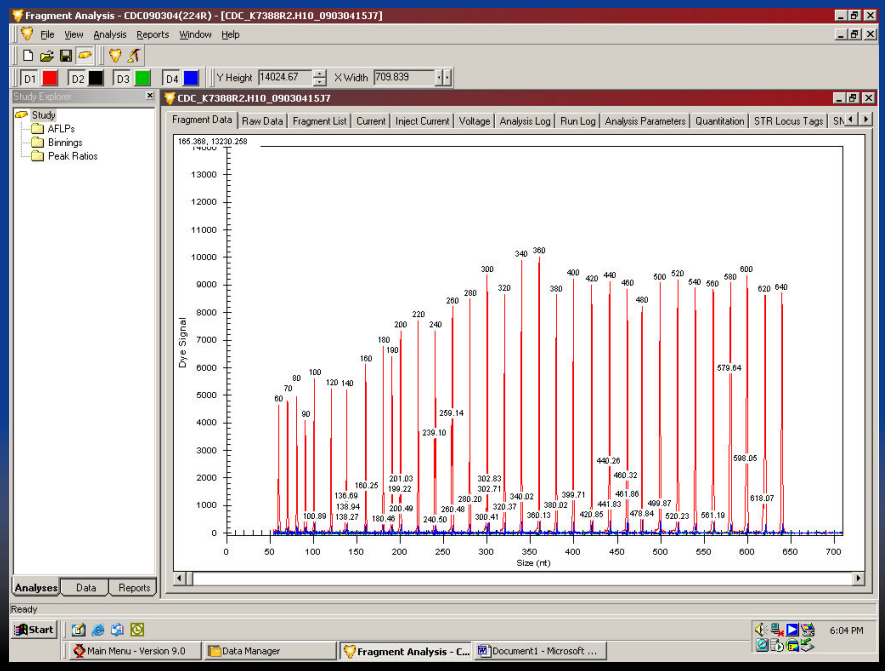
Troubleshooting laboratory aspects of MLVA

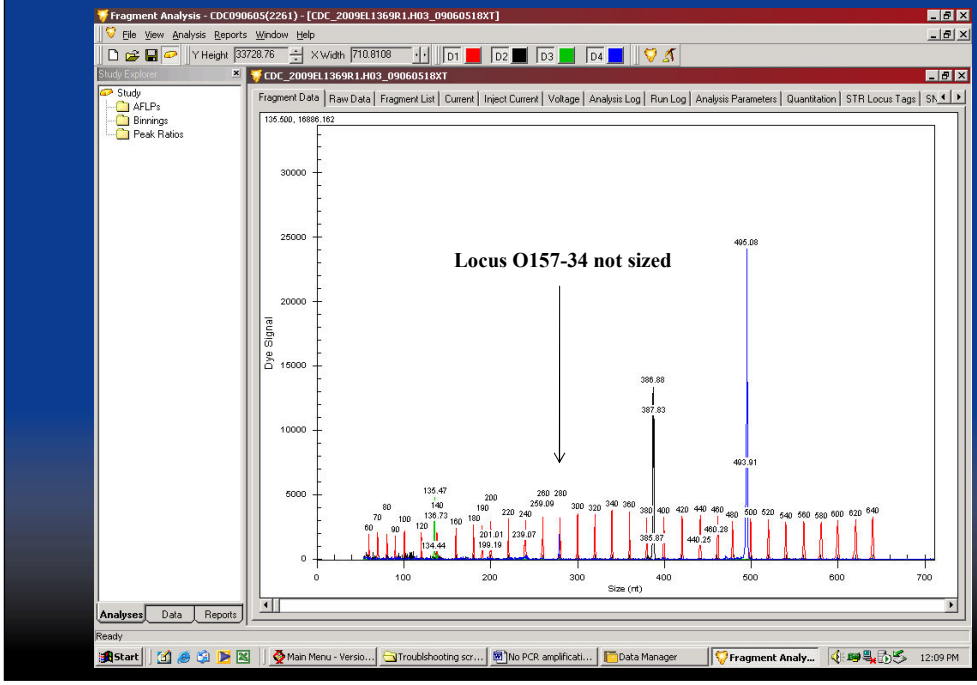
Eija Trees, Ph.D., D.V.M.
PulseNet Methods Development and Reference Unit
Enteric Diseases Laboratory Branch
CDC, Atlanta, GA

No PCR amplification due to a missing reagent or DNA - CEQ



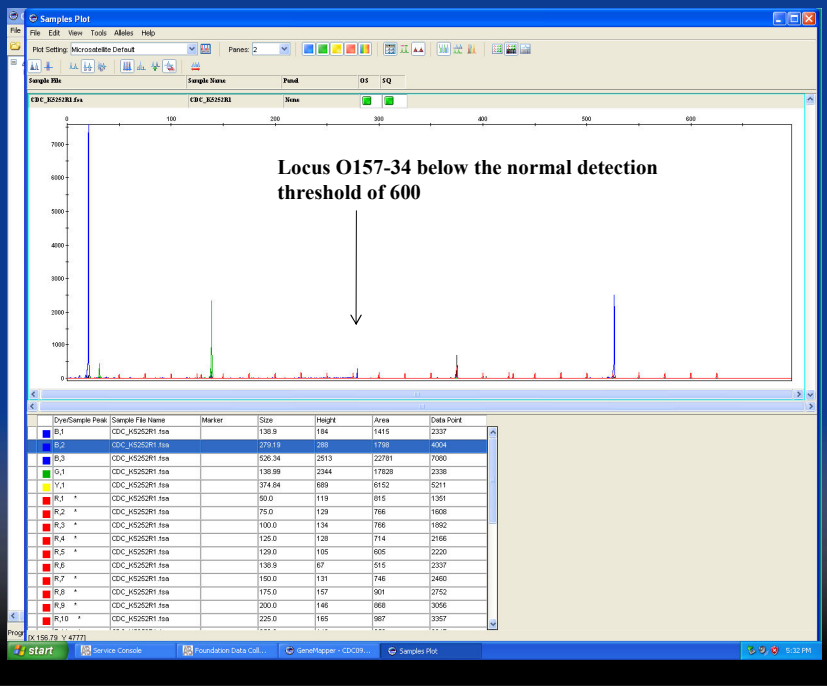
If all samples and controls fail to amplify, it is safe to assume that a critical component of the mastermix, such as Taq polymerase or $MgCl_2$ was not added to the mastermix.

Weak amplification of one locus due to degraded primers - CEQ



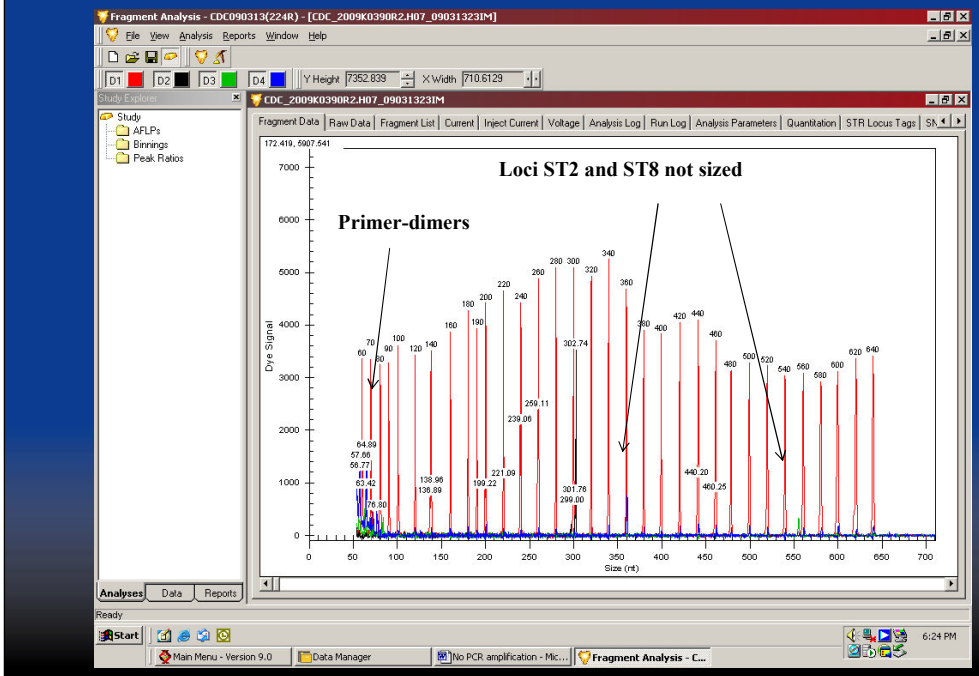
The primer working stocks degrade due to freeze-thaw cycles. New working stocks should be prepared from the concentrated stocks when a significant drop in the fluorescence intensity is observed. The concentrated stocks should be stored in small aliquots at -70ºC. The concentrated stocks typically store for 2 to 3 years.

Weak amplification of one locus due to pipetting error - ABI



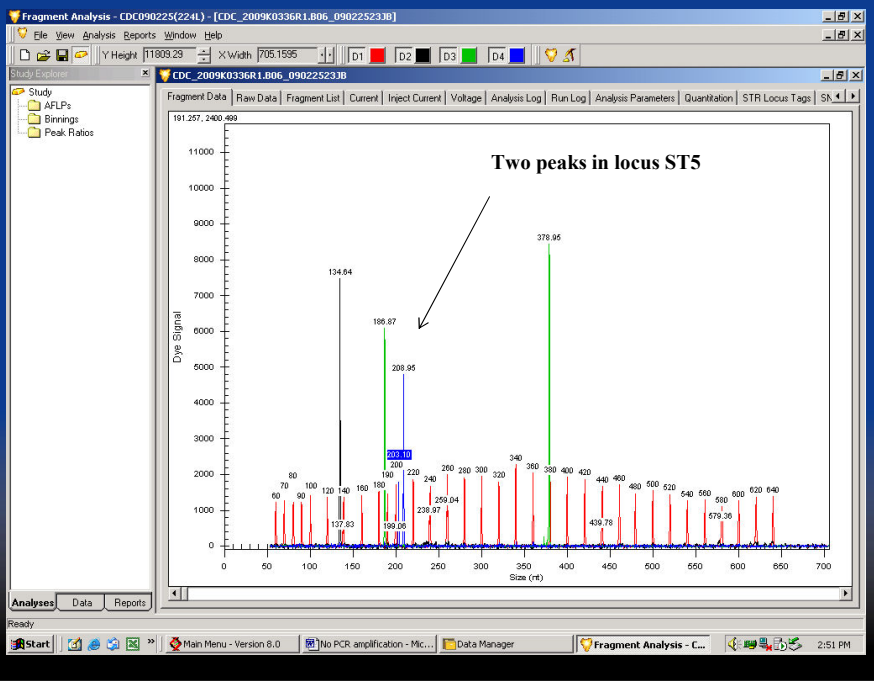
Since the pipetting volumes are very small, extra attention should be paid to careful pipetting techniques. Pipettes should also be regularly (every 6 to 12 months) calibrated.

Weak PCR amplification of all loci due to a thermocycler problem - CEQ

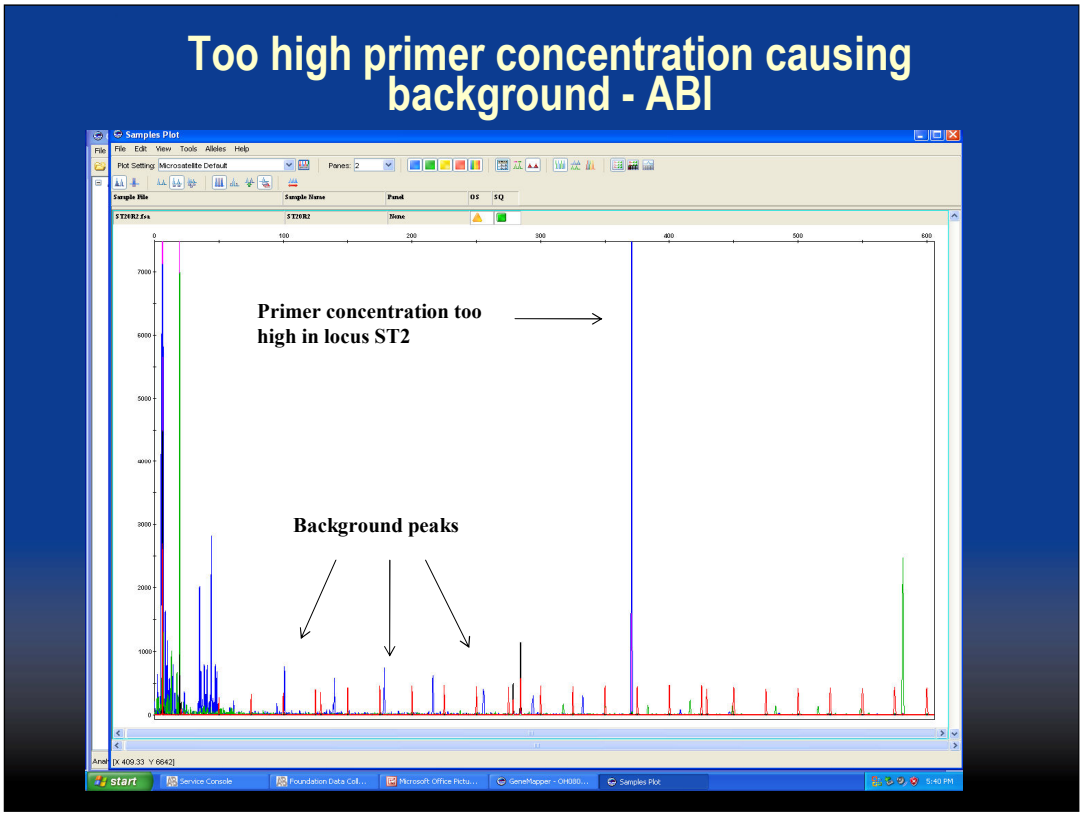


If the thermocycler is malfunctioning (for example not reaching the correct temperatures), all targets typically amplify weakly, and a pronounced primer-dimer peak is observed. In some thermocyclers PCR tubes work better than PCR plates due to more efficient temperature distribution.

Two peaks in the same locus – contamination or two alleles?

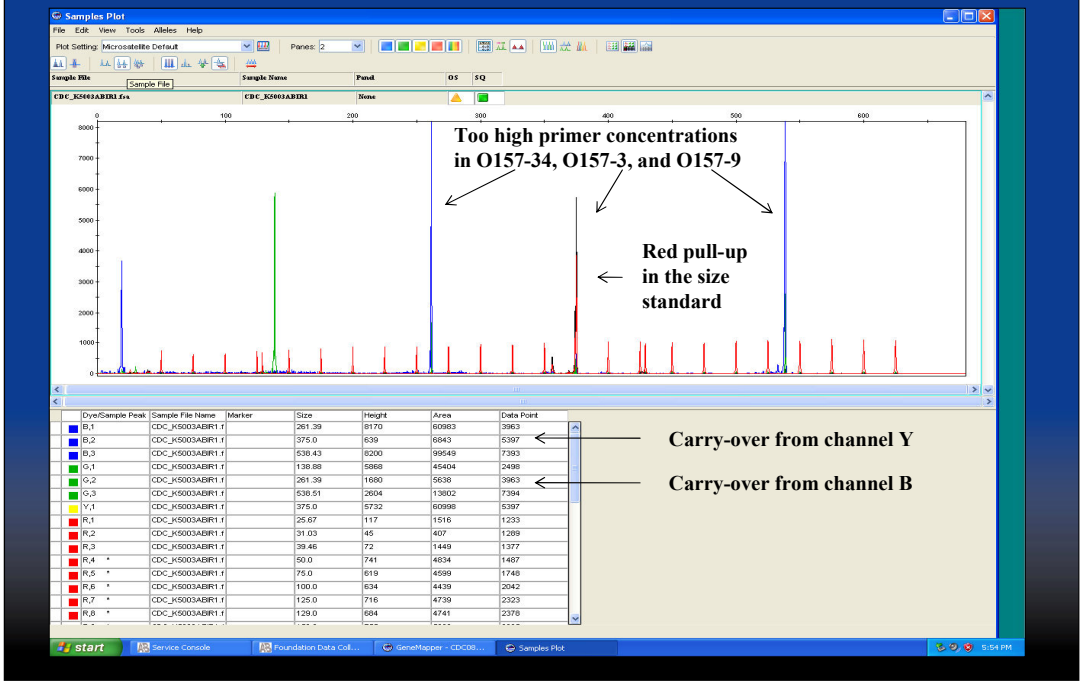


Cross contamination of the fragment analysis reaction or the DNA template may result in multiple peaks being observed in a single locus. In this case, the PCR should always be repeated with a fresh DNA template. In rare cases, an isolate may posses two copies of the same locus in its genome.

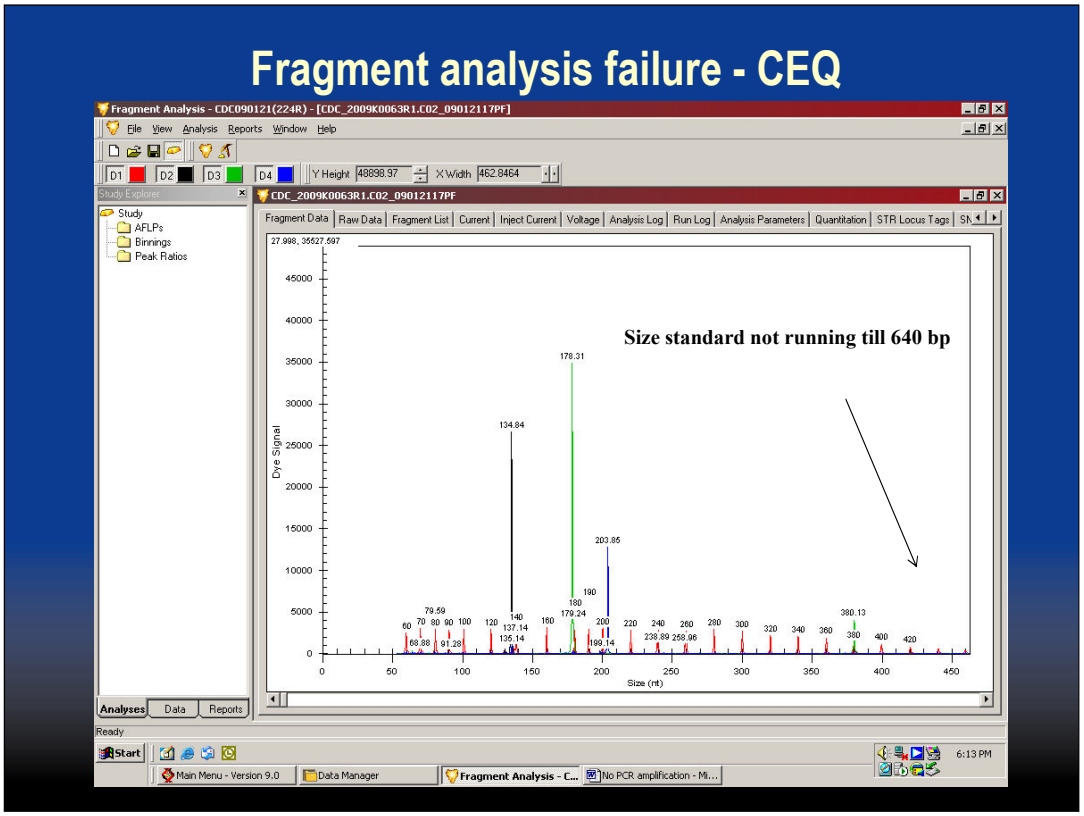


Too high primer concentration may be caused by a working stock that was prepared wrong or if a new primer stock was introduced to the mix. When primer stocks age, the fluorescence intensity gradually drops necessitating increases in primer concentrations in the mastermix. When a new stock is introduced in the mastermix it is often necessary to adjust the concentrations downwards in order to optimize the fluorescence intensity.

Too high primer concentrations causing fluorescence carry-over - ABI

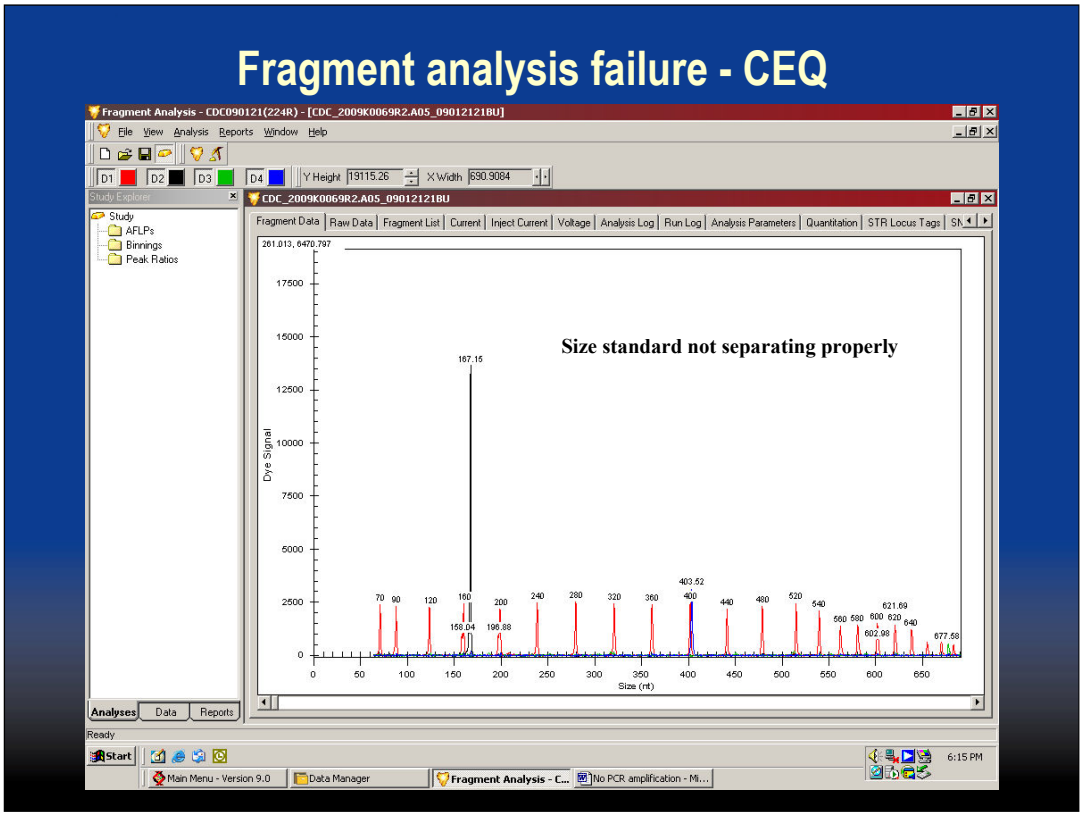


Too high primer concentrations very often cause fluorescence overflow between channels which is manifested as non-specific carry-over peaks. This problem is particularly pronounced in the Applied Biosystems sequencers. It is therefore important to have the primer concentrations well optimized since the carry-over peaks can interfere with the data analysis. A too high primer concentration can also cause red pull-up in the size standard which may interfere with sizing if the software labels the pull-up peak instead of the actual size standard peak.



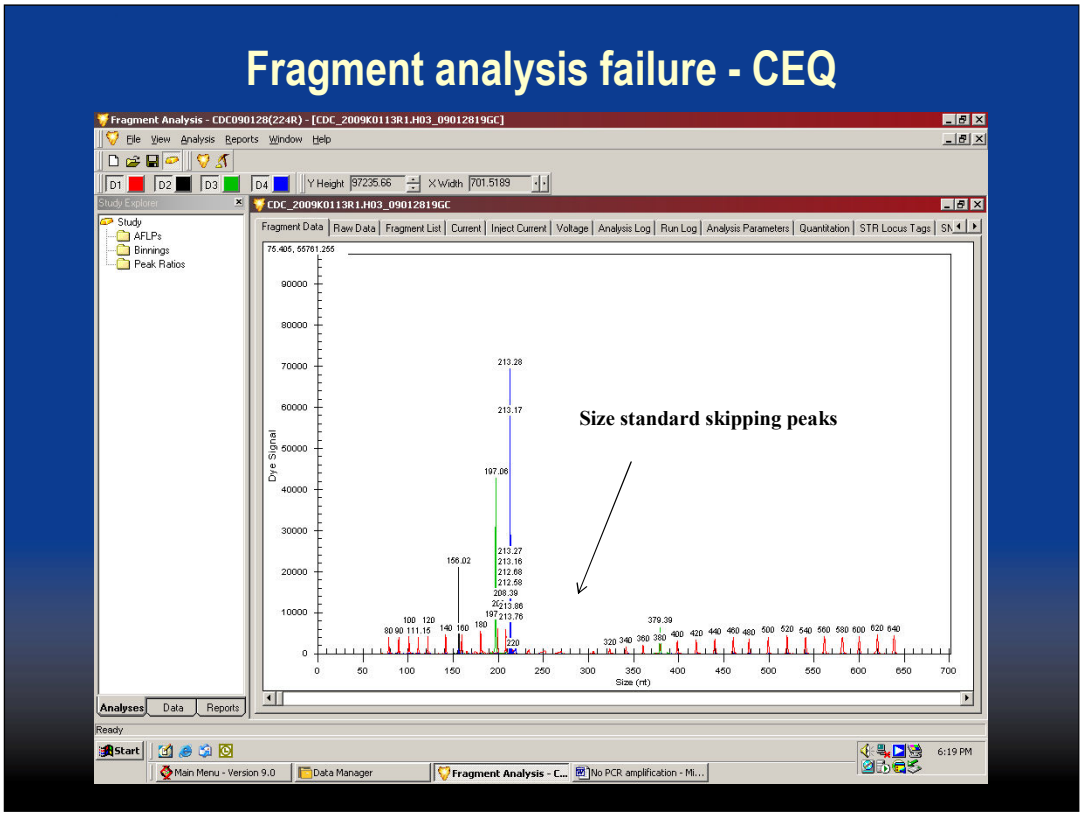
An example of a fragment analysis failure on the Beckman Coulter CEQ. Fragment analysis failures can be caused by expired or degraded size standard or sample loading solution or by a capillary array that is too old (>45-50 days at room temperature) or has too many (>100) runs on it. If reagent and array related issues can be ruled out, an increased failure rate can often be attributed to instrument related issues, and may require a service call.

Fragment analysis failure - CEQ



Another example of a fragment analysis failure on the Beckman Coulter CEQ.

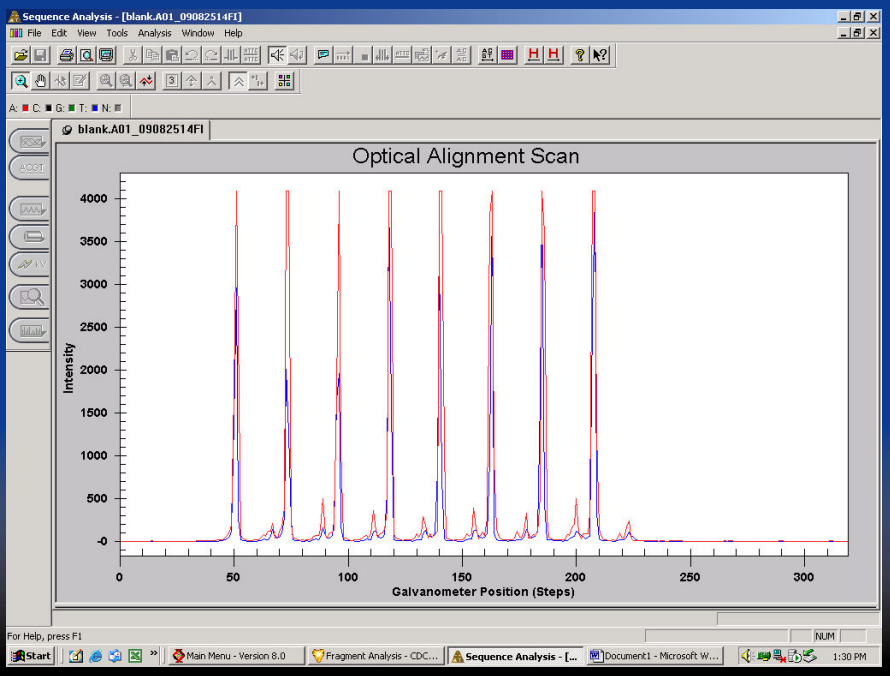
Fragment analysis failure - CEQ



Another example of a fragment analysis failure on the Beckman Coulter CEQ.

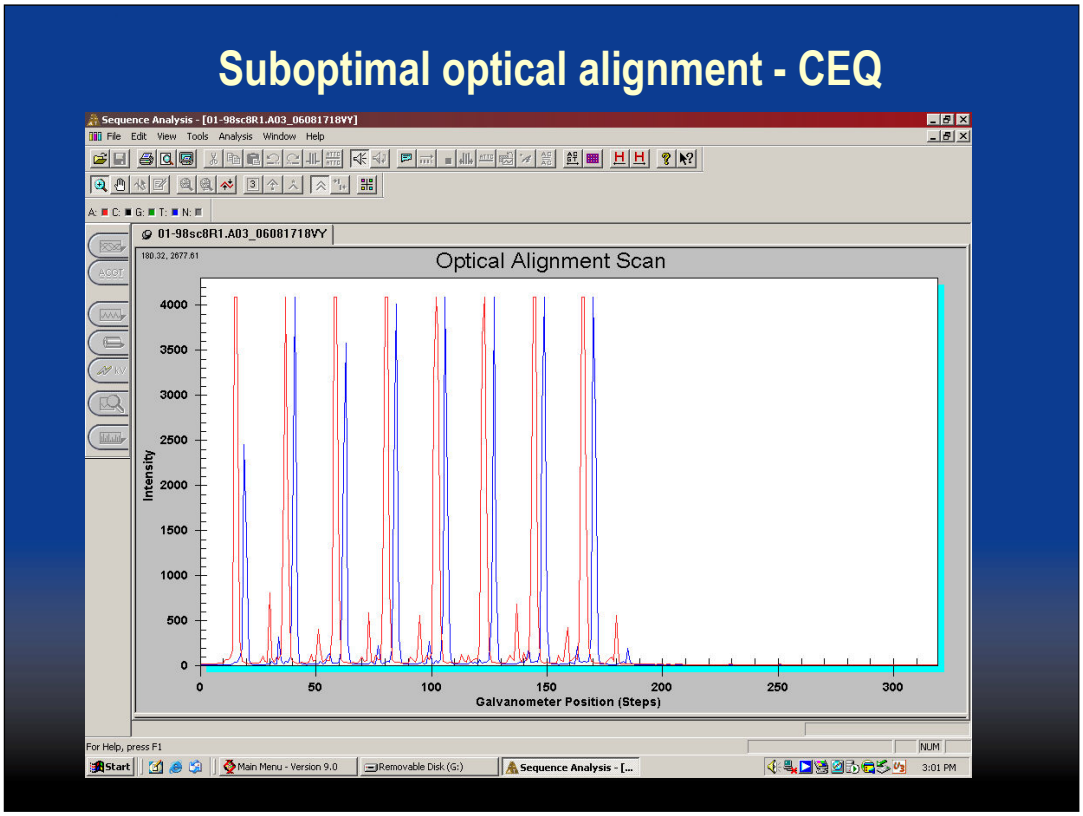
On the Beckman Coulter CEQ, too high background fluorescence can cause an increased fragment analysis failure rate. Background fluorescence may be high if the array is too old (>45-50 days at room temperature) or has too many runs (> 100) on it. If a new array has high background, cleaning the optical window with a swab moistened with distilled water typically brings down the background.

Acceptable optical alignment - CEQ

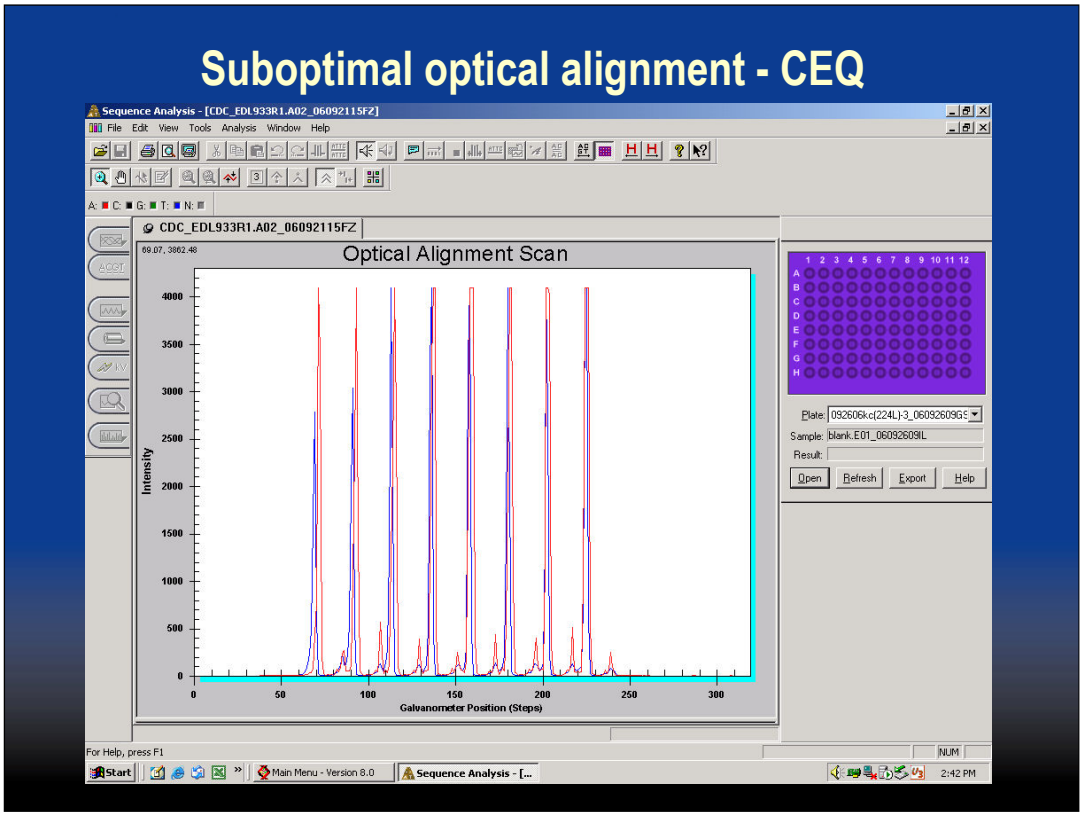


On the Beckman Coulter CEQ, suboptimal optical alignment can cause an increased fragment analysis failure rate. In an optimal optical alignment, the red and blue peaks for all eight capillaries are exactly superimposed and start at the galvanometer reading 50 and end at 210.

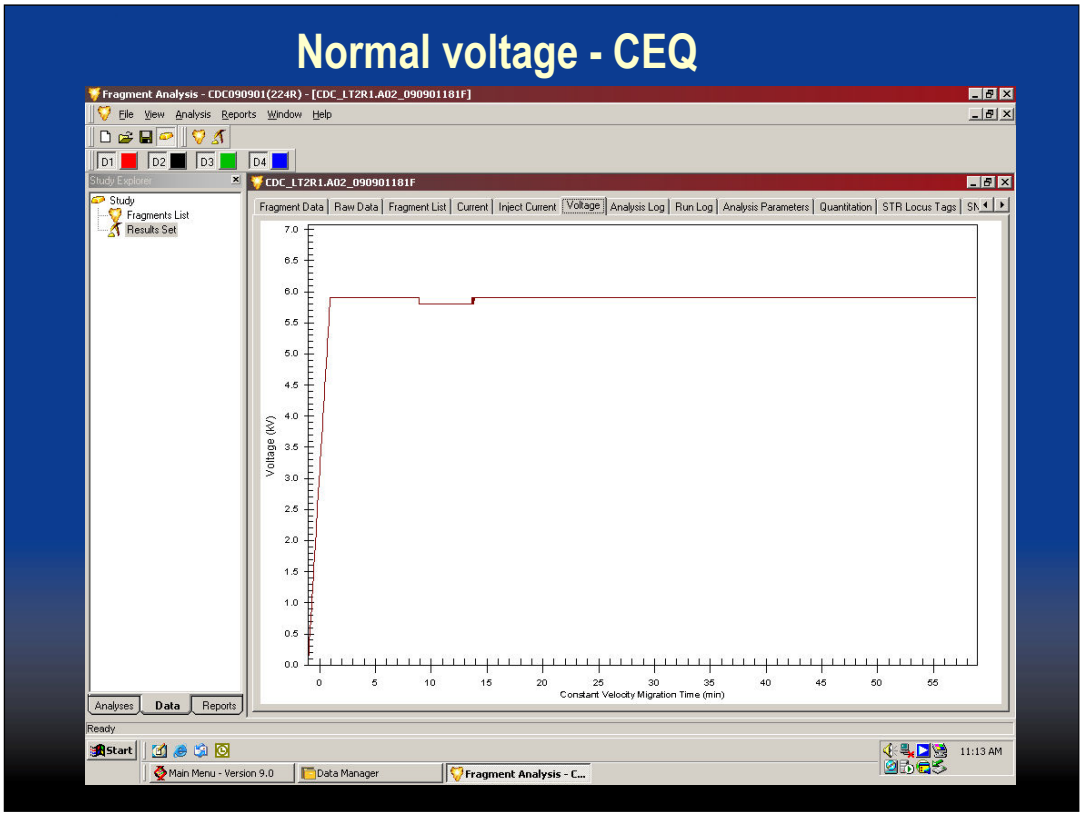
Suboptimal optical alignment - CEQ



An example of a bad optical alignment on the Beckman Coulter CEQ. The peaks start too early and the red and blue peaks are not superimposed.

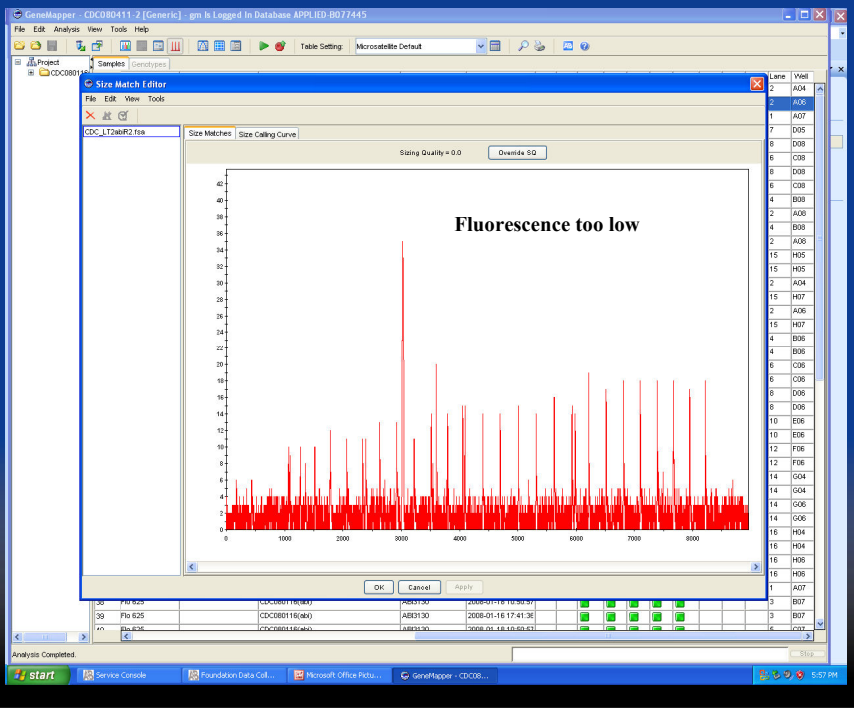


An example of a bad optical alignment on the Beckman Coulter CEQ. The peaks start too late.

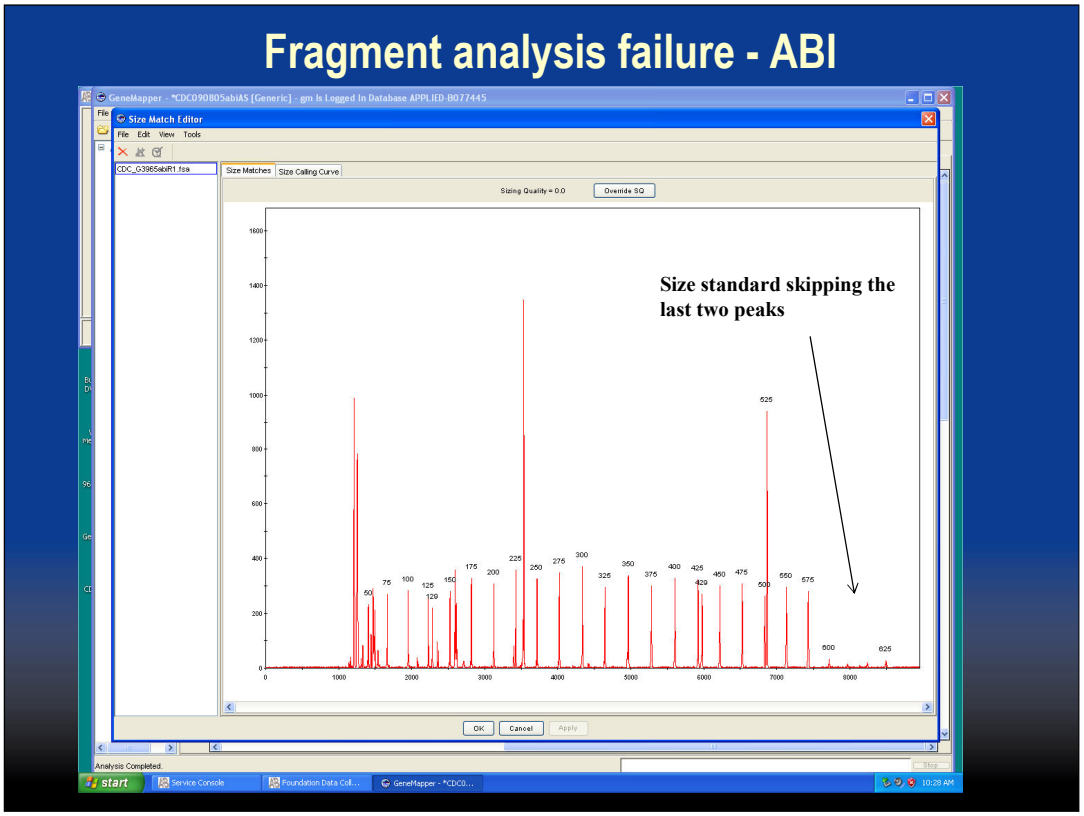


The normal voltage should be at around 6 kV. A voltage crash caused by for example an air bubble in the array can cause the fragment analysis reaction to fail.

Fragment analysis failure - ABI

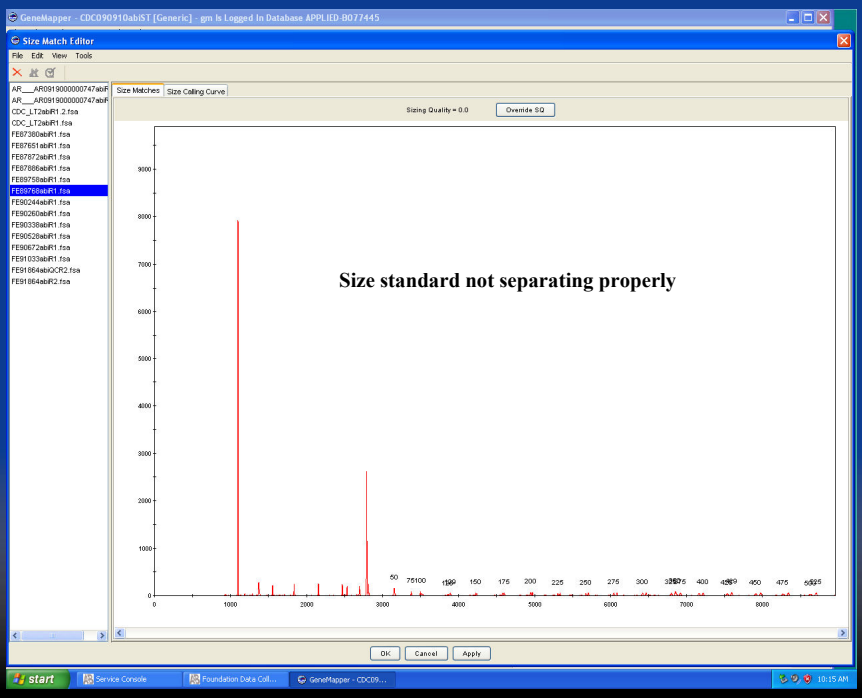


An example of a failed fragment analysis reaction on the Applied Biosystems Genetic Analyzer. Just like on the Beckman Coulter CEQ, fragment analysis failures can be caused by expired or degraded size standard or sample loading solution or by a capillary array that has too many runs on it (>80-100). If reagent and array related issues can be ruled out, increased failure rate can often be attributed to instrument related issues, and may require a service call.

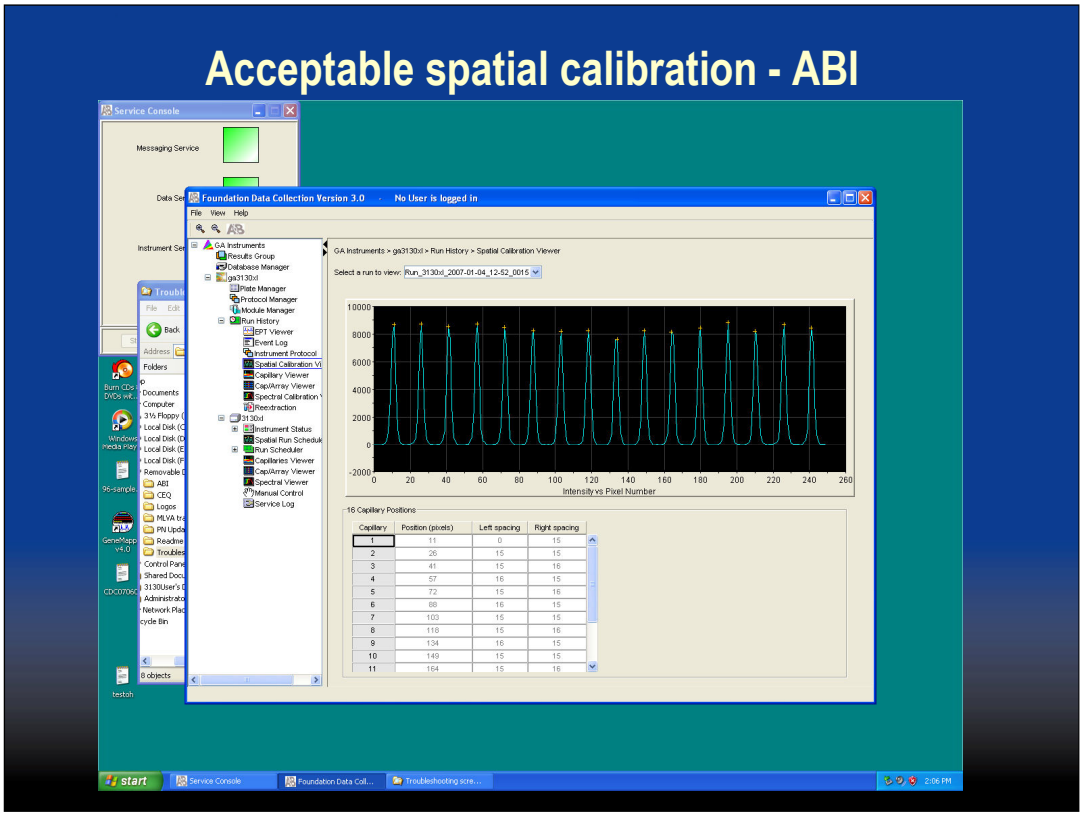


Another example of a failed fragment analysis reaction on the Genetic Analyzer

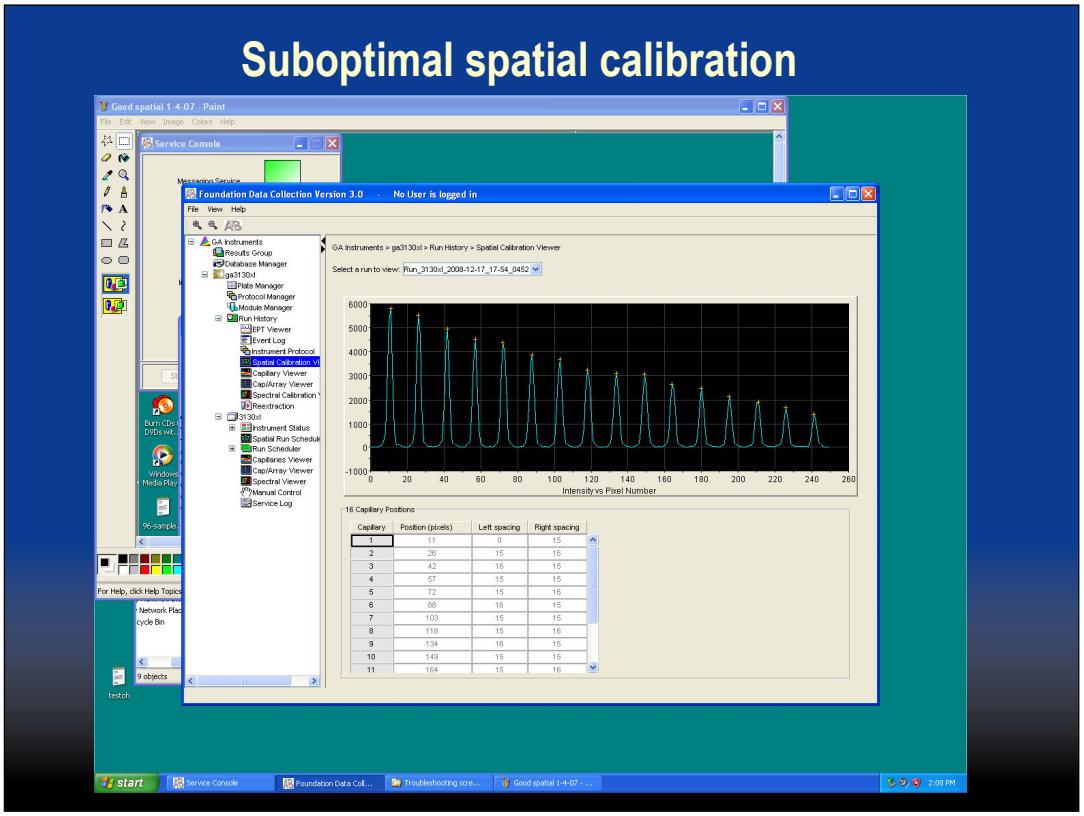
Fragment analysis failure - ABI



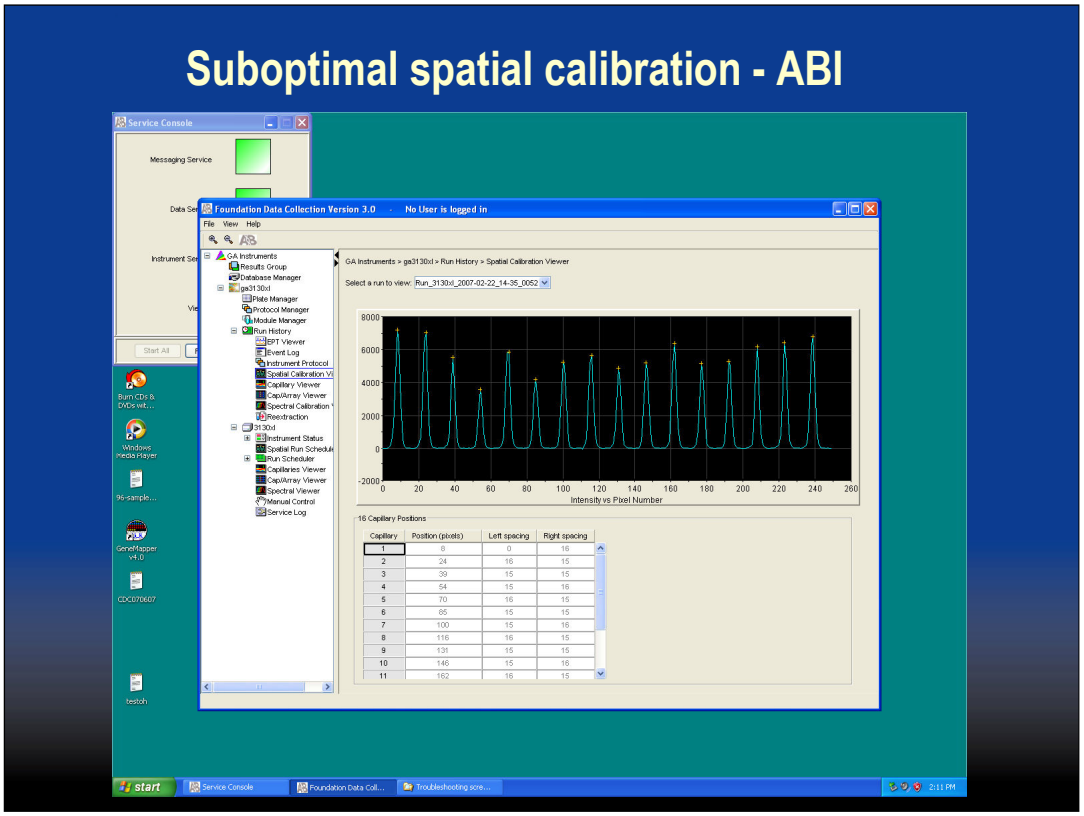
Another example of a failed fragment analysis reaction on the Genetic Analyzer



On the Applied Biosystems Genetic Analyzer, a suboptimal spatial calibration can cause an increased fragment analysis failure rate. In an acceptable spatial calibration, the peaks for each 16 capillaries are sharp, spaced equally and of approximate equal height.

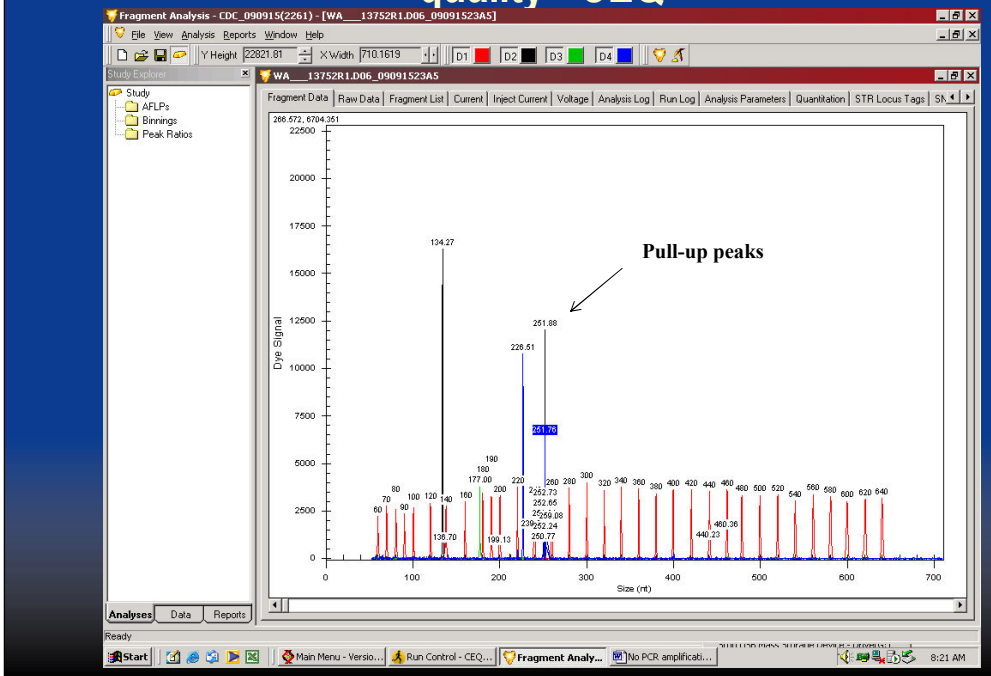


An example of a suboptimal spatial calibration on the Genetic Analyzer. Suboptimal spatial calibrations require a service call.



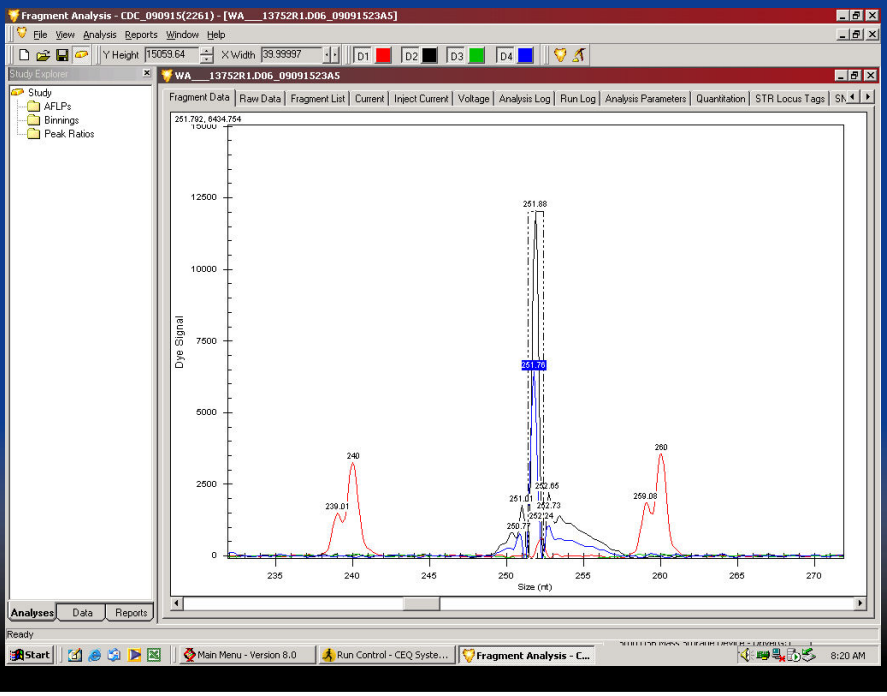
Another example of a suboptimal spatial calibration on the Genetic Analyzer.

Non-specific pull-up peaks due to suboptimal array quality - CEQ

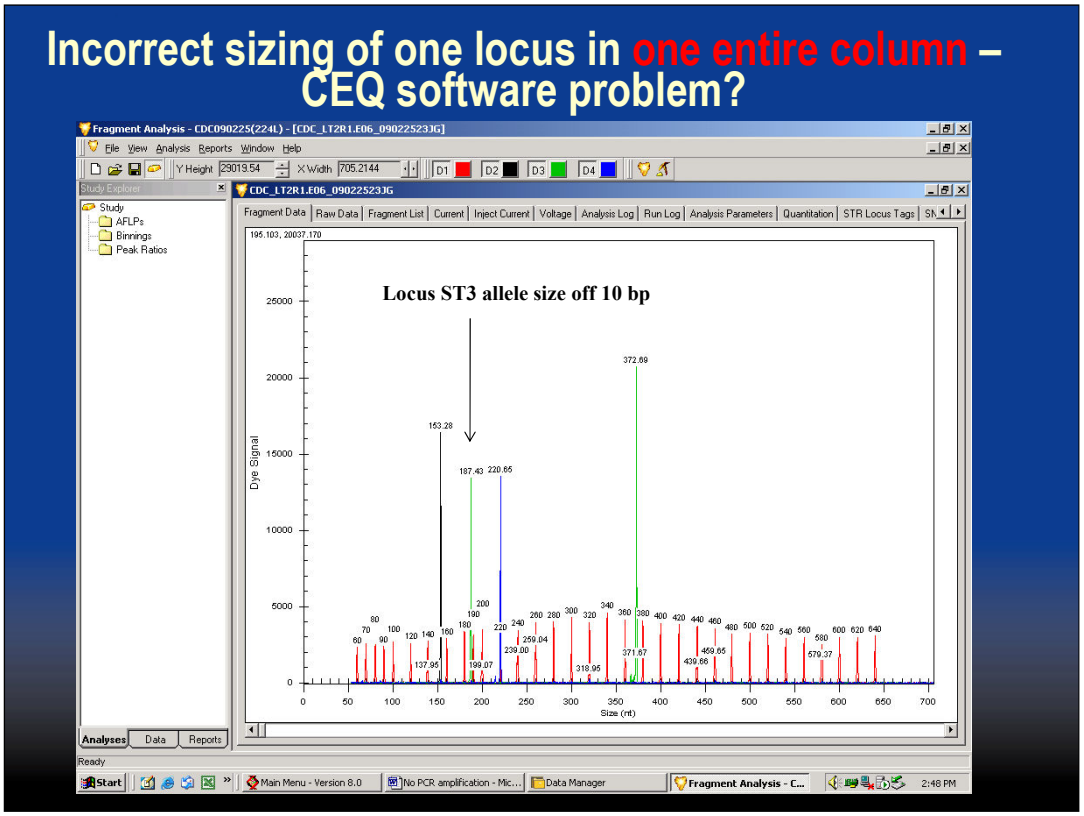


On the Beckman Coulter CEQ, when the array ages you often start seeing “pull-up peaks” which are manifested as non-specific peaks labeled with two or all three dyes that are exactly the same size. Please note that this phenomenon has nothing to do with PCR. If the same PCR reaction is tested with a newer array, the pull-up peaks would be gone.

Non-specific pull-up peaks due to suboptimal array quality - CEQ

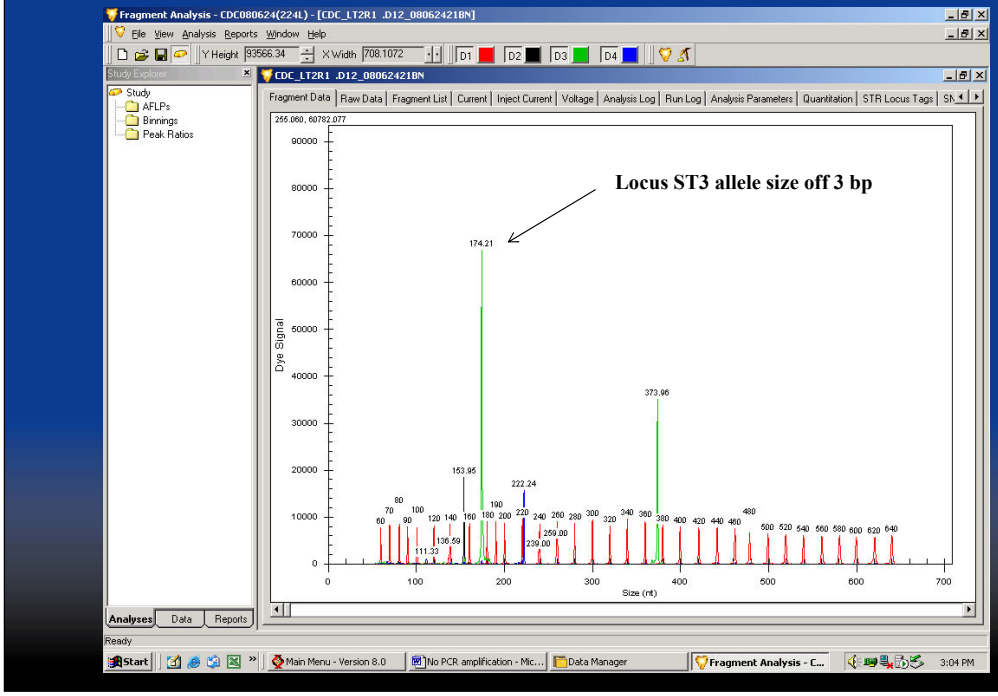


In this case the pull-up peaks were labeled with D2 (black) and D4 (blue) dyes



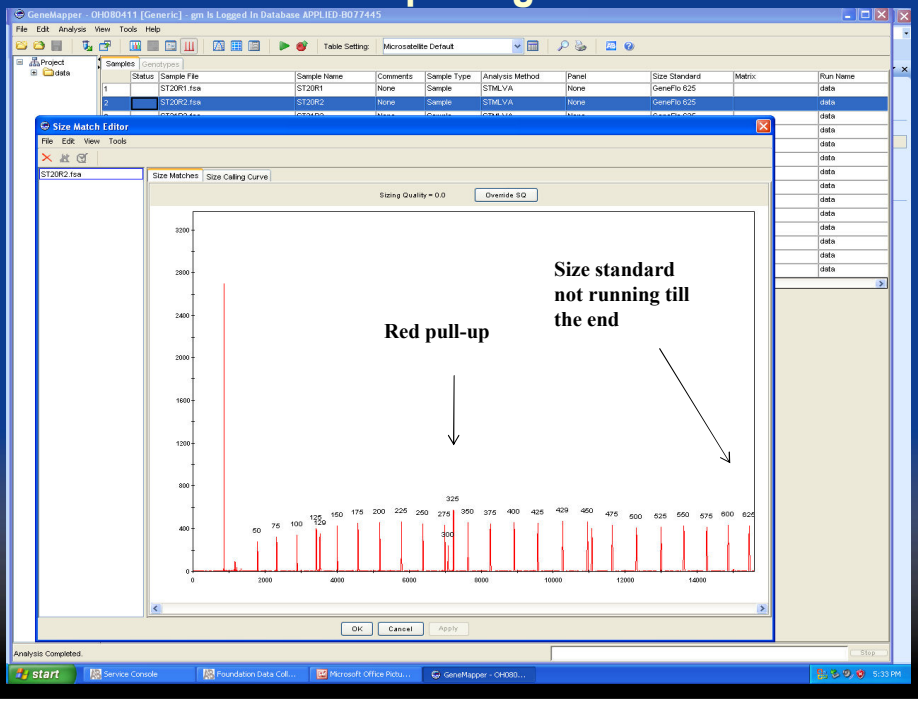
On the Beckman Coulter CEQ, a software malfunction in middle of run can cause some, but usually not all, loci be sized wrong. When this happens, the incorrect sizing usually occurs in entire column and then in the next column sizing appears to be correct. Any fragment analysis reactions in which the observed size does not fit the expected size ranges for known alleles stated in the look-up tables (included in the BioNumerics SOPs) should be repeated. If software malfunctions are frequent, a service call should be placed.

Incorrect sizing of one locus in whole run due to expired gel - CEQ



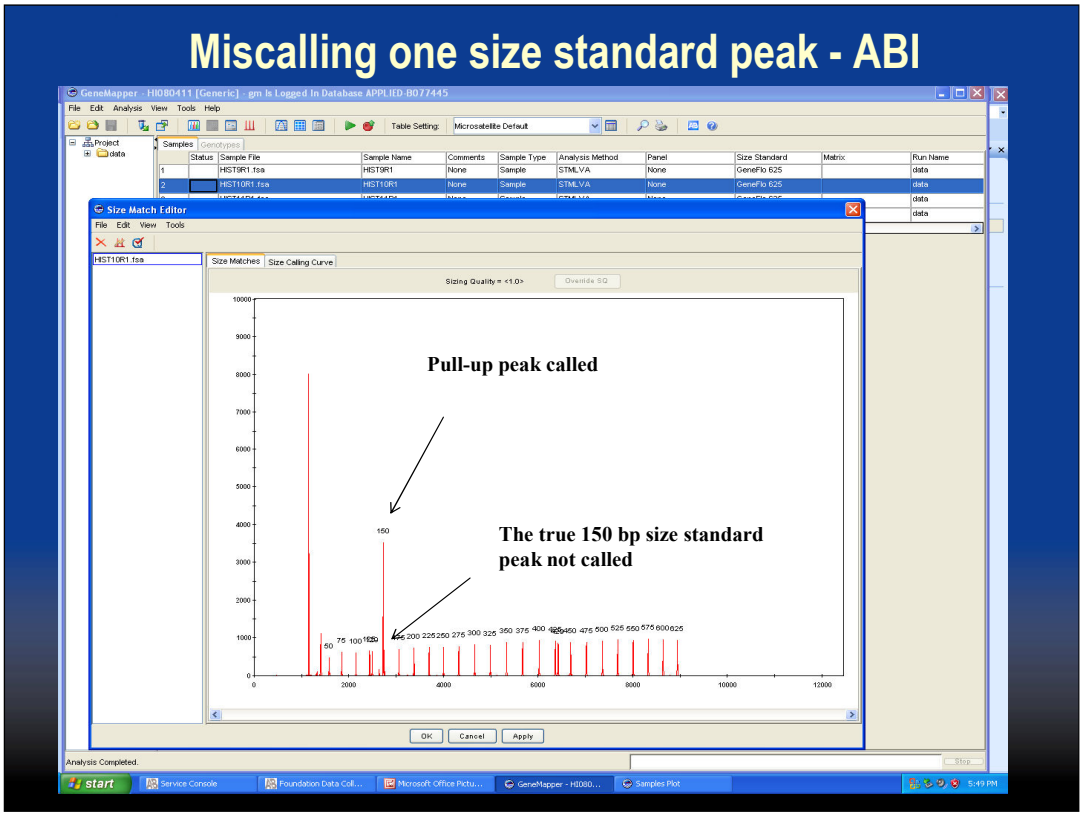
On the Beckman Coulter CEQ, using a gel that has spent more than 72 hours at the room temperature can cause inaccurate sizing.

Miscalled size standard peaks due to red pull-up and expired gel - ABI



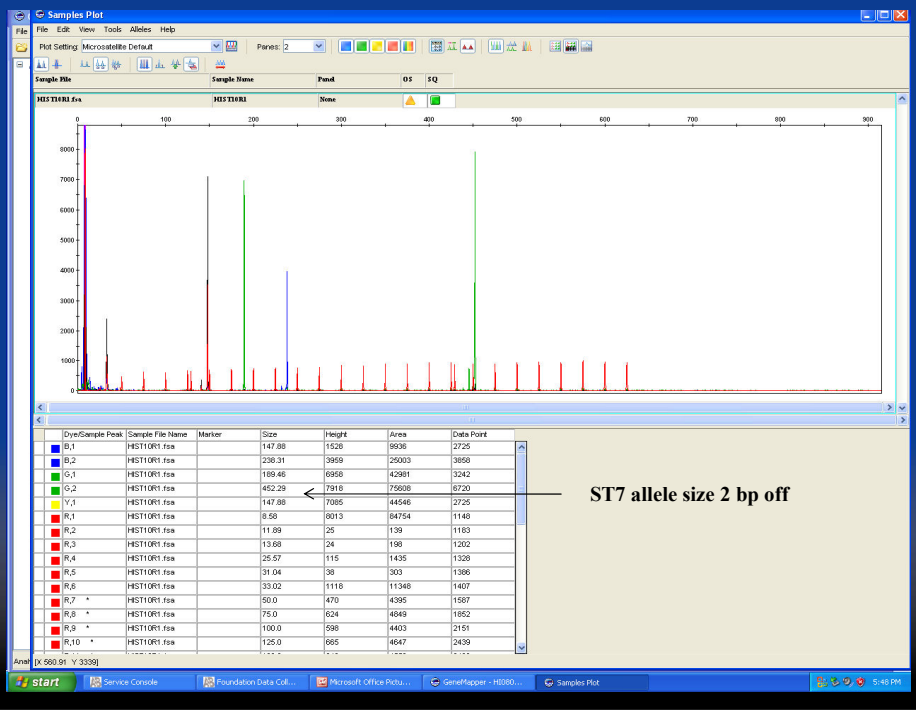
On the Applied Biosystems Genetic Analyzer, using a gel that has spent more than 8 days at the room temperature can cause the size standard to run short which may result in large fragments being missed. Additionally in this situation, pull-up peaks in the size standard (caused by too a high primer concentration) may be labeled resulting in incorrect sizing of the fragments.

Miscalling one size standard peak - ABI



On the Genetic Analyzer, pull-up peaks in the size standard may sometimes be labeled even when the size standard runs all the way till the end. Errors in the size standard labeling can be manually fixed with the GeneMapper software, and the sample can be re-analyzed without having to rerun the fragment analysis reaction.

Incorrect sizing due to miscalled size standard peak - ABI



In this case, erroneous labeling of a pull-up peak in the size standard resulted in one fragment being 2 bp outside the accepted size range.