
    - 8 Certification Strains
    - 1 Positive Control Strain (EDL933)
    - 2 Internal Ladder Strains (EC04PN0139 and EC04PN0570)

After the strains have been reconstituted according to the directions in Appendix PNQ06-1, streak each culture onto agar plates (overnight incubation at 37^{o}C), pick an isolated colony, and subculture to another plate. Use the growth from the second plate to make the DNA templates. Please let me know if this package does not arrive in satisfactory condition, or if the cultures are not viable. **Please, make a stock culture (freeze at -70°C) of each of the strains according to your laboratory's policy within 1 week from receiving them. Long-term storage of these cultures will ensure the availability of the PulseNet certification set for future use, including MLVA certification of additional personnel**.

The strain numbers of the *E. coli* cultures are as follows:

CDC# 01-98 CDC# 05-98 CDC# 07-98 CDC# 08-98 CDC# 12-98 CDC# 24-98

CD# 11 (G5286) CDC# 48 (G7602)

Please follow these supplemental instructions for testing the certification isolates by MLVA. Refer to PNL23 (3130) or PNL28 (3500) for detailed instructions.

1. Make DNA templates from each test isolate, positive control EDL933, and internal ladder isolates EC04PN0139 and EC04PN0570.

2. Perform PCR and fragment analysis following the instructions of the standard protocol with the possible exception of the primer concentration modifications your laboratory may have had to make to optimize the PCR assays.

    a. Make sure to include your PulseNet laboratory ID (the unique identifier code that was assigned to your laboratory by CDC PulseNet) in front of the CDC strain ID number and your initials after the strain ID number, i.e. follow the strain ID format *labID*_CDC01-98xx.

3. Export the peak file from the sequencer in the .txt (tab-delimited) format. Please notice that in addition to the certification set isolates, the peak file must also contain the positive control strain EDL933 in duplicate for each reaction, one negative control for each reaction, the internal ladder in duplicate, and the molecular size standard peaks (R peaks).

a. Name the peak files according to the standardized PulseNet naming system:

Use the laboratory ID that was assigned to your laboratory by CDC PulseNet for the first two to four letters of the file. The next 2 spaces will indicate the year the file was generated. The next 4 spaces indicate the month and the date the run was performed. For example: **GA090219.txt** is a peak file run on Feb 19th, 2009 at the GA Public Health Laboratory. If you perform multiple runs on a same day, differentiate the peak files using sequential numbers, for example GA090219-1, GA090219-2

4. Send the peak file to CDC PulseNet at pfge@cdc.gov within four weeks after receiving the strains.

a. In the email to CDC, include ***E. coli* O157 MLVA Certification** in the subject line.

Currently, for *E. coli* O157, an individual may be certified for peak file submission only. Once the *E. coli* O157 national MLVA database is available on-line, individuals may also be certified for analysis.

After the peak files are submitted, the PulseNet certification file evaluator will analyze the files and inform your laboratory of your results ("Satisfactory" or "Needs Improvement") within four weeks of receiving the files. If the peak file is satisfactory, the person who submitted the file will be eligible to send peak files to PulseNet for analysis. If the submitted certification files are not satisfactory, the individual will need to review the troubleshooting comments received from the evaluator and resubmit once results have improved. If the submitter fails certification three times, the individual will not be allowed to submit again for six months. Before resubmitting, the individual will be expected to work with CDC until satisfactory results are achieved.

Please let me know if you have questions or further clarification is needed.

Good luck,

Eija Trees, D.V.M., Ph.D.
Unit Chief
PulseNet Next Generation Subtyping Methods Unit
EDLB, DFWED, CDC
Tel: 404-639-3672
E-mail: EHyytia-Trees@cdc.gov

Appendix PNQ06-3

Instructions: PulseNet Certification for MLVA Peak Files of *S. enterica* serotype Typhimurium-Applied Biosystems Genetic Analyzer 3130/3500 Platform

Dear PulseNet Participant,

This package should contain:

- 14 vials of primers (please see Appendix PNQ06-5 for primer reconstitution instructions)
- 11 vials of lyophilized cultures of *Salmonella* Typhimurium
    - 8 Certification Strains
    - 1 Positive Control Strain (LT2)
    - 2 Internal Ladder Strains (2009K0825 and 2009K0826)

After the strains have been reconstituted according to the directions in Appendix PNQ06-1, streak each culture onto agar plates (overnight incubation at 37^{o}C), pick an isolated colony, and subculture to another plate. Use the growth from the second plate to make the DNA templates. Please let me know if this package does not arrive in satisfactory condition, or if the cultures are not viable. **Please, make a stock culture (freeze at -70°C) of each of the strains according to your laboratory's policy within 1 week from receiving them. Long-term storage of these cultures will ensure the availability of the PulseNet certification set for future use, including MLVA certification of additional personnel**.

The strain numbers of the *S. enterica* serotype Typhimurium cultures are as follows:

CDC# 61-99 CDC# 63-99 CDC# 76-99 CDC# 78-99 CDC# 80-99 CDC# 81-99

CDC# 83-99 CDC# H8290

Please follow these supplemental instructions for testing the certification isolates by MLVA. Refer to PNL24 (3130) or PNL29 (3500) for detailed instructions.

1. Make DNA templates from each test isolate, positive control LT2, and internal ladder isolates 2009K0825 and 2009K0826.

2. Perform PCR and fragment analysis following the instructions of the standard protocol with the possible exception of the primer concentration modifications your laboratory may have had to make to optimize the PCR assays.

    a. Make sure to include your PulseNet laboratory ID (the unique identifier code that was assigned to your laboratory by CDC PulseNet) in front of the CDC strain ID number and your initials after the strain ID number, i.e. follow the strain ID format *labID*_CDC61-99xx.

3. Export the peak file from the sequencer in the .txt (tab-delimited) format. Please notice that in addition to the certification set isolates, the peak file must also contain the positive control strain LT2 in duplicate for each reaction, one negative control for each reaction, the internal ladder in duplicate, and the molecular size standard peaks (R peaks).

a. Name the peak files according to the standardized PulseNet naming system:

Use the laboratory ID that was assigned to your laboratory by CDC PulseNet for the first two to four letters of the file. The next 2 spaces will indicate the year the file was generated. The next 4 spaces indicate the month and the date the run was performed. For example: **GA090219.txt** is a peak file run on Feb 19th, 2009 at the GA Public Health Laboratory. If you perform multiple runs on a same day, differentiate the peak files using sequential numbers, for example GA090219-1, GA090219-2.

4. Send the peak file to CDC PulseNet at pfge@cdc.gov within four weeks after receiving the strains.

a. In the email to CDC, include ***S.* Typhimurium MLVA Certification** in the subject line.

Currently, for *S. enterica* serotype Typhimurium, an individual may be certified for peak file submission only. Once the national *S.* Typhimurium MLVA database is available on-line, individuals may also be certified for analysis.

After the peak files are submitted, the PulseNet certification file evaluator will analyze the files and inform your laboratory of your results ("Satisfactory" or "Needs Improvement") within four weeks of receiving the files. If the peak file is satisfactory, the person who submitted the file will be eligible to send peak files to PulseNet for analysis. If the submitted certification files are not satisfactory, the individual will need to review the troubleshooting comments received from the evaluator and resubmit once results have improved. If the submitter fails certification three times, the individual will not be allowed to submit again for six months. Before resubmitting, the individual will be expected to work with CDC until satisfactory results are achieved.

Please let me know if you have questions or further clarification is needed.

Good luck,

Eija Trees, D.V.M., Ph.D.
Unit Chief
PulseNet Next Generation Subtyping Methods Unit
EDLB, DFWED, CDC
Tel: 404-639-3672
E-mail: EHyytia-Trees@cdc.gov

Appendix PNQ06-4
Instructions: PulseNet Certification for MLVA Peak Files of *S. enterica* serotype Enteritidis - Applied Biosystems Genetic Analyzer 3130/3500 Platform

Dear PulseNet Participant,

This package should contain:
- 14 vials of primers (please see Appendix PNQ06-5 for primer reconstitution instructions)
- 11 vials of lyophilized cultures of *Salmonella* Enteritidis
    - 8 Certification Strains
    - 1 Positive Control Strain (K1891)
    - 2 Internal Ladder Strains (H9560 and 2010K0017)

According to the directions in Appendix PNQ06-1, streak each culture onto agar plates (overnight incubation at 37^{o}C), pick an isolated colony, and subculture to another plate. Use the growth from the second plate to make the DNA templates. Please let me know if this package does not arrive in satisfactory condition, or if the cultures are not viable. **Please, make a stock culture (freeze at -70°C) of each of the strains according to your laboratory's policy within 1 week from receiving them. Long-term storage of these cultures will ensure the availability of the PulseNet certification set for future use, including MLVA certification of additional personnel**.

The strain numbers of the *S. enterica* serotype Enteritidis cultures are as follows:

CDC# K2148 CDC# H9654 CDC# 2009K0432 CDC# K3307 CDC# J0932

CDC# K4417 CDC# K2158 CDC# K0746

Please follow these supplemental instructions for testing the certification isolates by MLVA. Refer to PNL26 (3130) or PNL30 (3500) for detailed instructions.

1. Make DNA templates from each test isolate, positive control K1891, and internal ladder isolates H9560 and 2010K0017.

2. Perform PCR and fragment analysis following the instructions of the standard protocol with the possible exception of the primer concentration modifications your laboratory may have had to make to optimize the PCR assays.

    a. Make sure to include your PulseNet laboratory ID (the unique identifier code that was assigned to your laboratory by CDC PulseNet) in front of the CDC strain ID number and your initials after the strain ID number, i.e. follow the strain ID format *labID*_CDCK2148xx.

3. Export the peak file from the sequencer in the .txt (tab-delimited) format. Please notice that in addition to the certification set isolates, the peak file must also contain the positive control strain K1891 in duplicate for each reaction, one negative control for each reaction, the internal ladder in duplicate, and the molecular size standard peaks (R peaks).

a. Name the peak files according to the standardized PulseNet naming system:

Use the laboratory ID that was assigned to your laboratory by CDC PulseNet for the first two to four letters of the file. The next 2 spaces will indicate the year the file was generated. The next 4 spaces indicate the month and the date the run was performed. For example: **GA090219.txt** is a peak file run on Feb 19th, 2009 at the GA Public Health Laboratory. If you perform multiple runs on a same day, differentiate the peak files using sequential numbers, for example GA090219-1, GA090219-2.

4. Send the peak file to CDC PulseNet at pfge@cdc.gov within four weeks after receiving the strains.

a. In the email to CDC, include ***S.* Enteritidis MLVA Certification** in the subject line.

Currently, for *S. enterica* serotype Enteritidis, an individual may be certified for peak file submission only. Once the national *S.* Enteritidis MLVA database is available on-line, individuals may also be certified for analysis.

After the peak files are submitted, the PulseNet certification file evaluator will analyze the files and inform your laboratory of your results ("Satisfactory" or "Needs Improvement") within four weeks of receiving the files. If the peak file is satisfactory, the person who submitted the file will be eligible to send peak files to PulseNet for analysis. If the submitted certification files are not satisfactory, the individual will need to review the troubleshooting comments received from the evaluator and resubmit once results have improved. If the submitter fails certification three times, the individual will not be allowed to submit again for six months. Before resubmitting, the individual will be expected to work with CDC until satisfactory results are achieved.

Please let me know if you have questions or further clarification is needed.

Good luck,

Eija Trees, D.V.M., Ph.D.
Unit Chief
PulseNet Next Generation Subtyping Methods Unit
EDLB, DFWED, CDC
Tel: 404-639-3672
E-mail: EHyytia-Trees@cdc.gov

Appendix PNQ06-5

Instructions: Reconstitution of Primers for *E. coli* O157:H7 and *Salmonella enterica* serotypes Typhimurium and Enteritidis

E. coli O157:H7:

Reconstitution of the primers:

The amount of primer in each vial is indicated on the label. In order to prepare a **100 µM** stock, please reconstitute the primers by adding following amounts of distilled water:

Amount of primer in the vial	**Water needed for a 100 µM stock**
10 nM	100 µl
40 nM	400 µl

Preparation of the working concentrations from the 100 µM stock:

Working concentration:	**Water (µl) + primer (µl)**
25 µM	30.0 + 10.0
5 µM	47.5 + 2.5
2.5 µM	48.75 + 1.25
1 µM	99.0 + 1.0

Salmonella Typhimurium:

Reconstitution of the primers:

The amount of primer in each vial is indicated on the label. In order to prepare a **100 µM** stock, please reconstitute the primers by adding following amounts of distilled water:

Amount of primer in the vial	**Water needed for a 100 µM stock**
10 nM	100 µl
20 nM	200 µl
40 nM	400 µl

Preparation of the working concentrations from the 100 µM stock:

Working concentration:	**Water (µl) + primer (µl)**
25 µM	30.0 + 10.0
5 µM	47.5 + 2.5
2.5 µM	48.75 + 1.25

***Salmonella* Enteritidis:**

Reconstitution of the primers:

The amount of primer in each vial is indicated on the label. In order to prepare a **100 µM** stock, please reconstitute the primers by adding following amounts of distilled water:

Amount of primer in the vial	**Water needed for a 100 µM stock**
10 nM	100 µl
20 nM	200 µl

Preparation of the working concentrations from the 100 µM stock:

Working concentration:	**Water (µl) + primer (µl)**
12.5 µM	43.75 + 6.25
2.5 µM	48.75 + 1.25

READING CONTROL SHEET FOR:
STANDARD OPERATING PROCEDURES FOR THE PULSENET QA/QC PROGRAM (PNQ)

NAME	DATE	COMMENTS	SIGNATURE

By signing above, you are indicating that you have read and understood all SOPs included in the PNQ section of this manual.