Advanced Topics in STR DNA Analysis

CE Troubleshooting

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CE Troubleshooting

Outline for This Section

1. Chemistry/molecular biology problems – stutter, -A, degradation, inhibition, low copy #
2. Sample and buffer problems – formamide, urea, water, salt concentration, free dye ("dye blobs")
3. External factors – power supply, room temperature, cleanliness, voltage leaks
4. Instrument problems – optical system, capillary clogging, air bubbles, syringe leaks
5. Troubleshooting benchmarks/QC monitoring

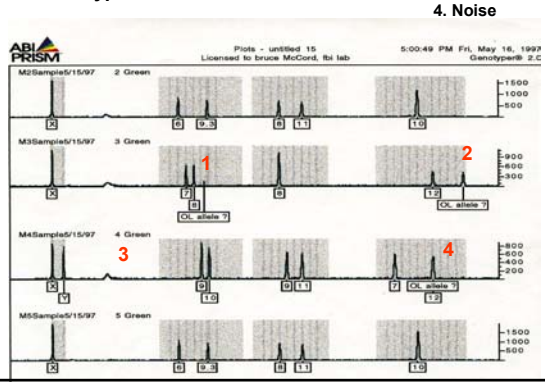
1. Chemistry/Molecular Biology Problems

- **PCR amplification issues**
  - **Adenylation**
  - **Stutter**
  - **Non Specific Amplification**
  - **Primer dimers**
  - **Pipetting small amounts**
- **Degradation/Inhibition**
  - **Allele dropout**
  - **Over amplification**
  - **Ski slope effect**
  - **Mitigation Steps for inhibition**

Off-ladder alleles

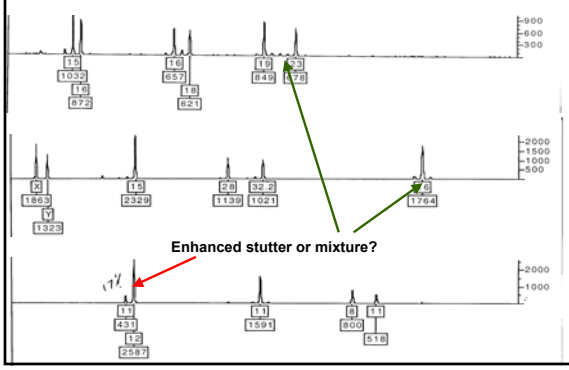
Four types

1. Spike
2.OL Allele
3. Free Dye
4. Noise

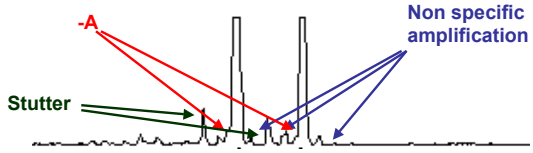


Extract of Pistol Grip and Trigger

Relatively low amount of amplified DNA?



Overloaded peaks will also show relatively high stutter



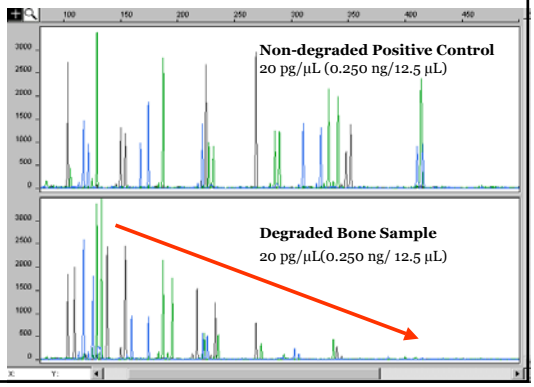
Truncated peaks give wrong ratios for peak stutter
Why else is overloading bad?

1. **raised baseline**
2. **non specific amplification**
3. **peak height ratios**
4. **-A**

Degradation and PCR Inhibition

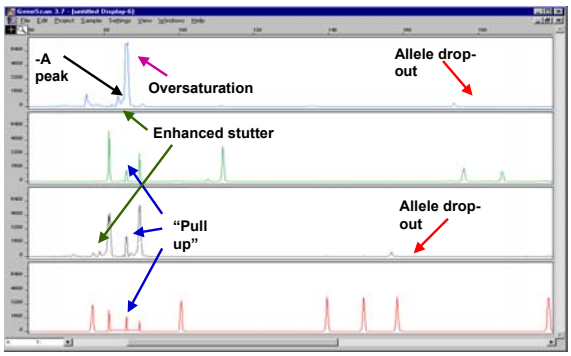
- Degradation affects larger alleles more, however there is no published study on the "threshold at which degradation is apparent"
  - The amplification efficiency of each set of alleles varies independently and differential amplification across loci can occur – Moretti, JFS 2001
  - Low quality formamide can mimic the degradation effect
  - Inhibition generally affects certain loci more than others and may or may not produce a slope effect- McCord, unpublished
  - There are several likely mechanisms for inhibition including DNA aggregation, Protein-DNA binding, chelation of Mg, interference with primer binding, etc.

DNA Degradation



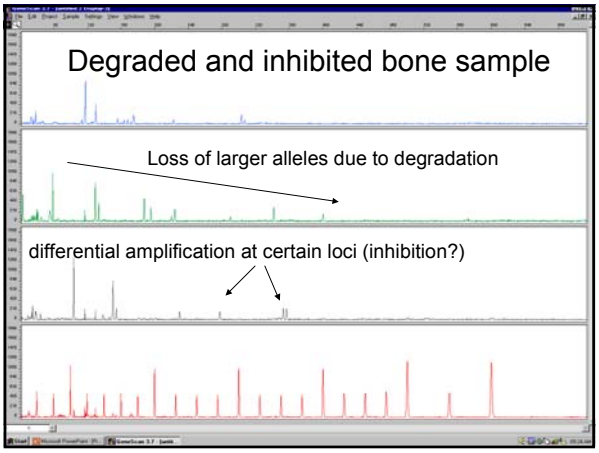
Degraded DNA and Amplification

With degraded DNA two injections may be necessary to keep data on-scale



Non-DNA Contamination/Inhibition

- Anything that is water soluble may co-extract with DNA unless a capture technique is used.
- For capture techniques anything with a similar chemical property to DNA may co-extract
- Detergents, metal ions, humic substances are all potent contaminant/inhibitors
- Can cause all sorts of strange effects including
  - Spikes, dye blobs, elevated baselines, loss of signal, odd current effects

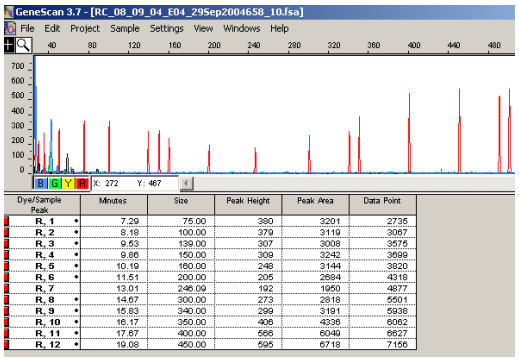


2. Sample Issues

- Formamide Conductivity
- Excessive salt in sample due to evaporation
- Metal ion contamination
- Sensitivity issues with Microcon cleanup (salt removal)
- Dye "blobs" – artifacts from primer synthesis

Golden Gate Effect

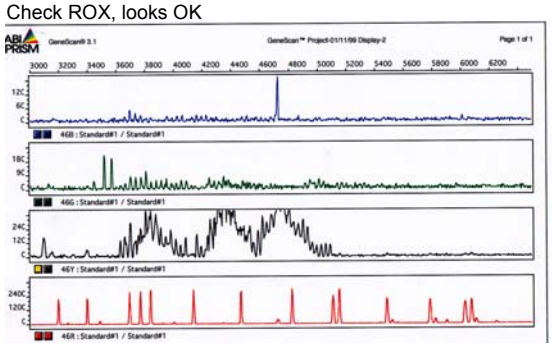
Attributed to poor formamide



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
R, 1	7.29	75.00	380	3201	2735
R, 2	8.18	100.00	379	3119	3067
R, 3	9.53	139.00	307	3008	3575
R, 4	9.86	150.00	309	3242	3699
R, 5	10.19	160.00	248	3144	3820
R, 6	11.51	200.00	205	2684	4318
R, 7	13.01	246.09	192	1950	4877
R, 8	14.67	300.00	273	2818	5501
R, 9	15.83	340.00	299	3191	5938
R, 10	16.17	350.00	406	4336	6062
R, 11	17.67	400.00	566	6049	6627
R, 12	19.08	450.00	595	6718	7156

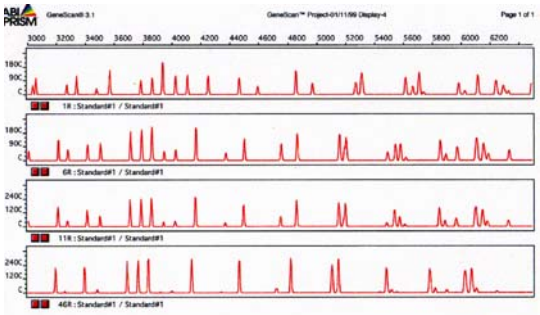
Sample Problem?.

Check ROX, looks OK



320 V/cm 47 cm uncoated capillary
POP4 Polymer

Answer: Incomplete denaturation of standard due to poor quality formamide



320 V/cm 47 cm uncoated capillary
POP4 Polymer

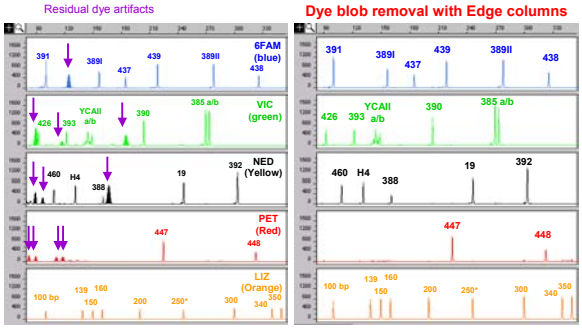
Post PCR manipulation

- Reprocessing post PCR to concentrate samples can improve signal but be careful
  - PCR sample is concentrated but:
    - Spin filtration may result in removal of background salts,
    - This can greatly enhance sensitivity due to the stacking process
    - Best idea- remake sample up in buffer, not water to avoid reading stochastic effects.

Example of an Interpretational Guideline

1or 3 ul product:	microcon enhancement:	Action:
50RFU < peak < 150RFU	peak > 150RFU	Report peak
peak <50RFU peak <50RFU	peak > 150RFU 50RFU < peak < 150RFU	Report activity (A) only Report activity (A) only
no peak detected no peak detected	peak > 150RFU 50RFU < peak < 150RFU	Not reported Not reported

Dye Blobs and their Removal



NIST Y-STR 20plex assay

Butler, J.M. (2005) Constructing STR multiplex assays. *Methods in Molecular Biology: Forensic DNA Typing Protocols* (Carracedo, A., ed.), Humana Press: Totowa, New Jersey, *in press*.

3. External Factors

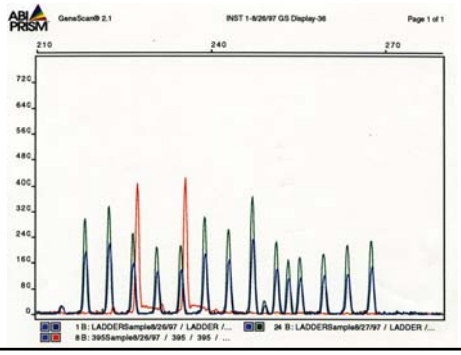
- Room temperature
  - Variations in room temperature can cause mobility shifts with band shifts and loss of calibration
  - Temperature is also important due to effects of high humidity on electrical conductance
- Cleanliness
  - Urea left in sample block can crystallize and catalyze further crystal formation causing spikes, clogs and other problems.
  - Best bet is to keep polymer in system and not remove or change block until polymer is used up.

Temperature effects

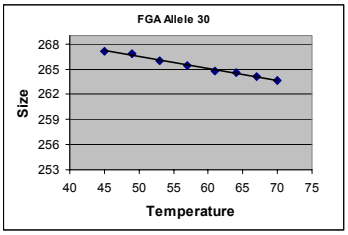
- Viscosity – mobility shift
  - $\mu_{ep} = q/6\pi\eta r$
- Diffusion – band broadening
  - ← DNA →
- Conformation – DNA size based sieving
  - vs $\mu_{ep} = q/6\pi\eta r$
- Current – Power
  - $P = VI = I^2R$
  - Increased current → internal temperature rise→ diffusion → band broadening

Band shift in the FGA locus

Likely the result of temperature or viscosity induced mobility change

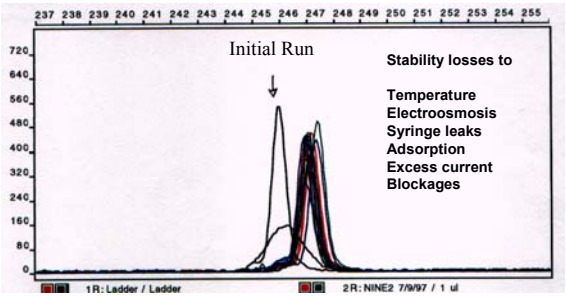


Effect of Temperature on allele size

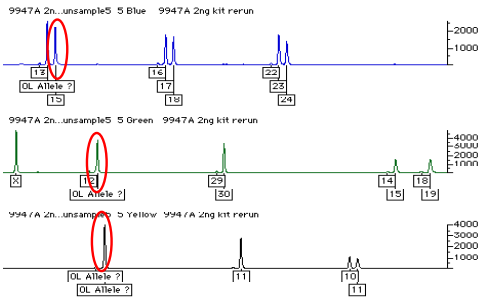


Slope is 0.14 bases/degree centigrade
Therefore a small change in temperature has a big effect
(A 1-2 degree shift in temperature of the heat plate can produce an OL allele)

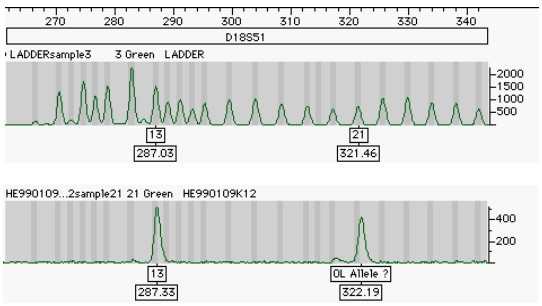
Due to its structure and its non-calibration, the "250"peak can be used to indicate stability



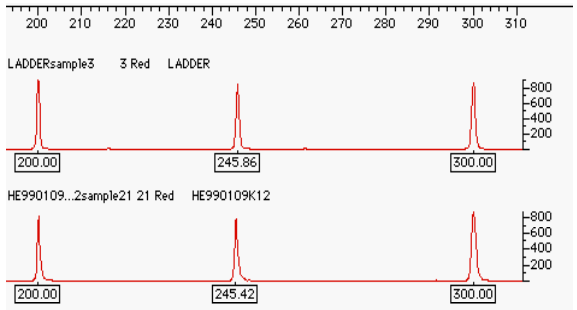
Temperature Effects: "OL" Alleles



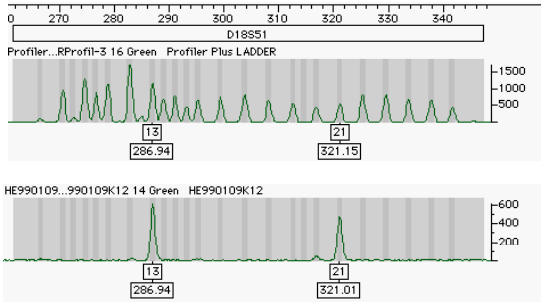
"OL Alleles"



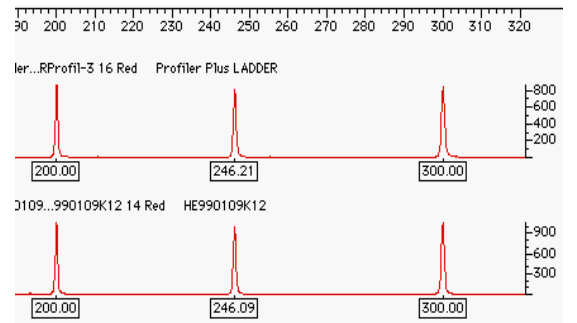
"OL alleles " - look at the 250 peak



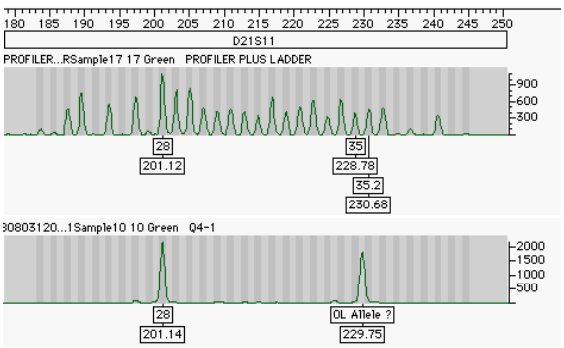
"OL allele re-injected"



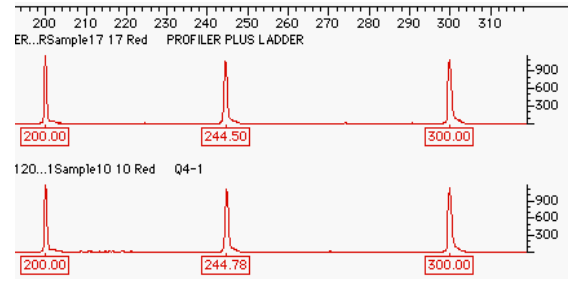
And the 250 peak...



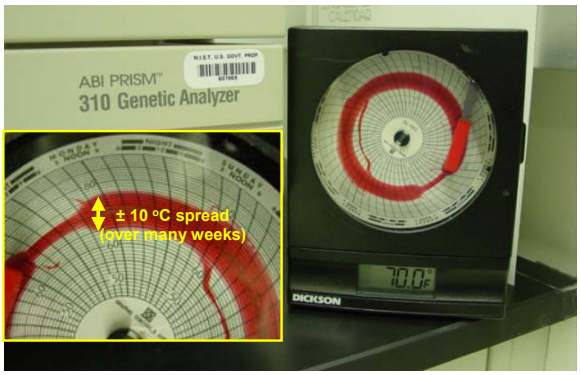
True off-ladder alleles



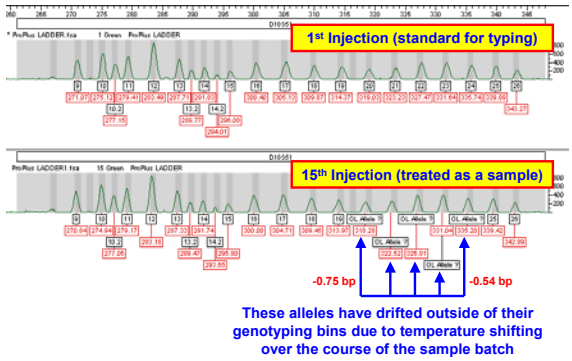
True off-ladder allele: the 250 peak



Monitoring Room Temperature Over Time



Use of Second Allelic Ladder to Monitor Potential Match Criteria Problems



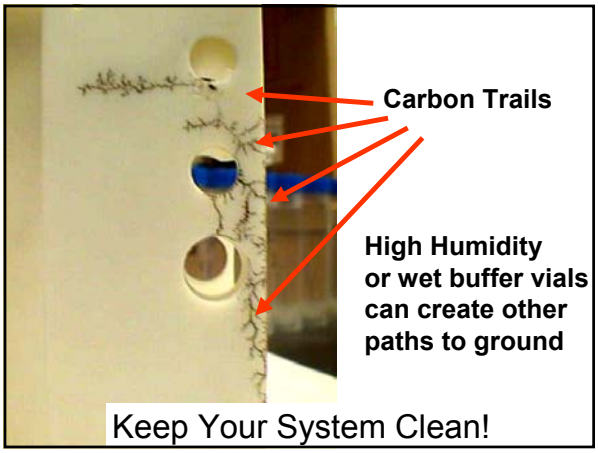
What to do if calibration is lost?

The 310 only calibrates to the first run ladder
this ladder sample may have been run at a different temperature!

- If protocol permits
  - Go to the next ladder
  - Rerun sample
  - Check current
  - Check allelic ladder
- Always check the ROX size standard
  - Look for extra bands
  - Check peak height
  - Check parameters and alignment

Cleanliness

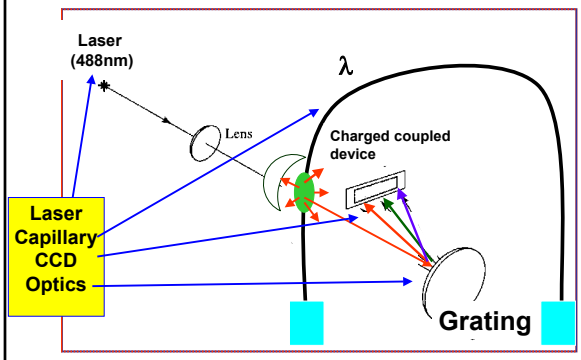
- Urea sublimates and breaks down to ionic components - these find a path to ground
- Similarly wet buffer under a vial creates paths to ground
- Capillary windows must be clear or matrix effects will occur
- Laser will often assist in this process
- Vial caps will transfer low levels of DNA to capillary



4. Instrumental Factors

- Optical System
  - Sensitivity changes with age, capillary diameter, capillary cleanliness, instrument calibration
- Fluidic System
  - Effects of bubbles, dust, urea crystals, leaks in syringe and capillary ferrule
- Matrix Calculations
  - Changes in buffer, optics, sample dye can alter the software calibrations
- Capillary Problems
  - Chemisorbed materials on capillary surface can produce osmotic flow, DNA bandbroadening and inconsistant resolution (meltdowns)

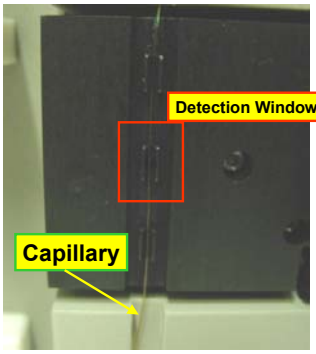
Consider the optical system



Issues with the Optical System

- **Pay attention to signal to noise, not absolute peak intensity**
- Argon Ion lasers outgas and eventually loose intensity; **take note of laser current and monitor it over time**
- Fluorescence expression:
  $I_f = I_0 k\varepsilon b C \varphi$ - changes in input intensity, I_0
  - changes in capillary diameter, b
  - cleanliness of capillary, k
  - All these things directly affect peak RFUs, however, baseline noise is more affected by detector.
- **Thus by monitoring signal to noise, you can get a better picture of your optical system.**

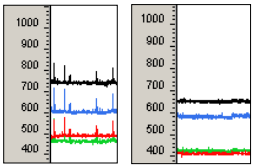
The Detection Window



Make sure that the capillary window is lined up (if it is not, then no peaks will be seen)

Window may need to be cleaned with ethanol or methanol

Review Start of Raw Data Collection



Little spikes indicate need to change buffer… check current

Fluidic Problems

- Syringe leaks
  - At the barrel
  - At the capillary nut
  - At the capillary window
- Viscosity changes
  - Water in the block
  - Bubbles
  - Temperature
- Capillary conditioning
  - Preelectrophoresis
  - clogging

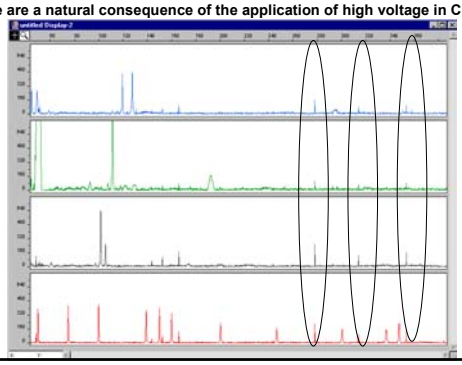
Buffer Issues

- The buffer and polymer affect the background fluorescence- affecting the matrix
- Urea crystals and dust may produce spikes
- High salt concentrations may produce reannealing of DNA
- High salt concentrations affect current
- Low polymer concentrations affect peak resolution

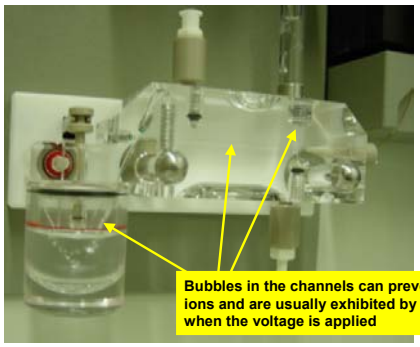
Current Spikes

Generally appear in all lanes and are sharper than regular peaks

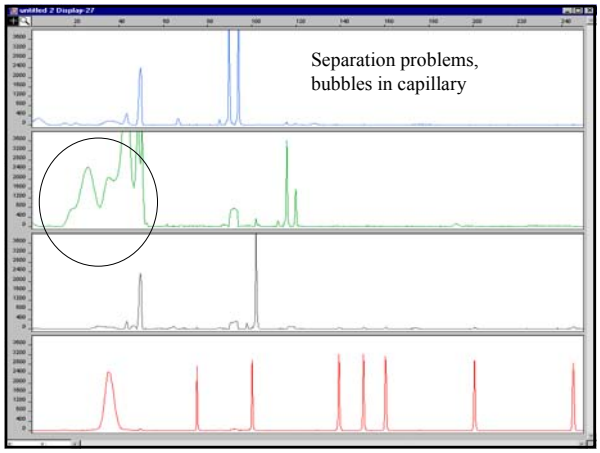
These are a natural consequence of the application of high voltage in CE

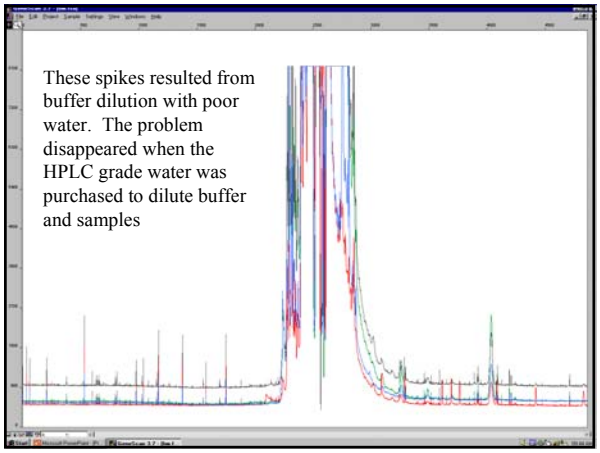


Remove all bubbles from the channels

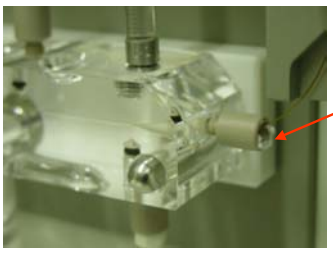


Bubbles in the channels can prevent flow of ions and are usually exhibited by zero current when the voltage is applied





Beware of Urea Crystals



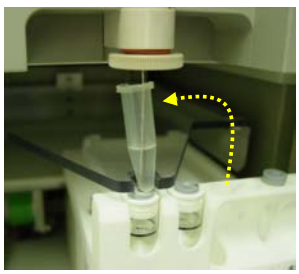
Urea crystals have formed due to a small leak where the capillary comes into the pump block

Urea sublimates and can evaporate to appear elsewhere

Use a small balloon to better grip the ferrule and keep it tight

Pump block should be well cleaned to avoid problems with urea crystal formation

Storage when ABI 310 is not in use

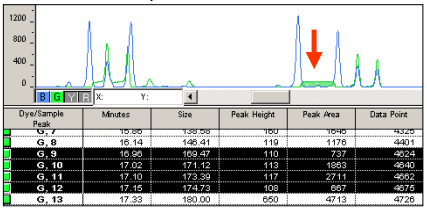


Remember that the water in the open tube will evaporate over time…

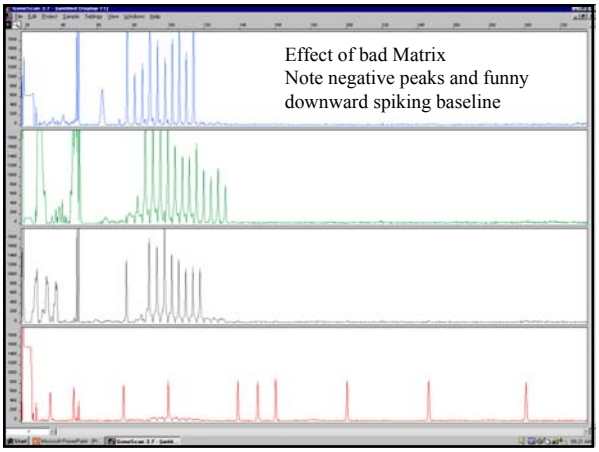
- Keep inlet of capillary in water…if it dries out then urea crystals from the polymer will clog the opening
- The waste vial (normally in position 3) can be moved into position
- A special device can be purchased from Suppelco to rinse the capillary off-line
- Store in distilled water
- Note that the laser is on when the instrument is on

Matrix Problems

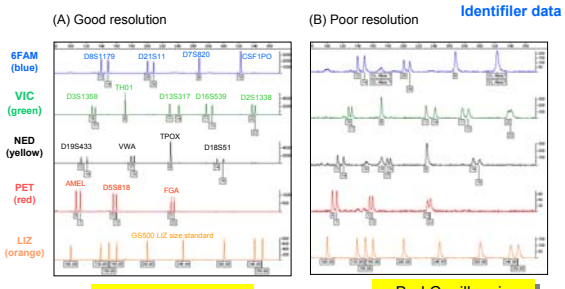
- A poor matrix can lead to raised baseline and therefore calling of too many peaks
- Larger sized alleles will not be identified as peaks because the GeneScan table for a particular dye color has filled up



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
G, 7	15.86	138.58	760	1646	4325
G, 8	16.14	146.41	119	1176	4401
G, 9	16.96	169.47	110	737	4624
G, 10	17.02	171.12	113	1863	4640
G, 11	17.10	173.39	117	2711	4662
G, 12	17.15	174.73	108	667	4675
G, 13	17.33	180.00	650	4713	4726



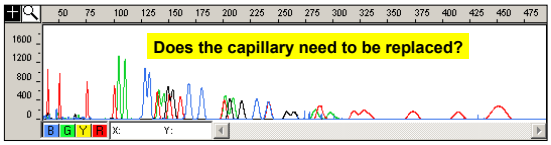
Capillary Meltdowns



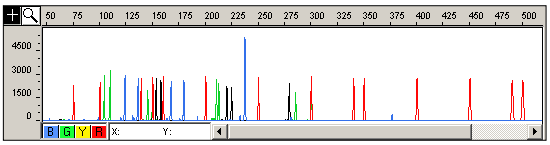
Butler, J.M., Buel, E., Crivellente, F., McCord, B.R. (2004) Forensic DNA typing by capillary electrophoresis: using the ABI Prism 310 and 3100 Genetic Analyzers for STR analysis. *Electrophoresis*, 25: 1397-1412.

Meltdowns can be permanent or transitory

as we have seen these may result from sample contamination effects



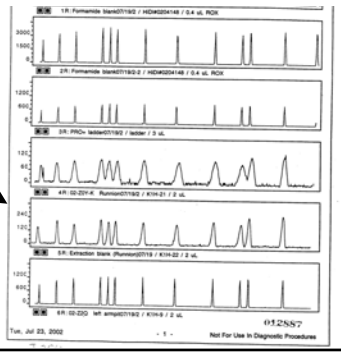
No! The next injection looks fine…



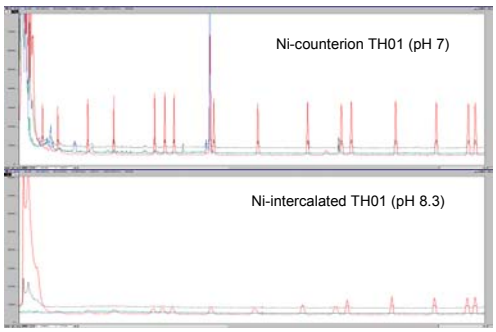
Effect of contaminant in reference sample

Contamination results in problems in subsequent analyses

Effect is transitory



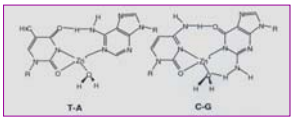
Effect of Ni Cations on a DNA Separation



1 µl TH01 added to 10 µl of 3.0 mM $NiCl_2$ in 10 mM Tris, pH 7 or pH 8.3. Sample allowed to interact for 1 hr and then 1 µl added to ROX/formamide.

Transition metal ions

Metal cations present in degraded samples represent a different type of contamination

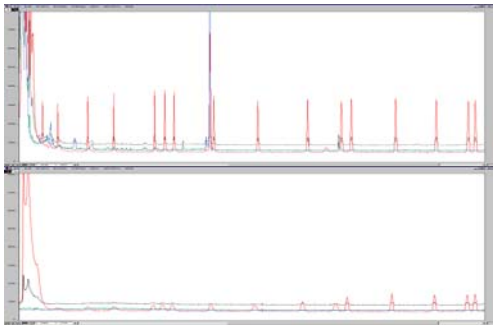


Zn^{2+}, Co^{2+}, and Ni^{2+} form DNA-metal ion complexes, termed M-DNA.at pH conditions above 8,

These cations produce severe effects in CE injection and analysis

Hartzell and McCord, *Electrophoresis*, in press

CE: Effect of pH 7 vs. 8.3



1 µl TH01 added to 10 µl of 3.0 mM $NiCl_2$ in 10 mM Tris, pH 7 or pH 8.3. Sample allowed to interact for 1 hr and then 1 µl added to ROX/formamide.

Meltdowns may be the result of

- Bad formamide
- Excess salt in sample/renaturation
- Water in the polymer buffer
- Syringe leak or bottom out
- Poisoned capillary
- Conductive polymer buffer due to urea degradation
- Crack/shift in capillary window
- Detergents and metal ions

A permanent loss of resolution may mean

- Adsorptive sites on a capillary
- Initiation of electroosmotic flow
- Conductivity changes in buffer
- Wrong molecular weight or concentration of sieving polymer (viscosity)

5. Troubleshooting benchmarks

- **Monitor run current**
- Observe syringe position and movement during a batch
- Examine ILS (ROX) peak height with no sample
- Observe "250 bp" peak in GS500 size standard
- Monitor resolution of TH01 9.3/10 in allelic ladder and size standard peak shapes
- **Keep an eye on the baseline signal/noise**
- Measure formamide conductivity
- Reagent blank – are any dye blobs present?
- See if positive control DNA is producing typical peak heights (along with the correct genotype)

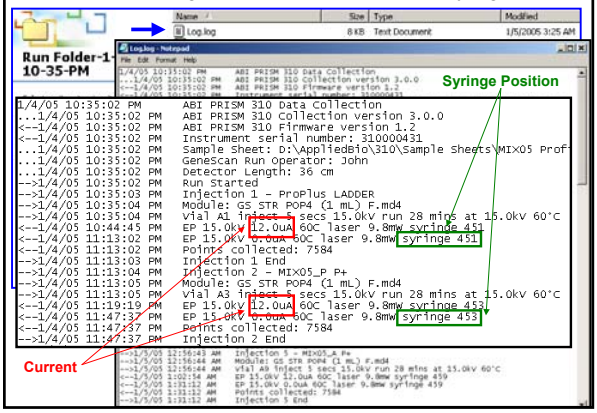
Measurement of Current

- V/I = R where R is a function of capillary diameter, [buffer], and buffer viscosity
- In a CE system the voltage is fixed, thus changes in resistance in the capillary will be reflected in the current observed
- Air bubbles, syringe leaks, alternate paths to ground, changes in temperature, changes in zeta potential, and contamination, will be reflected in the current
- A typical current for a CE system with POP4 buffer is **8-12 µA** (microamps)

Syringe Travel

- The ABI 310 instrument also keeps track of the position of the syringe (in the log file)
- Depending on the resistance to flow, the syringe will travel different lengths
- Syringe leaks may be reflected in a longer distance traveled prior to each injection
- These leaks occur around the barrel of the syringe and at the connection to the capillary block

Use of ABI 310 Log File to Monitor Current and Syringe Travel

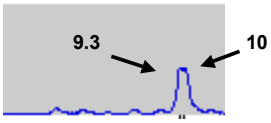


Measurement of Resolution for QC

% Valley (works best if peak heights are equal)

%valley = 100 x 1 – (Ave peak height/height to valley)

For the THO1 sample value = 0%
For the sample below, value = 20%



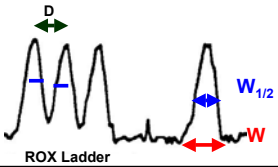
THO1 9.3/10

Chromatographic Resolution
The proper way to measure this
Expand the scale and focus on two peaks

R = 1.18 ($W_{1/2}$ + $W_{1/2}$)/D

Resolution in basepairs =
Distance between peaks/R

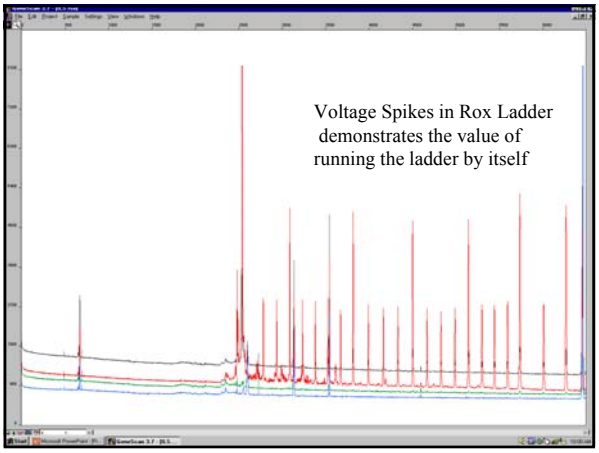
ie for R=2 and distance between peaks
Is 4 then resolution is 2 base pairs



ROX Ladder

ROX Ladder QC procedures

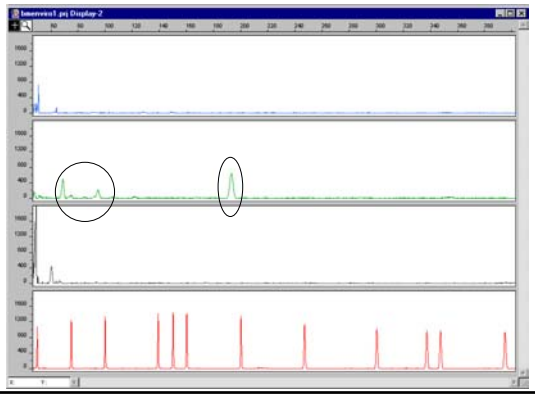
- A recommended sequence for initial operation of the 310
  - Rox ladder – initial injection - throwaway
  - Rox ladder- QC to test peak intensity and look for problems in blank
  - Allelic ladder- to determine resolution and to provide standard
  - 10-15 samples
  - Allelic ladder
  - 10-15 samples
  - Allelic ladder



Measurement of Signal and Noise Ratio

- You can also use the ROX size standard to keep track of sensitivity
  - For a given set of runs determine the average peak height of the Rox standard
  - Monitoring this signal level will help determine if any major loss of sensitivity has occurred
  - You can also measure the P-P noise level in the same way and compare the two values.

Dye Blobs in the Negative Control Sample



Question: What is a real blank?

- Because of the stacking effect, injections of pure water or formamide can produce extreme sensitivity
- This will allow you to detect small amounts of DNA clinging to the capillary, leading to a false impression that carry-over is a problem
- Instead, inject ROX plus formamide as your blank. In this case the added salt and fluorescent DNA drowns out these spurious peaks

Measuring Formamide Conductivity

(not this way)

The key is to measure the bottle when it comes in or buy the good stuff and immediately pipette it out into small tubes with or without ROX already added. Then freeze the tubes.

Do not ever open a cold bottle of formamide. Water will condense inside and aid in the formation of conductive formic acid.

Conclusion:

Troubleshooting is more than following the protocols

It means keeping watch on all aspects of the operation

1. Monitoring conductivity of sample and formamide
2. Keeping track of current and syringe position in log.
3. Watching the laser current
4. Watching and listening for voltage spikes
5. Monitoring room temperature and humidity

Just Remember