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:

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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	

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 PNL23-3b**

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).