

# Troubleshooting: Strategies and Solutions

Bruce McCord  
Professor of Chemistry  
Florida International University  
Miami, FL 33199  
[mccordb@fiu.edu](mailto:mccordb@fiu.edu)



Hang in there.....

## TECH TIPS

# Troubleshooting Capillary Electrophoresis Systems

By Bruce McCord

Associate Professor of Forensic Chemistry, Ohio University, Athens, Ohio

*The key to producing good DNA separations is to understand the principles underlying the injection, separation and detection of each allele.*

### INTRODUCTION

The development of capillary electrophoresis (CE) has played a key role in bringing about the modern application of DNA typing. Forensic laboratories are the beneficiaries of this new technology, but many practitioners are not fully aware of the underlying principles of the CE system. This article attempts to address the important issues in CE separations to aid analysts in troubleshooting problematic separations. The key to producing good DNA separations is to understand the principles underlying the injection, separation and detection of each allele. These points are addressed below.

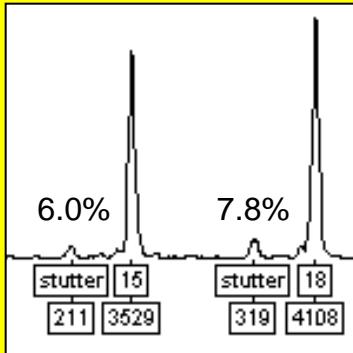
### SEPARATION

DNA analysis by CE is performed using entangled polymer buffers (Figure 1). These buffers can be easily pumped into a capillary prior to a separation and pumped out at its conclusion, providing a fresh separation matrix for each run. A typical buffer for forensic DNA separation contains 4% polydimethyl acrylamide (pDMA), buffered to pH 8

# 1. Deciphering Artifacts from the True Alleles

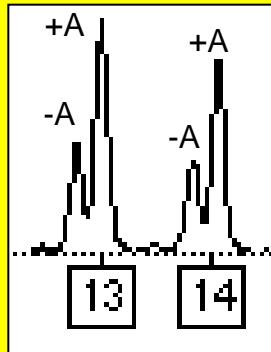
## Biological (PCR) artifacts

### Stutter products



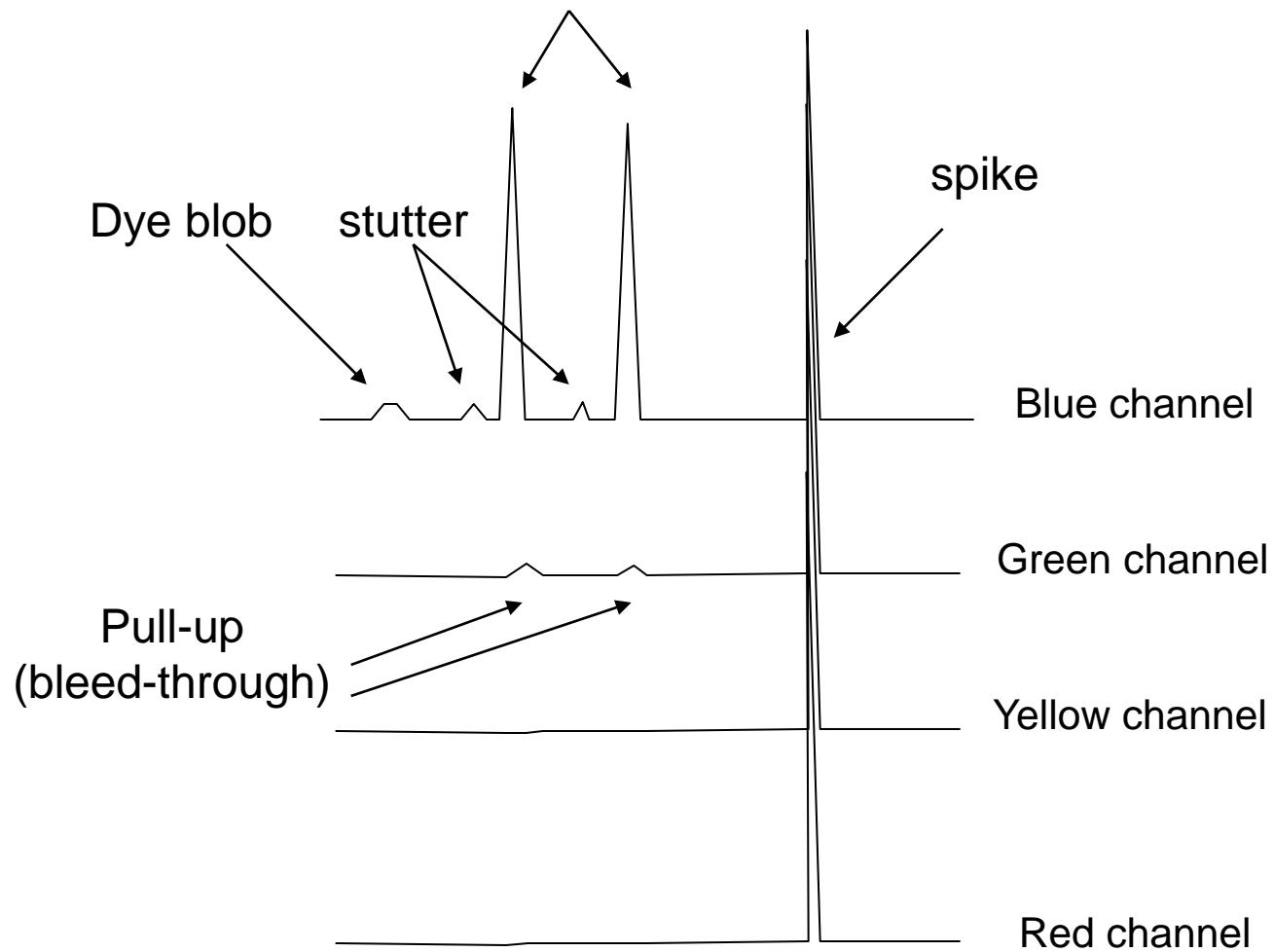
D3S1358

### Incomplete adenylation



D8S1179

## STR alleles



## Forensic News

October 2007

FAS Corner

[http://marketing.appliedbiosystems.com/images/forensic/volume11/docs/52808\\_FN\\_FAS\\_r3.pdf](http://marketing.appliedbiosystems.com/images/forensic/volume11/docs/52808_FN_FAS_r3.pdf)

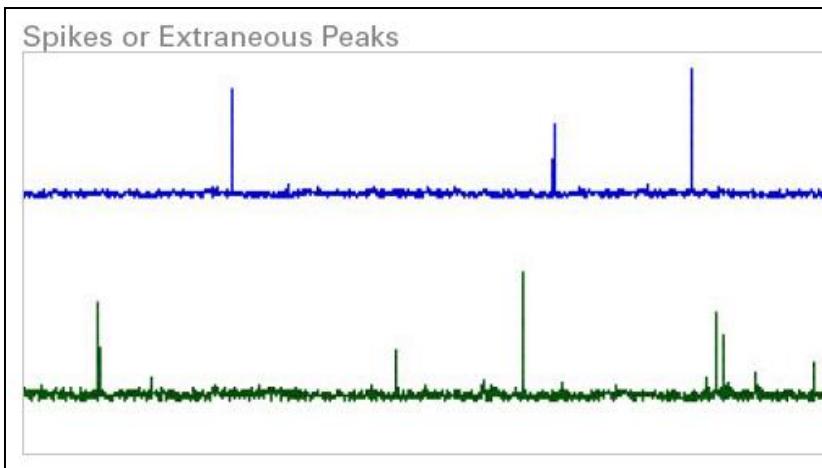
## Troubleshooting Amplification and Electrophoresis of the AmpFℓSTR® Kits

One of the key responsibilities of our Human Identification Field Application Specialists is to troubleshoot results obtained using any of the AmpFℓSTR® kits on any Applied Biosystems validated instrument platform.

## Troubleshooting Electrophoresis

Below are some common observations that may be seen during electrophoresis of AmpFℓSTR® kit PCR products:

- Spikes/Extraneous peaks
- No signal or low signal
- Loss of resolution
- Arcing
- Low reproducibility
- Contamination
- Baseline issues
- Poor peak morphology



# Off-ladder alleles

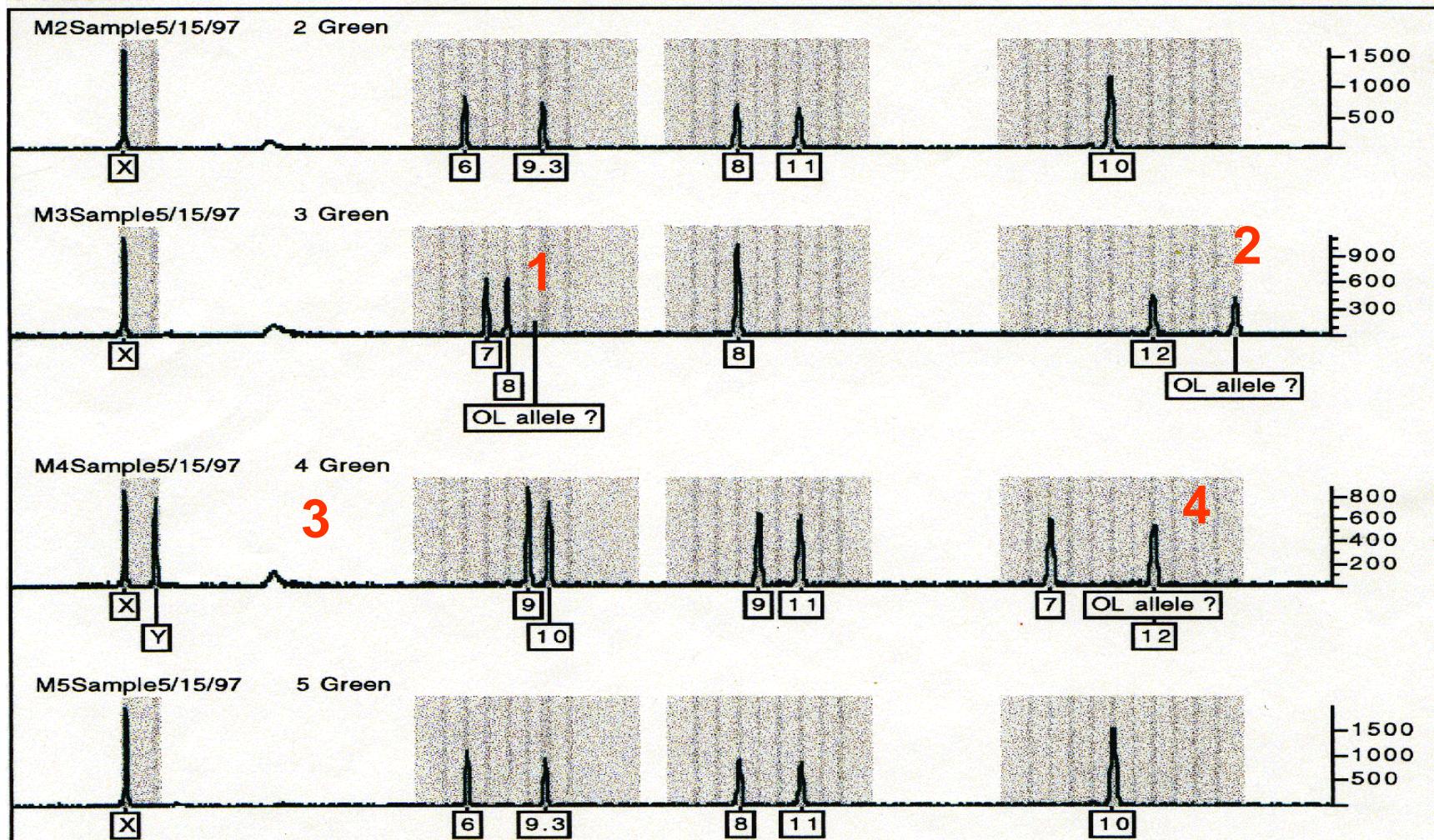
## Four types

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

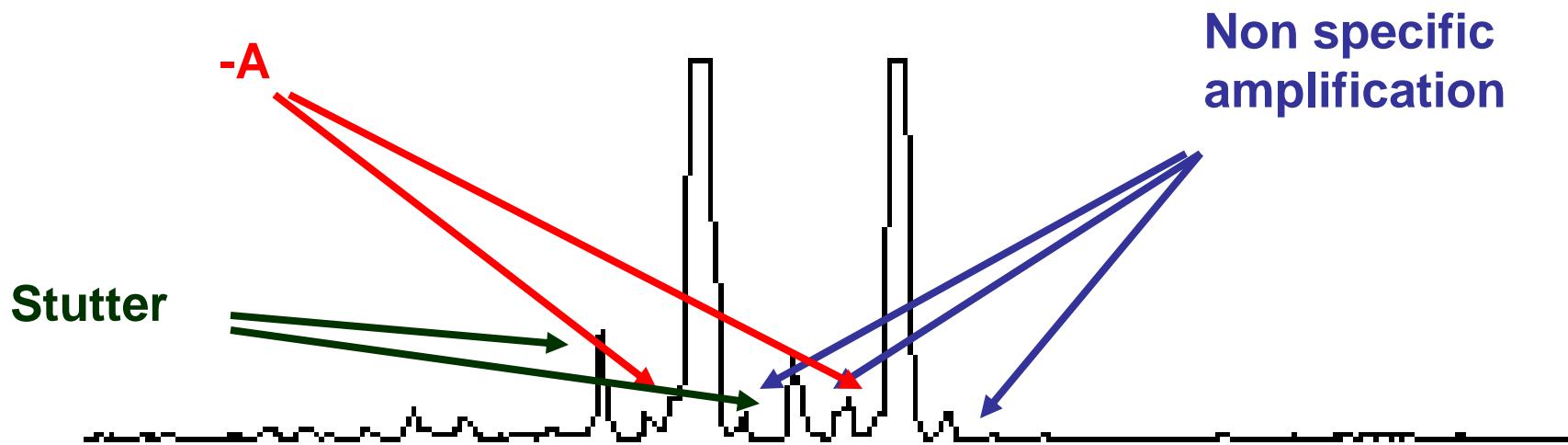


Plots - untitled 15  
Licensed to bruce McCord, fbi lab

5:00:49 PM Fri, May 16, 1997  
Genotyper® 2.0



Overloaded peaks will also show relatively high stutter and possibly artefacts



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

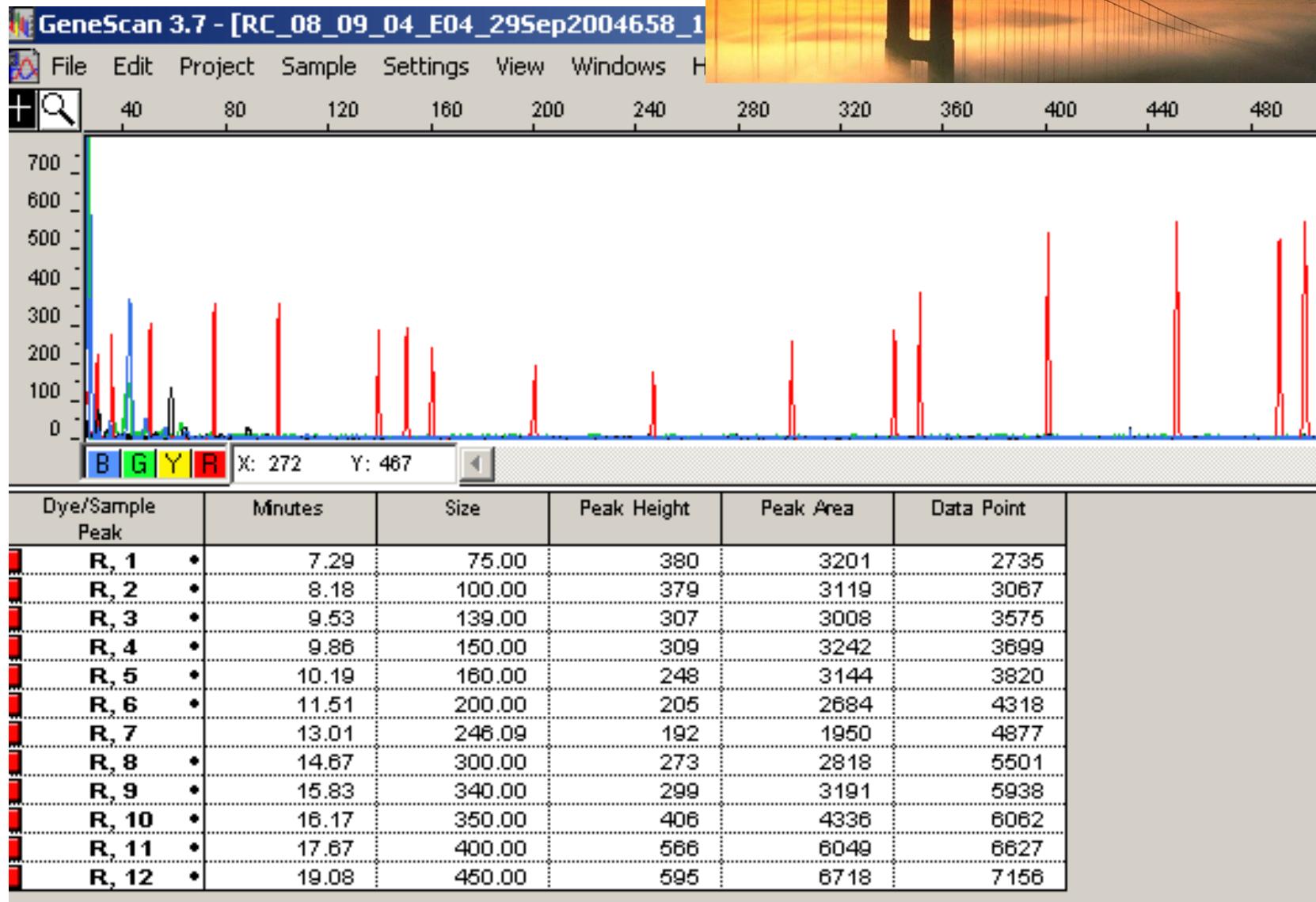
1. raised baseline
2. non specific amplification
3. peak height ratios
4. -A

## 2. Sample Issues

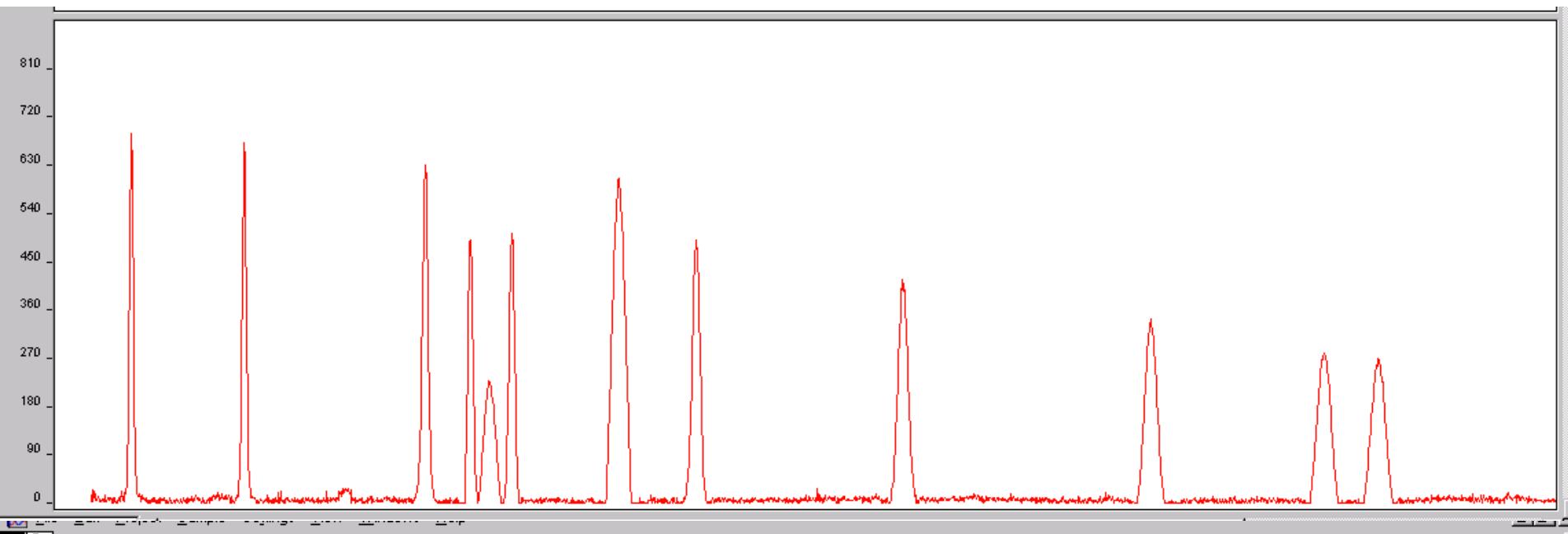
- Formamide Conductivity
- Excessive salt in sample due to evaporation
- Metal ion contamination
- Sensitivity issues with Microcon cleanup (salt removal)
- Dye “blobs” – artifacts from primer synthesis

# Golden Gate Effect

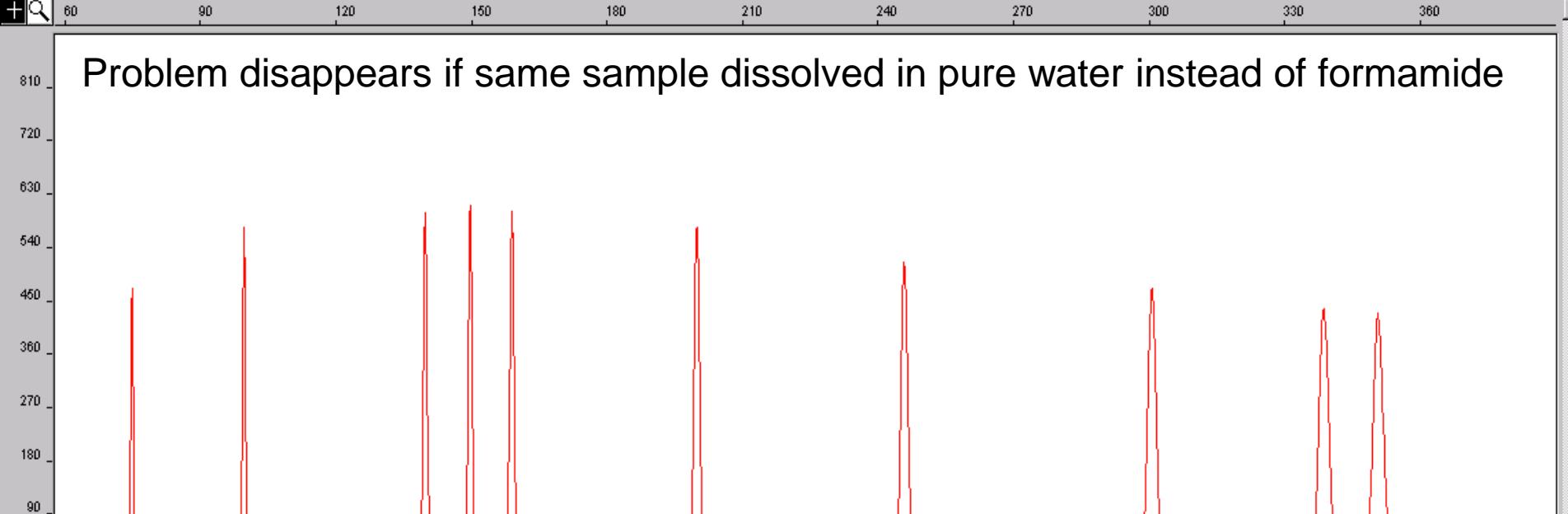
Attributed to poor formamide



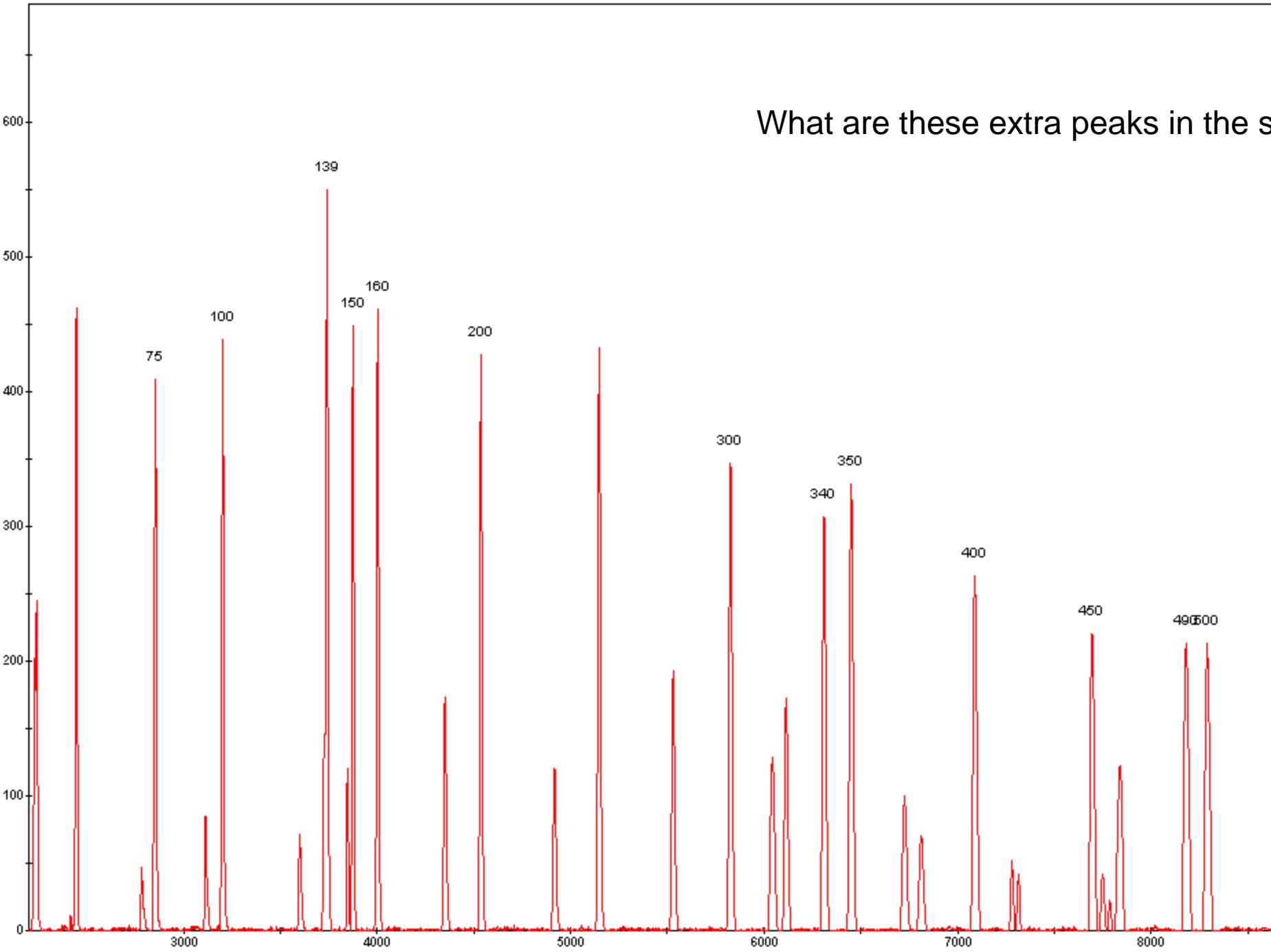
Effect of bad HI-Dye Formamide - Note broad peaks and extra bands



Problem disappears if same sample dissolved in pure water instead of formamide



What are these extra peaks in the spectrum?



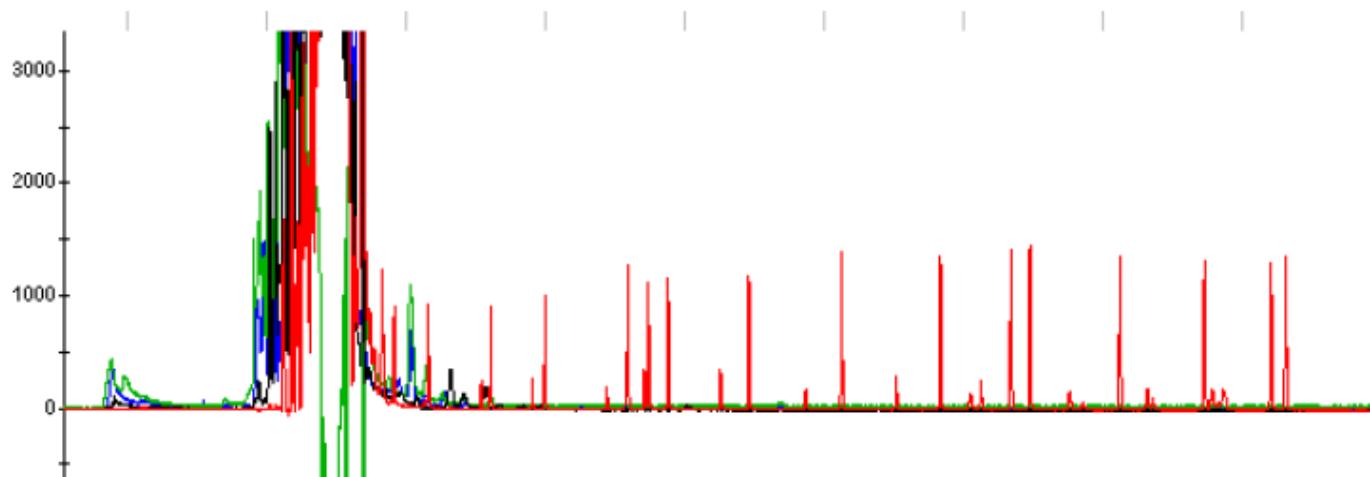
850 Lincoln Centre Driv  
Foster City, CA 94404 |  
T 650.570.6667 F 650.5  
[www.appliedbiosystems.com](http://www.appliedbiosystems.com)

## What does ABI Say?

Dear Valued Customer,

We are writing this letter in response to inquiries from customers regarding artifact peaks that appear as "shadow peaks" to true DNA peaks observed in the electropherogram. In most cases, these artifacts appear to be the most prevalent in the dye channel corresponding to the size standard and do not affect accurate sizing of the size standard peaks.

An example electropherogram is shown below:

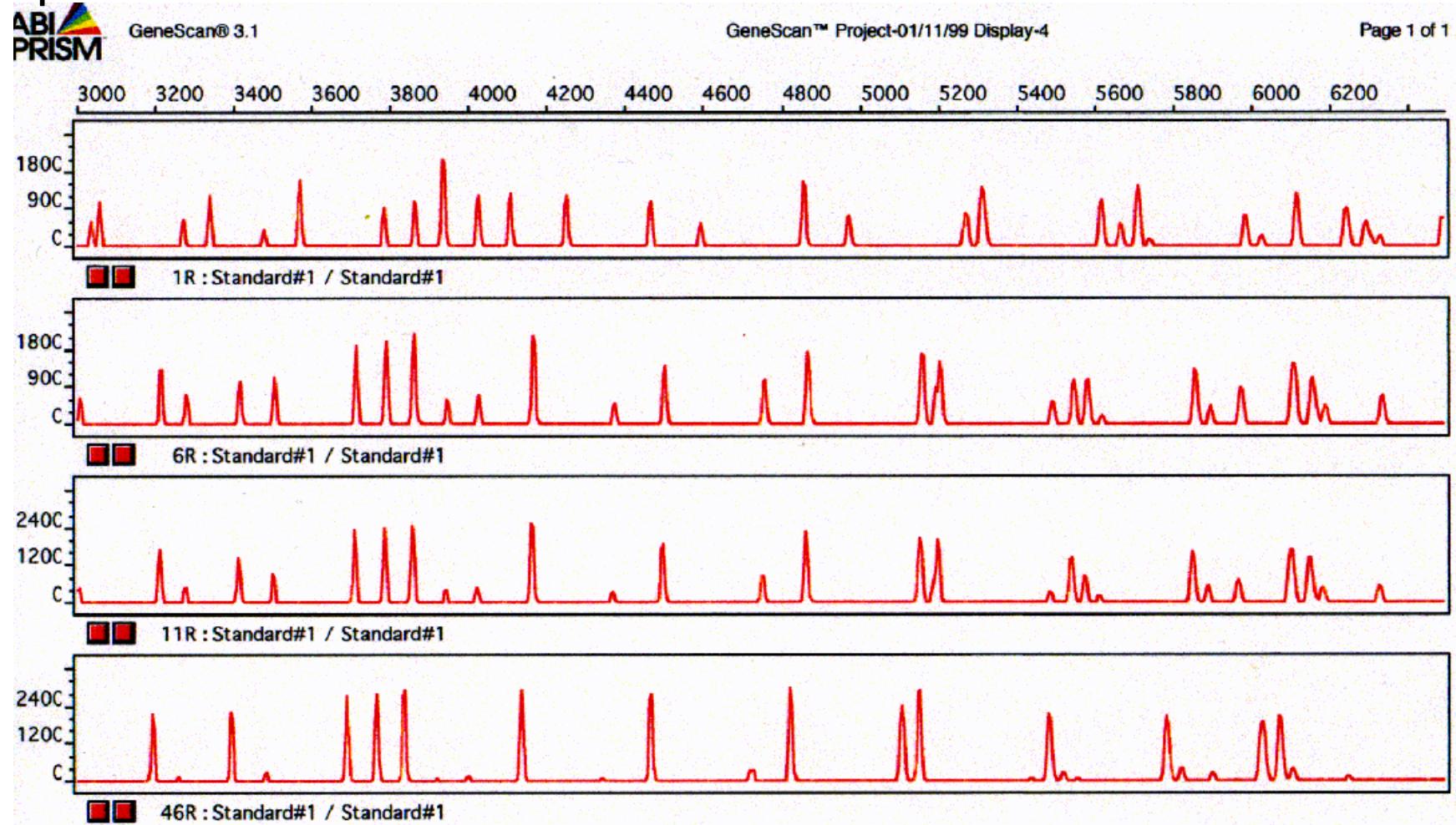


Electropherogram showing shadow peaks in GS500 ROX

The occurrence of these "shadow peaks" has been replicated at Applied Biosystems. We also observed during the testing process that higher shadow peak heights result from longer injection times. We are in the process of investigating the occurrence of these "shadow peaks" to determine the root cause and address the issue.

Applied Biosystems is committed to providing the highest quality products available for use in DNA typing. Thank you for your valued feedback. Your input is extremely valuable to us in our efforts to improve the quality of our products. Please feel free to contact HID Technical Support at 1.888.821.4HID (4443), #1 for further information.

What is it really?  
Incomplete denaturation of standard due to excessive salt in sample or in formamide



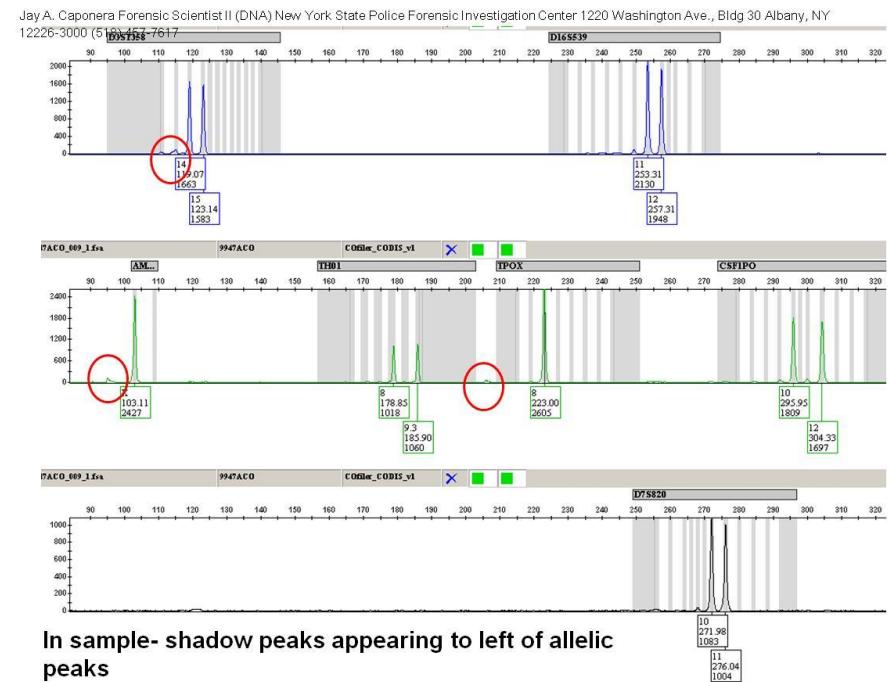
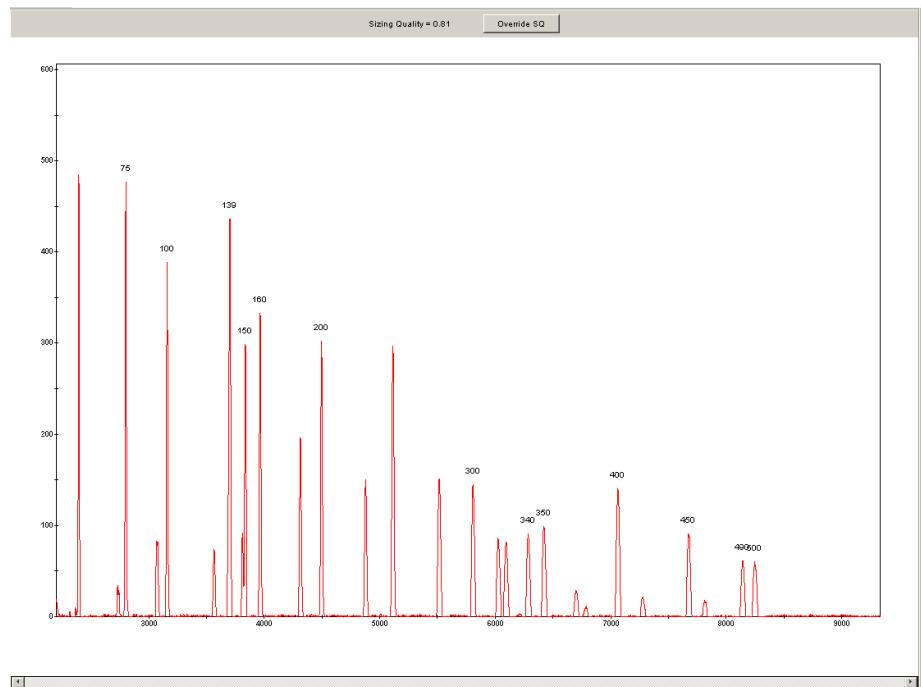
ds DNA migrates faster and over time with this set of runs ds DNA replaced the ssDNA

# Shadow peaks

Shadow peaks result from incomplete denaturation or from rehybridization.

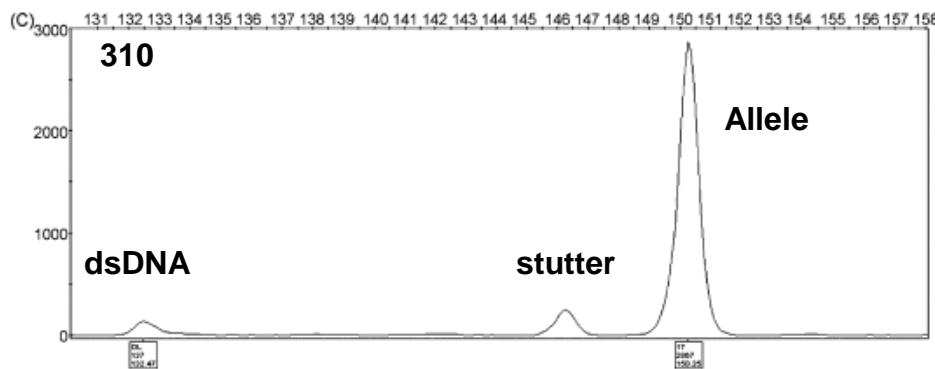
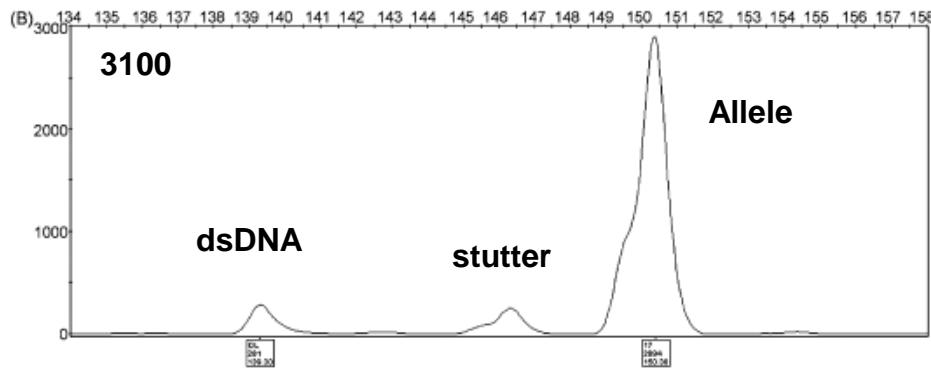
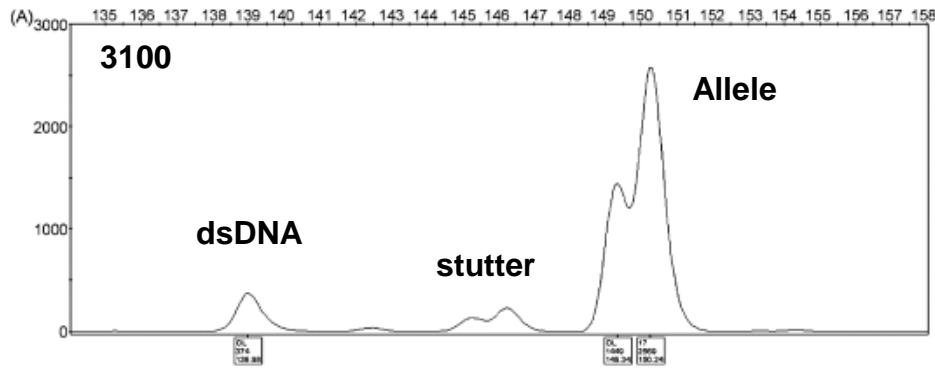
dsDNA migrates faster than ssDNA and the extra peaks appear ahead of the main peaks

They are most visible in the size standard but can appear in other dye lanes



# Hybridization due to leftover primers

R.S. McLaren et al. / Forensic Science International: Genetics 2 (2008) 257–273



Artifacts in the Powerplex 16 amplification of the vWA locus. Two artifacts occur.

1. The doubled peaks and shoulders are the result of primer hybridization to PCR amplicons not adenylation.
2. The additional peak eluting earlier is the result of renaturation of the ssDNA amplicon.

The first two slides are performed on a 3100 system. The second is on a 310. The 310 denatures the samples better due to its heat plate and eliminates the splitting, however, the dsDNA product is still present.

# Recent Promega Solution to Eliminating vWA Artifacts in PowerPlex 16 Results



Available online at [www.sciencedirect.com](http://www.sciencedirect.com)



Forensic Science International: Genetics 2 (2008) 257–273



[www.elsevier.com/locate/fsig](http://www.elsevier.com/locate/fsig)

## Post-injection hybridization of complementary DNA strands on capillary electrophoresis platforms: A novel solution for dsDNA artifacts

Robert S. McLaren <sup>a,\*</sup>, Martin G. Ensenberger <sup>a</sup>, Bruce Budowle <sup>b</sup>, Dawn Rabbach <sup>a</sup>, Patricia M. Fulmer <sup>a</sup>, Cindy J. Sprecher <sup>a</sup>, Joseph Bessetti <sup>a</sup>, Terri M. Sundquist <sup>a</sup>, Douglas R. Storts <sup>a</sup>

<sup>a</sup> Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711, United States

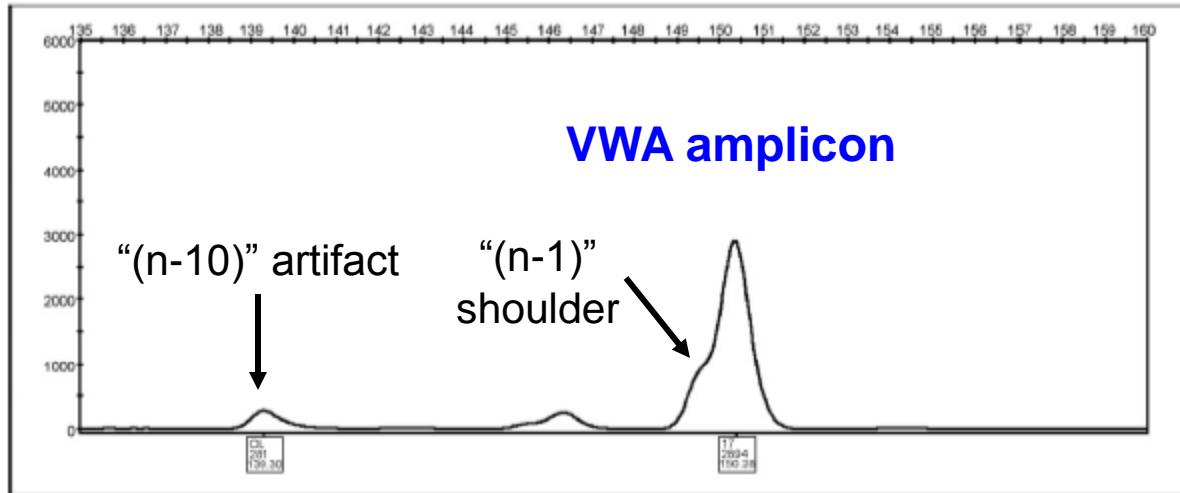
<sup>b</sup> Federal Bureau of Investigation, 2501 Investigation Parkway, Quantico, VA 22135, United States

Received 5 February 2008; received in revised form 11 March 2008; accepted 13 March 2008

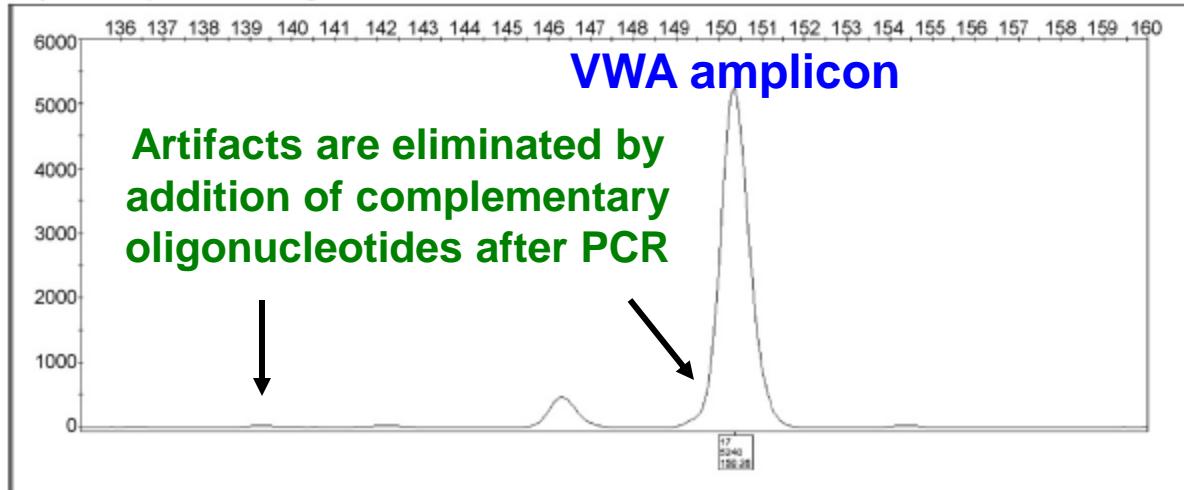
Several laboratories have reported the occurrence of a split or n -1 peak at the vWA locus in PowerPlex 16... The root cause of this artifact is post-PCR reannealing of the unlabeled, unincorporated vWA primer to the 30-end of the tetramethylrhodamine (TMR)-labeled strand of the vWA amplicon. **This reannealing occurs in the capillary post-electrokinetic injection.** The split peak is eliminated by incorporation into the loading cocktail of a sacrificial hybridization sequence (SHS) oligonucleotide that is complementary to the vWA primer. The SHS preferentially anneals to the primer instead of the TMR-labeled strand of the vWA amplicon...

# Impact of Added Oligos to vWA Amplicon Peaks

No COTplusSHS Oligonucleotide



20 $\mu$ M COTplusSHS Oligonucleotide

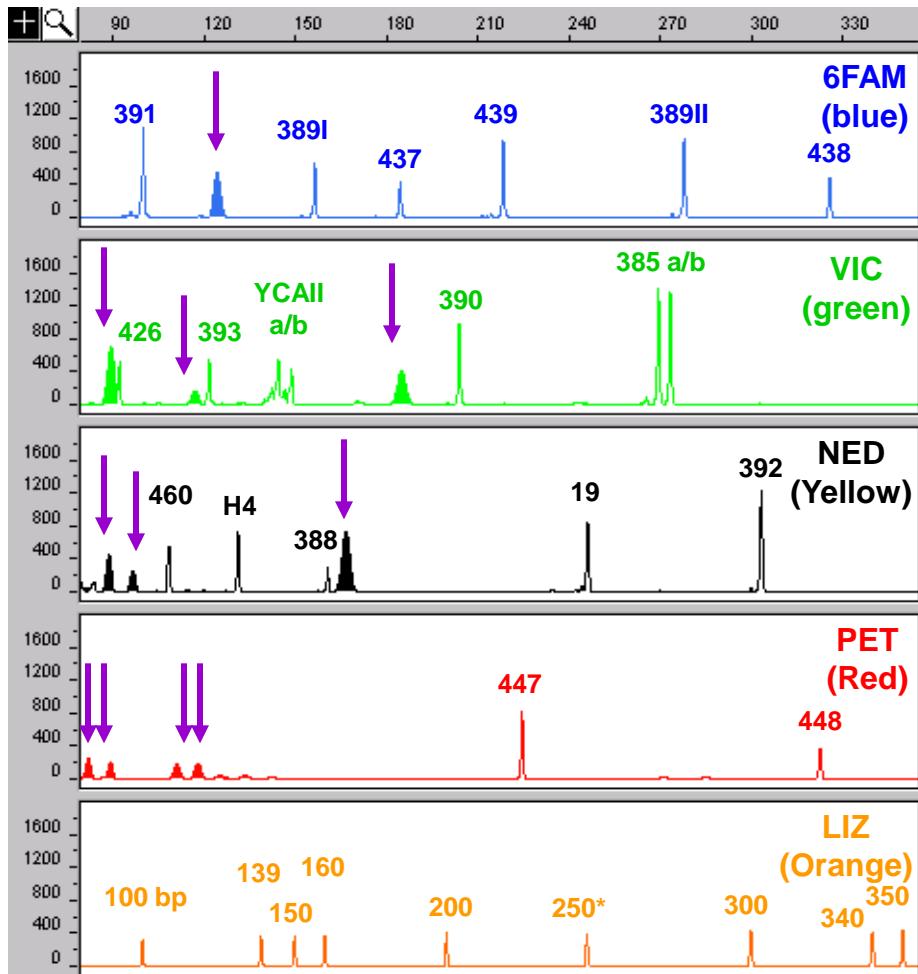


From Figure 5

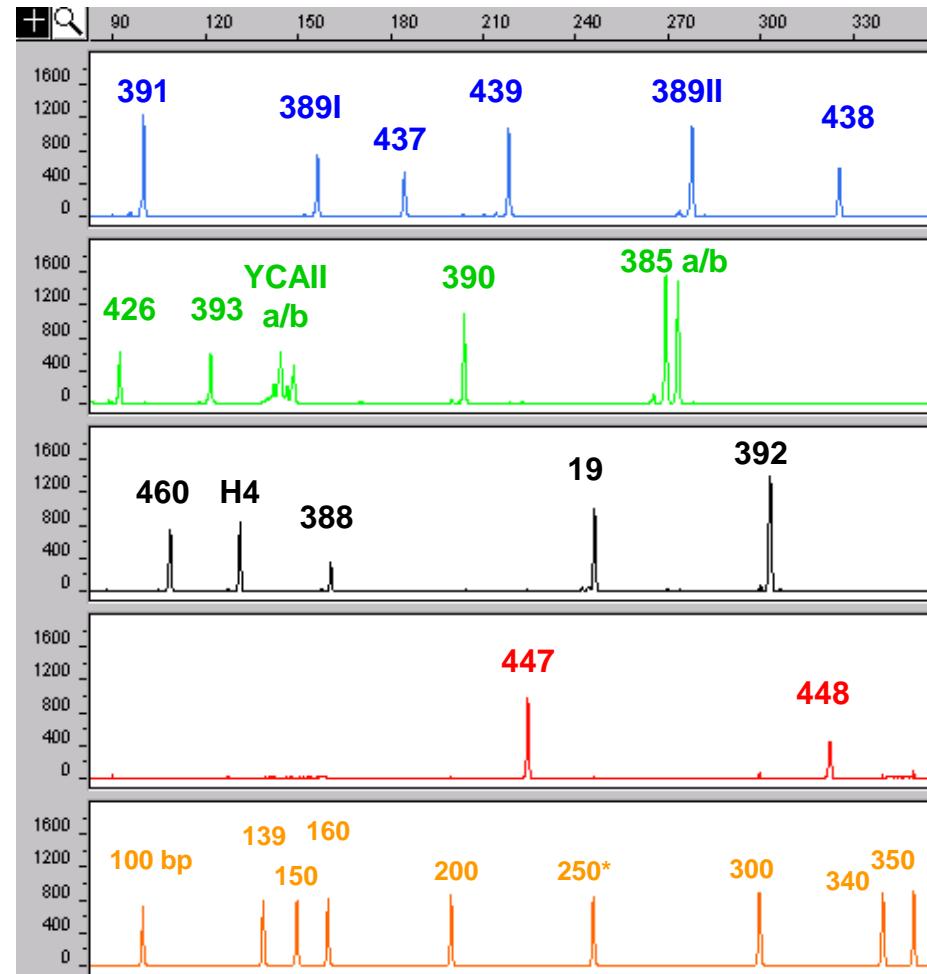
McLaren et al. (2008) *Forensic Science International: Genetics* 2: 257–273

# Dye Blobs and their Removal

Residual dye artifacts



Dye blob removal with Edge columns



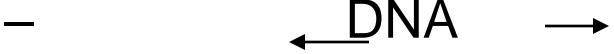
NIST Y-STR 20plex assay

Butler, J.M. (2005) Constructing STR multiplex assays. *Methods in Molecular Biology: Forensic DNA Typing Protocols* (Carracedo, A., ed.), Humana Press: Totowa, New Jersey, 297: 53-66.

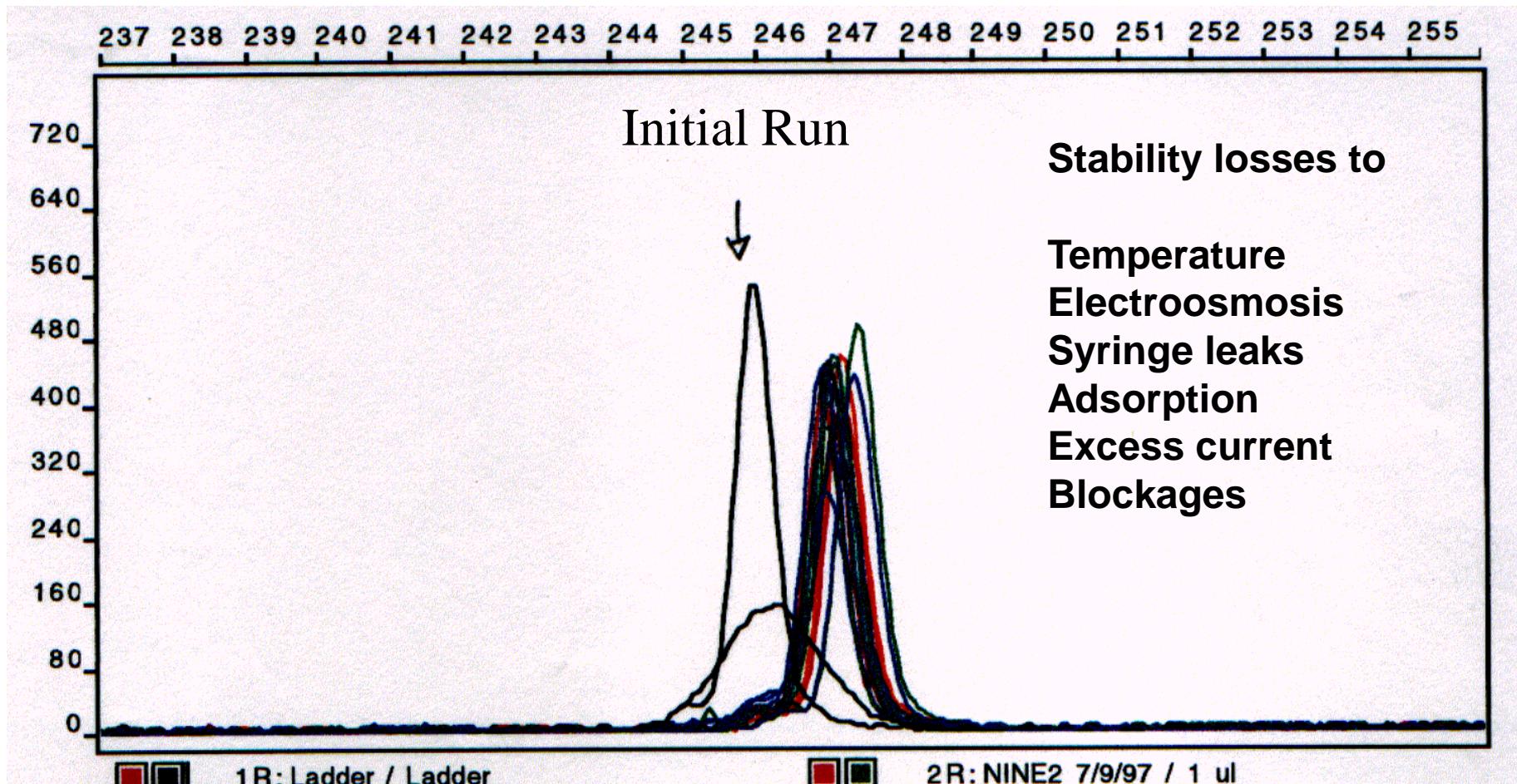
### 3. External Factors

- 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.

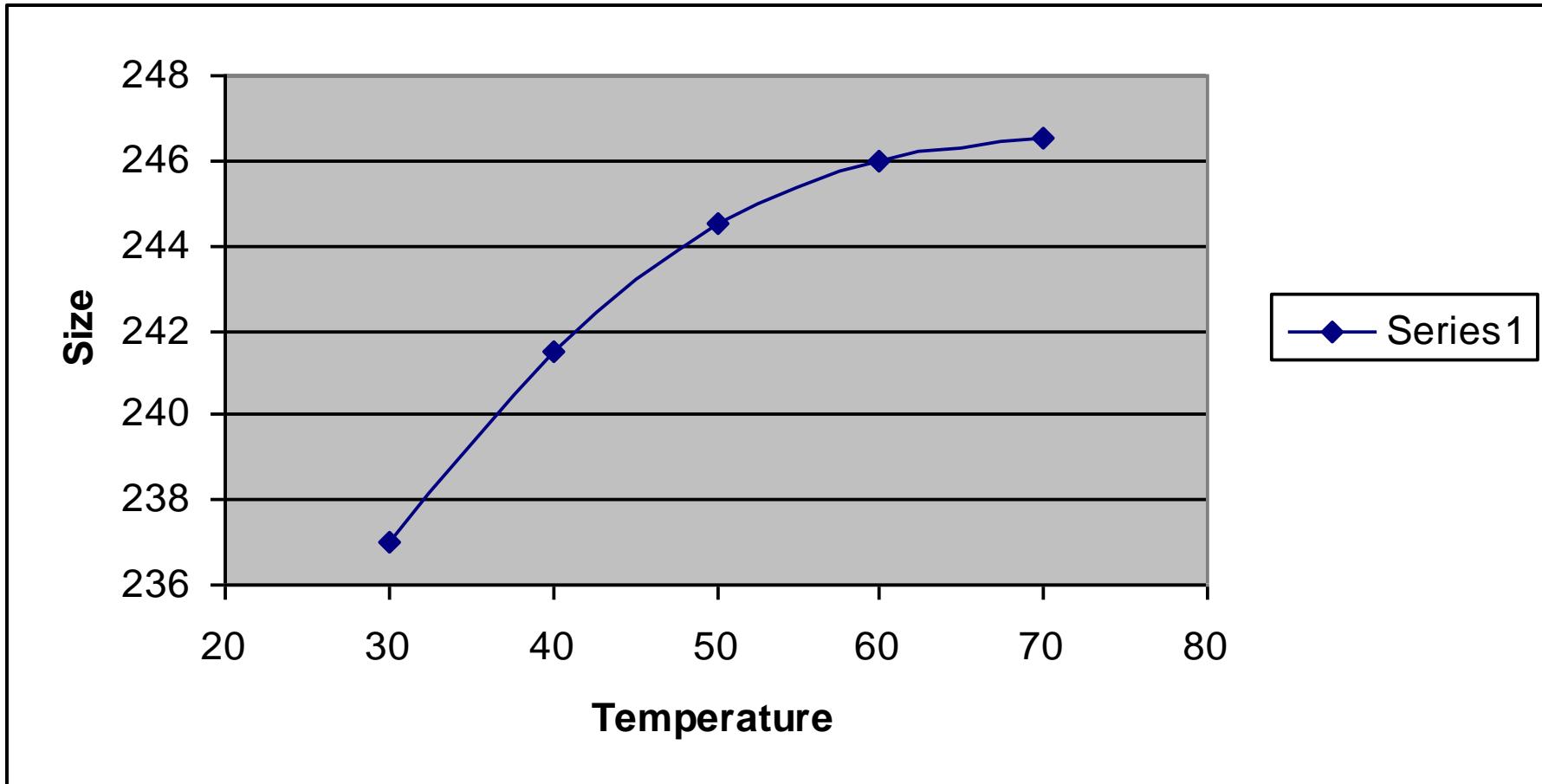
# Temperature effects

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

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

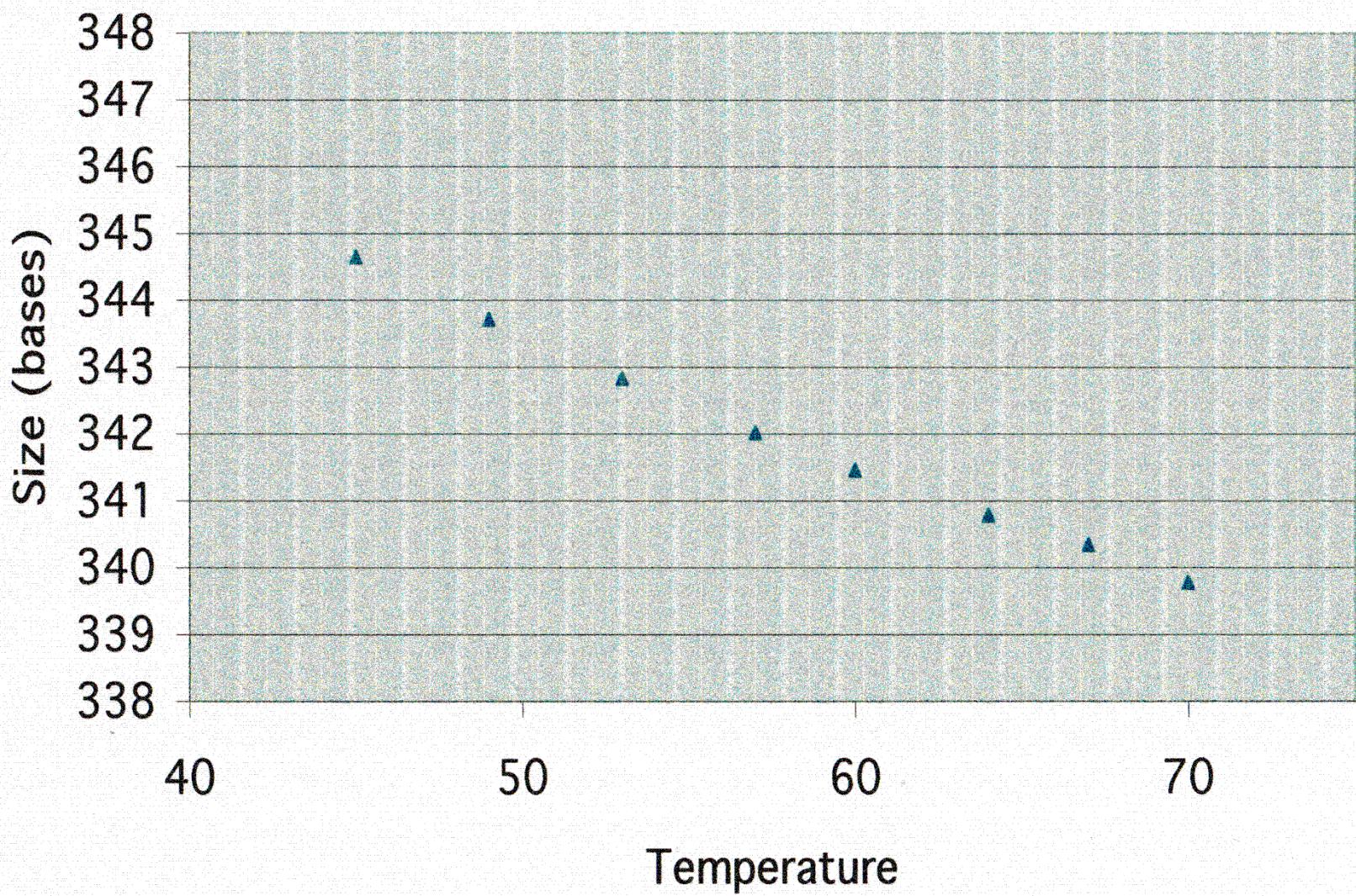


# Change in size of GS 250 peak with Temperature (Tamra Std)



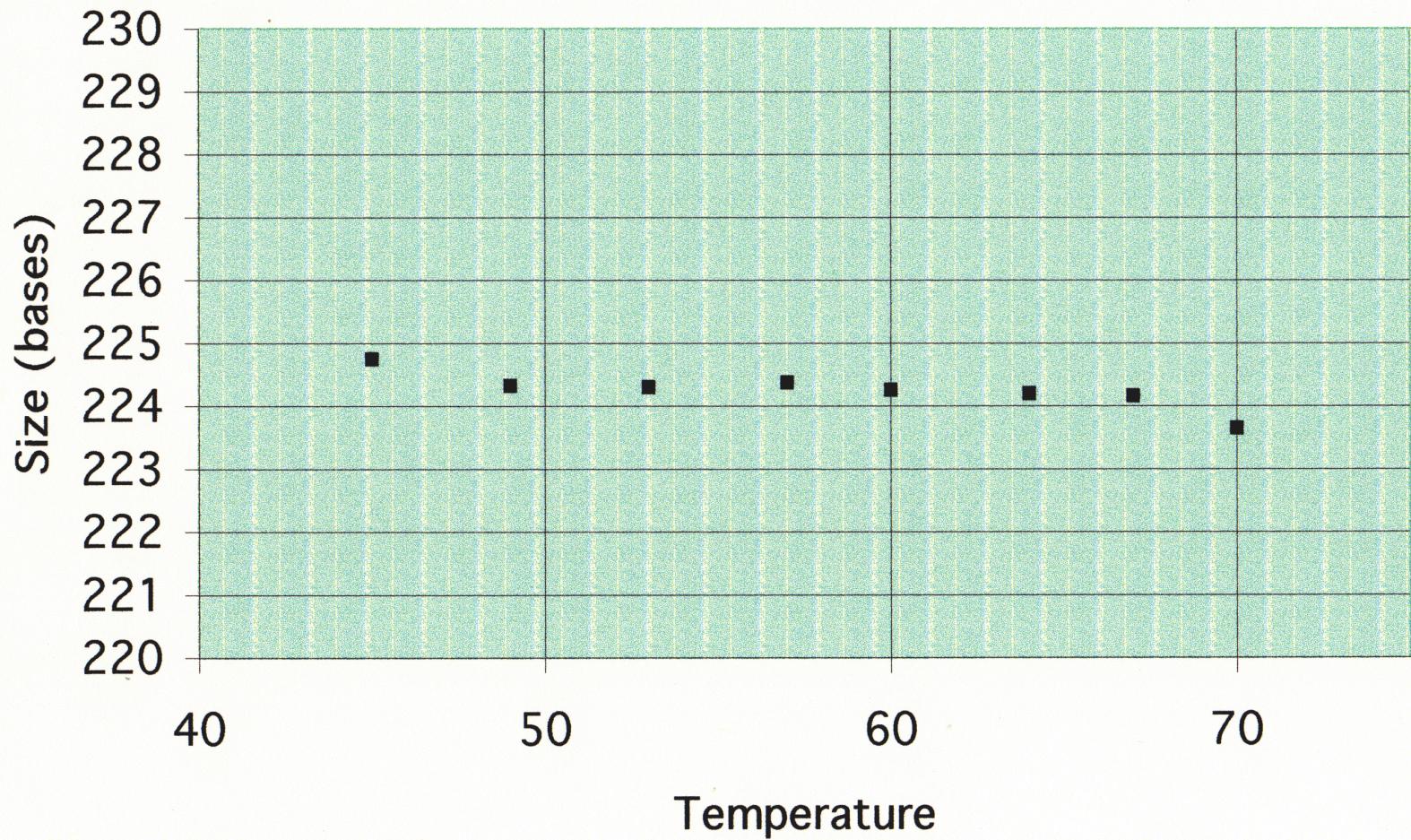
**4% pDMA with 8M urea and 5% 2-pyrrolidinone**  
**\*Rosenblum et al., Nucleic Acids Res.(1997) 25,19, 2925**

# D18S51

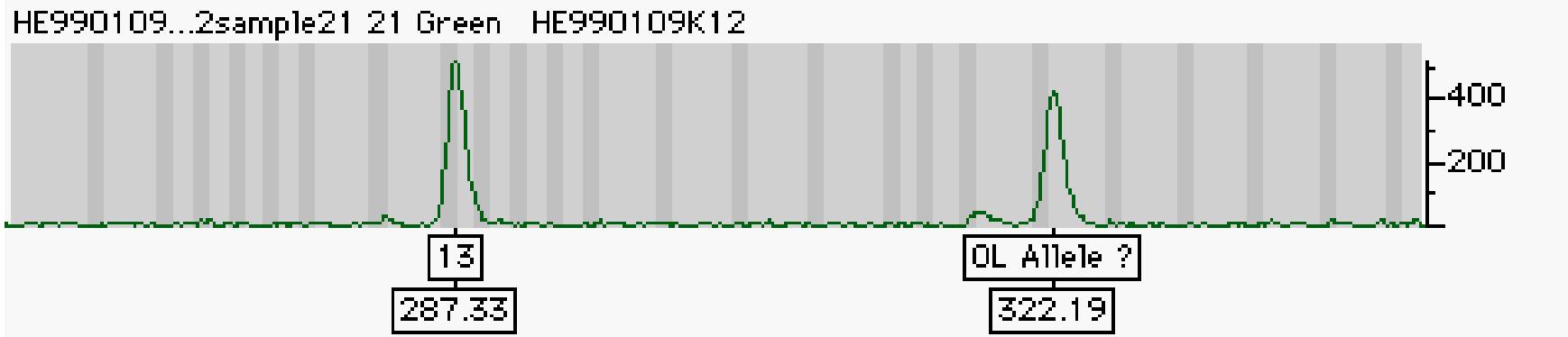
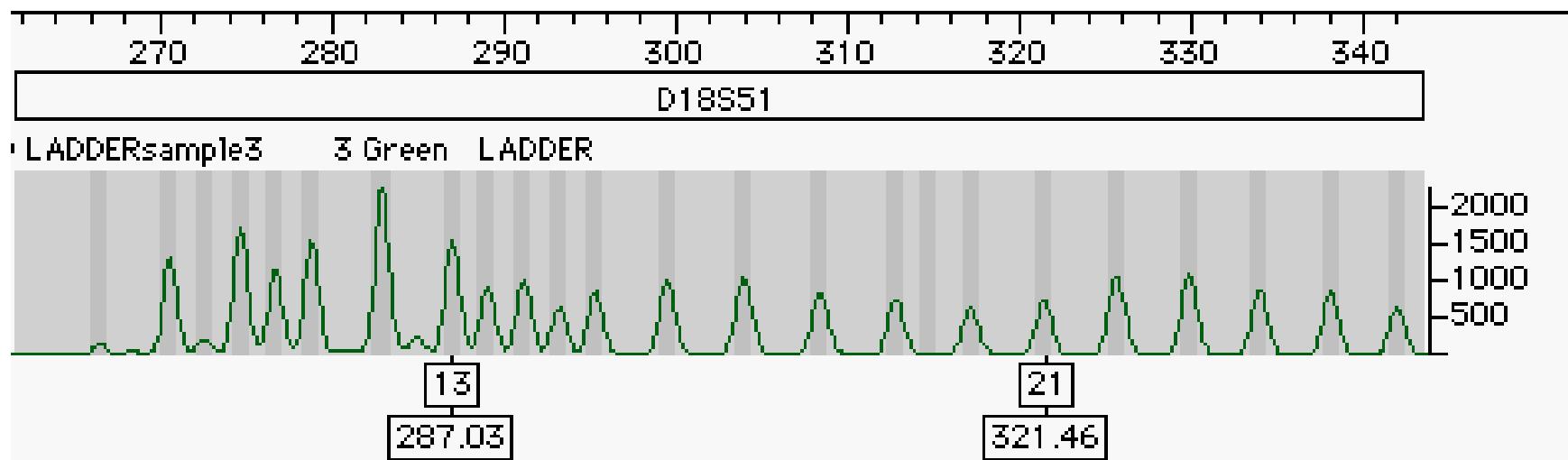


allele 34

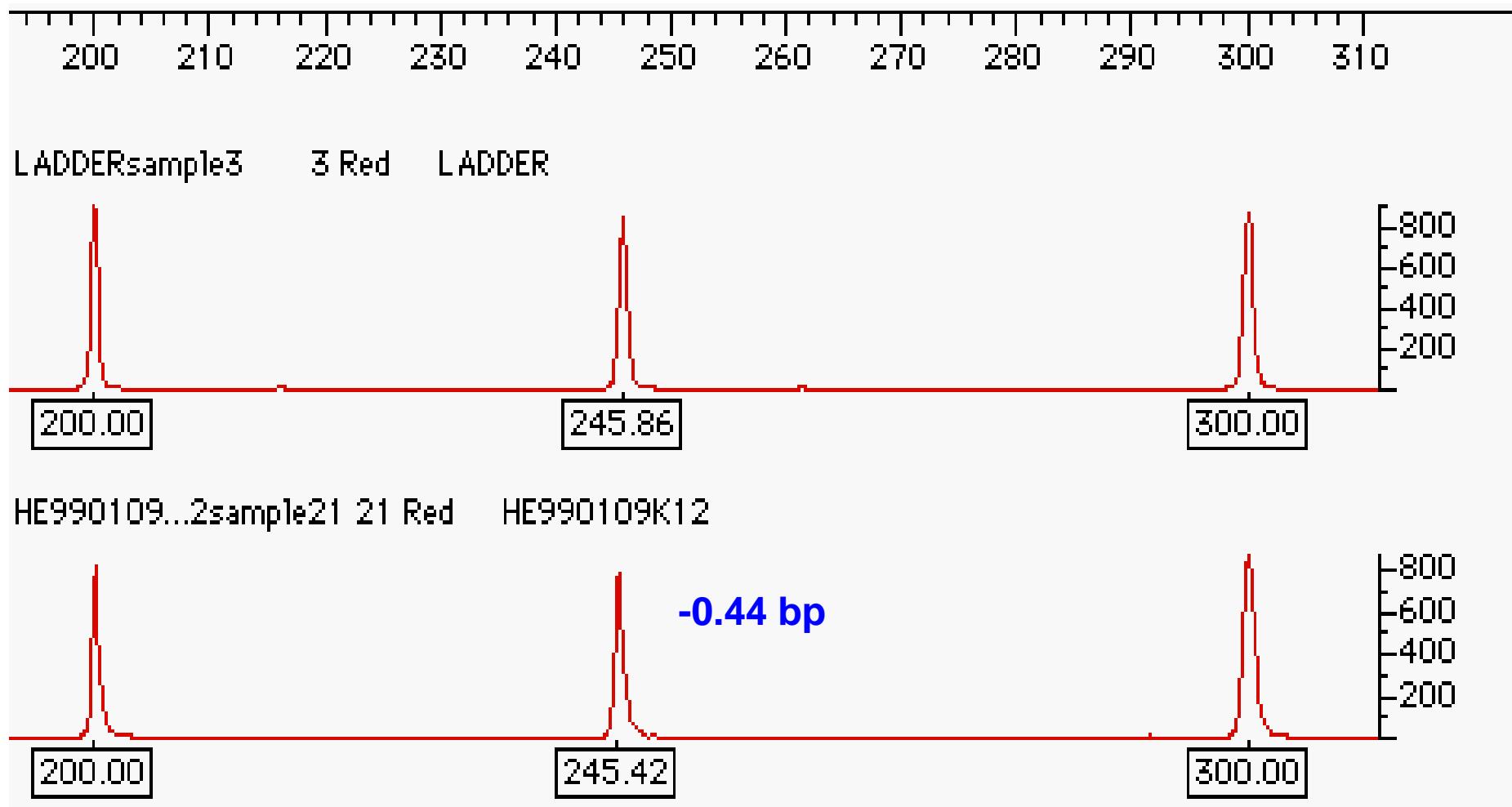
# D21S11



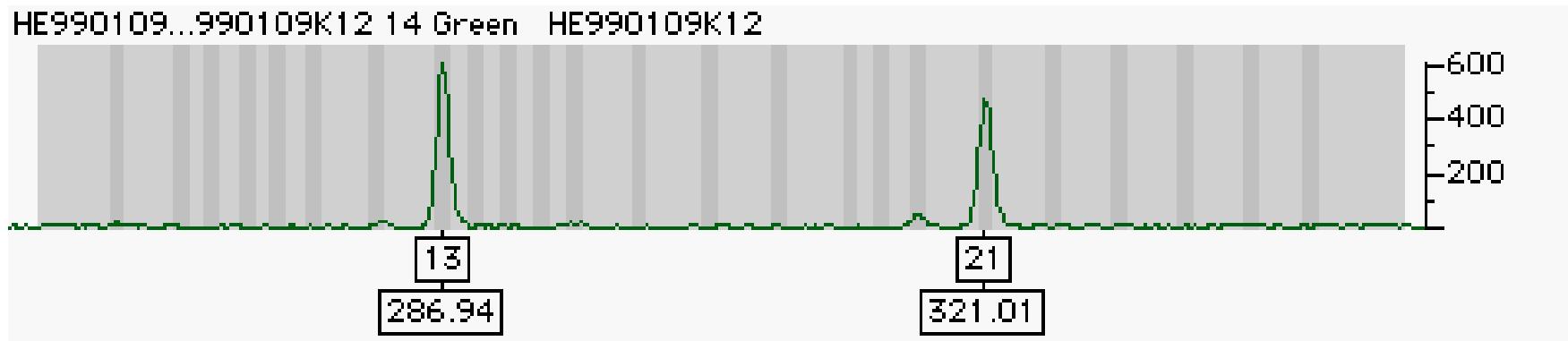
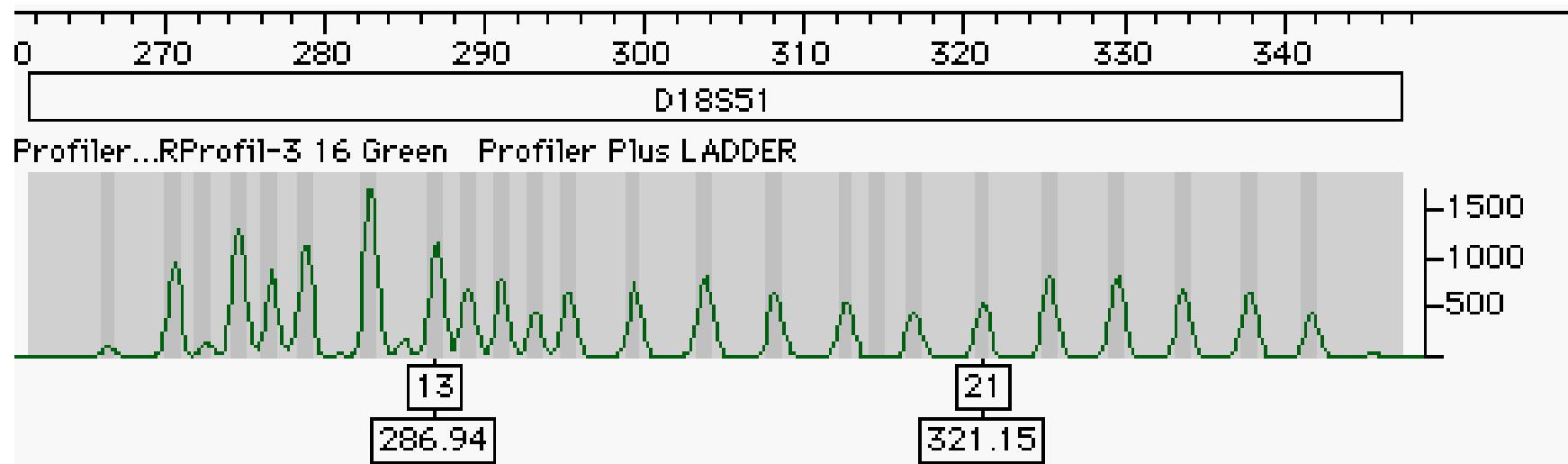
# “OL Alleles”



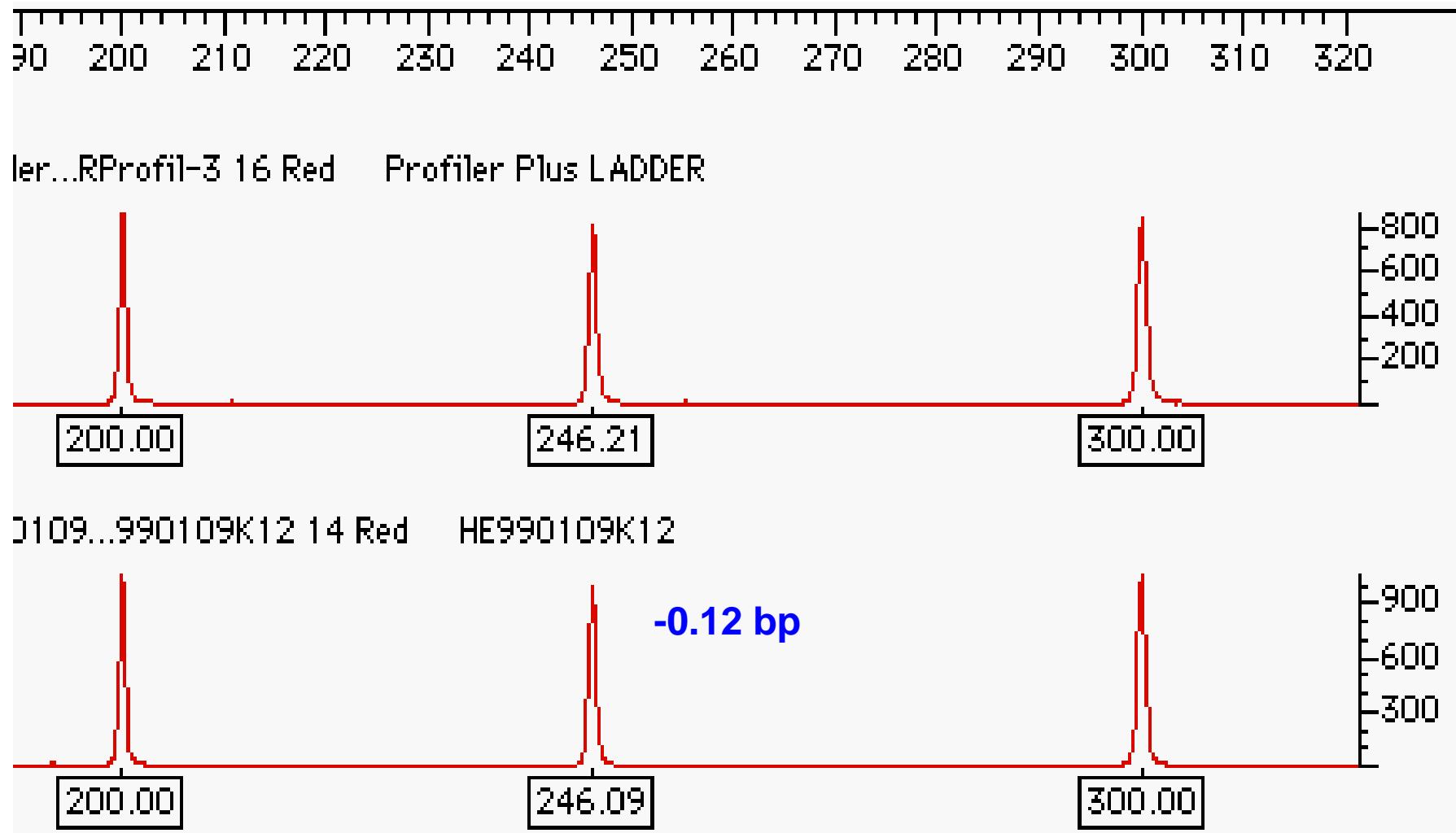
# “OL alleles” - look at the 250 peak



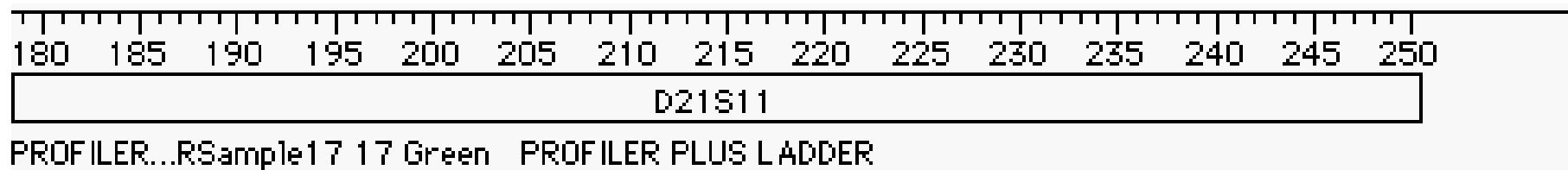
# “OL allele re-injected”



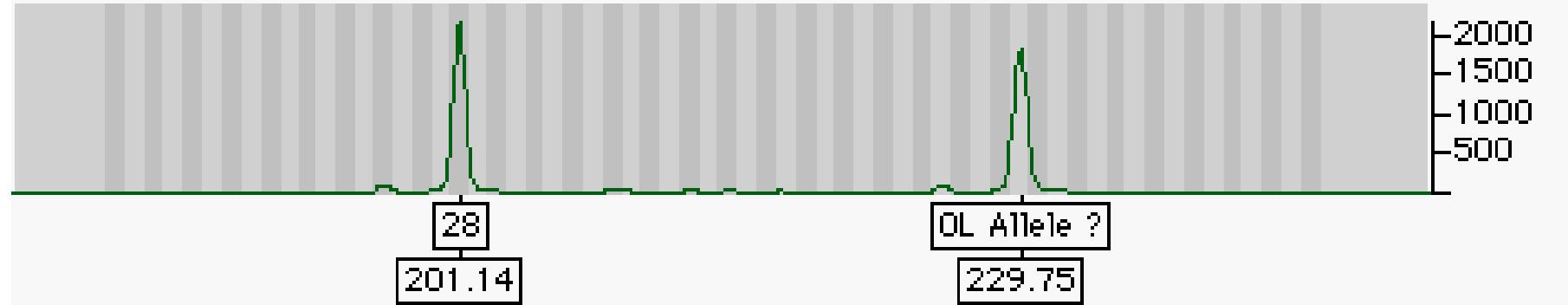
# And the 250 peak...



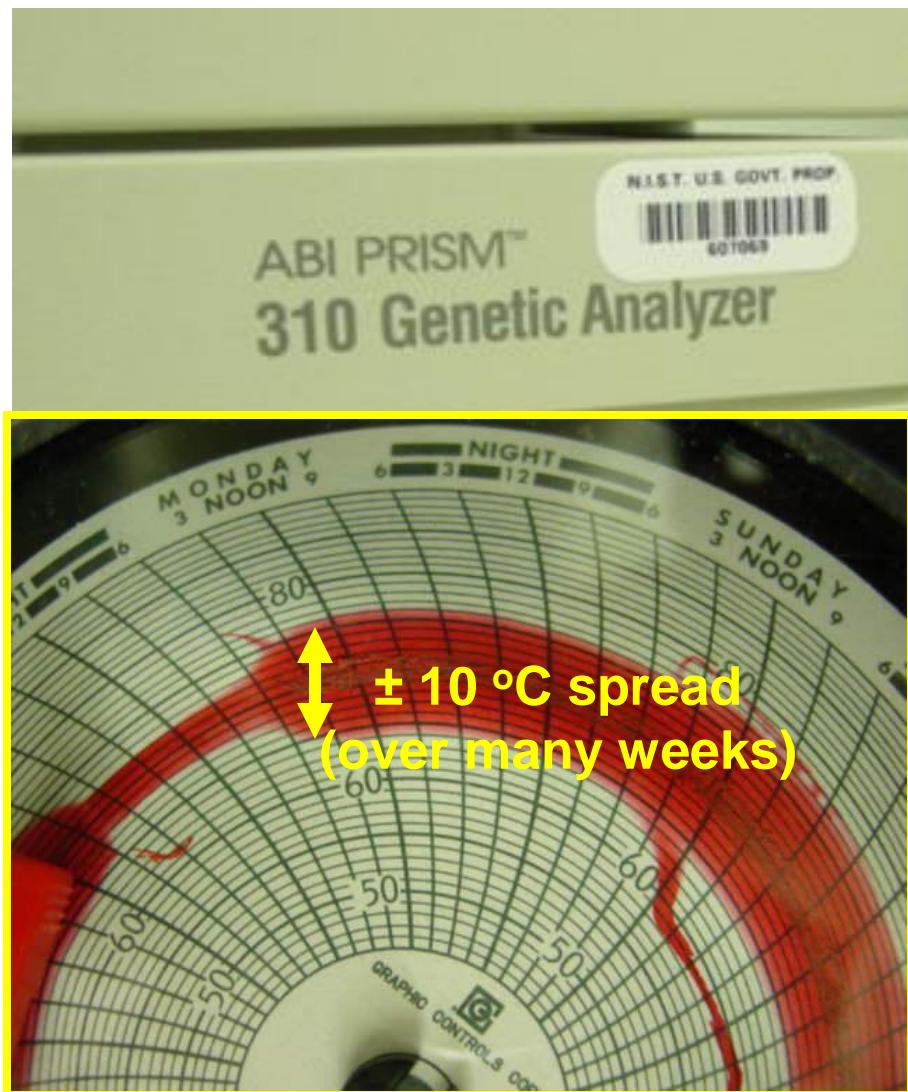
# True off-ladder alleles



30803120...1Sample10 10 Green Q4-1



# Monitoring Room Temperature Over Time





Refrigerator and freezer monitoring



# Temperature Probes

Frig/Freeze Monitors \$240

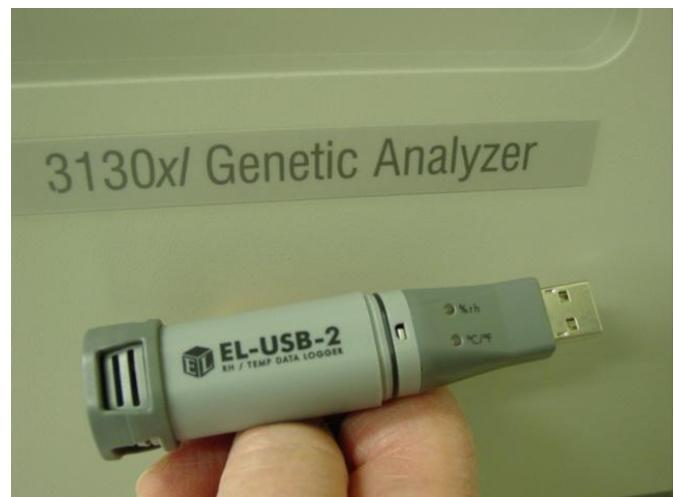
#DT-23-33-80 – USB Temperature Datalogger

PLUS Software \$79.00 (#DT-23-33-60)

Room Monitors, # DT-23039-52 – USB  
Temperature-Humidity Datalogger \$91.00

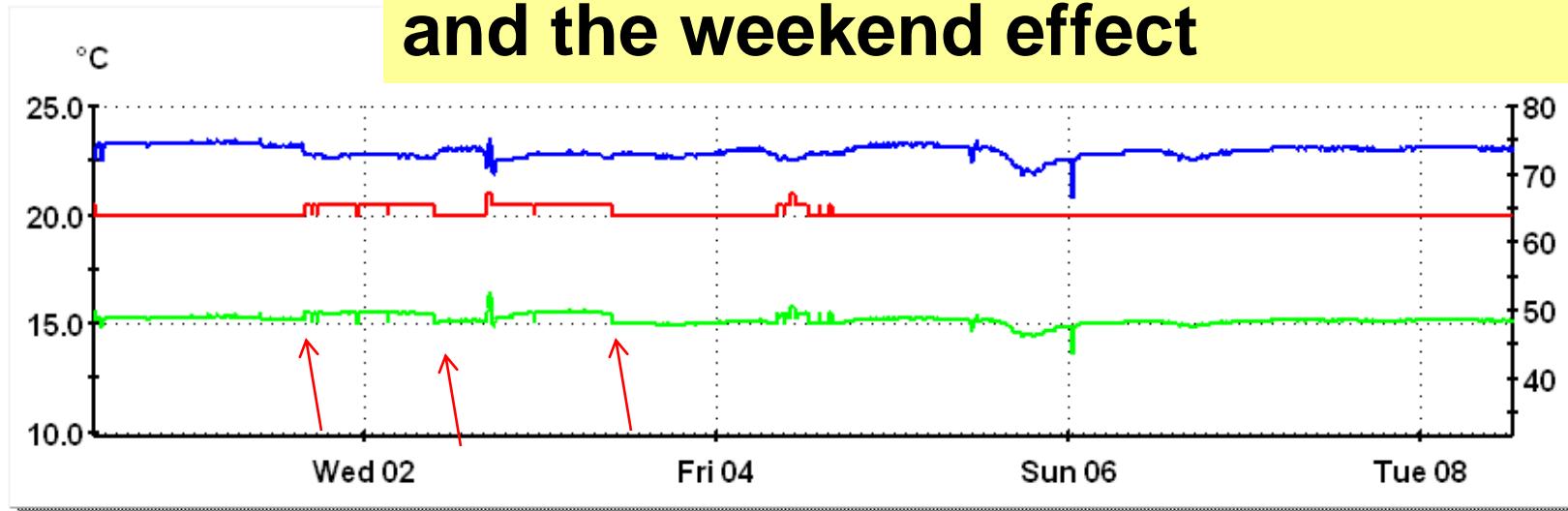
( Cole Parmer, Vernon Hills IL)

Room temperature monitoring



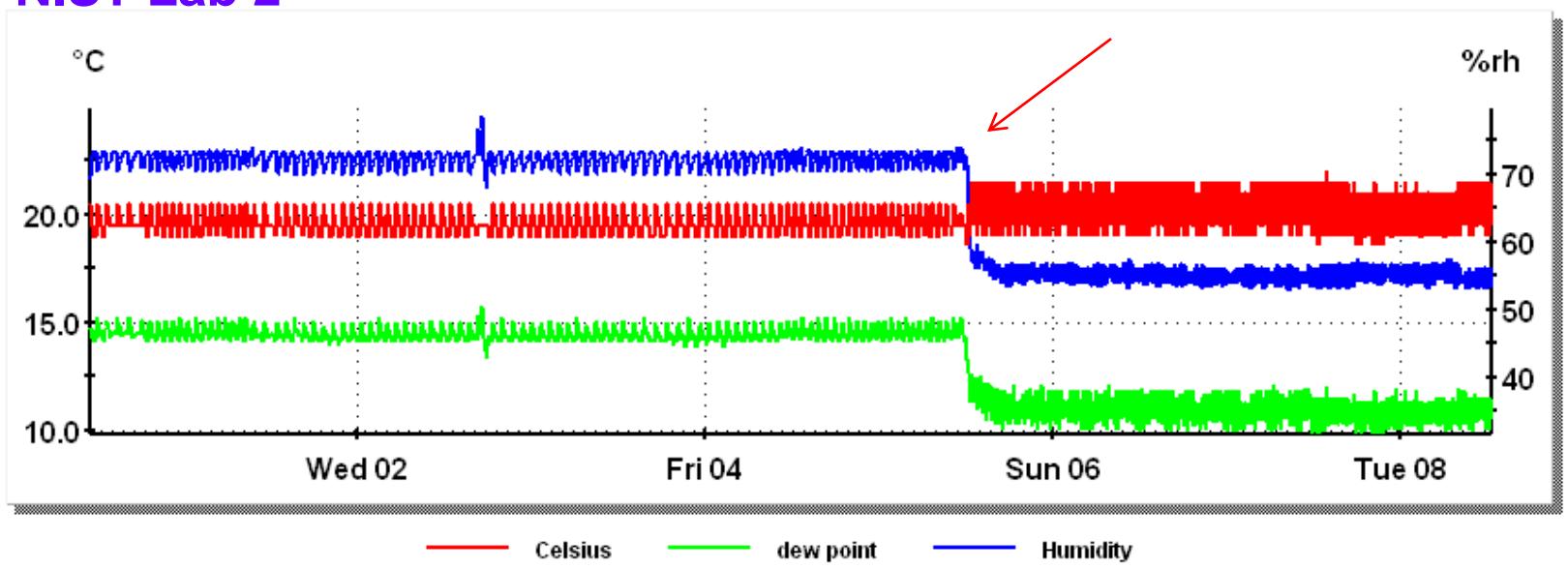
## NIST Lab 1

# Watch out for the 5 o'clock effect and the weekend effect



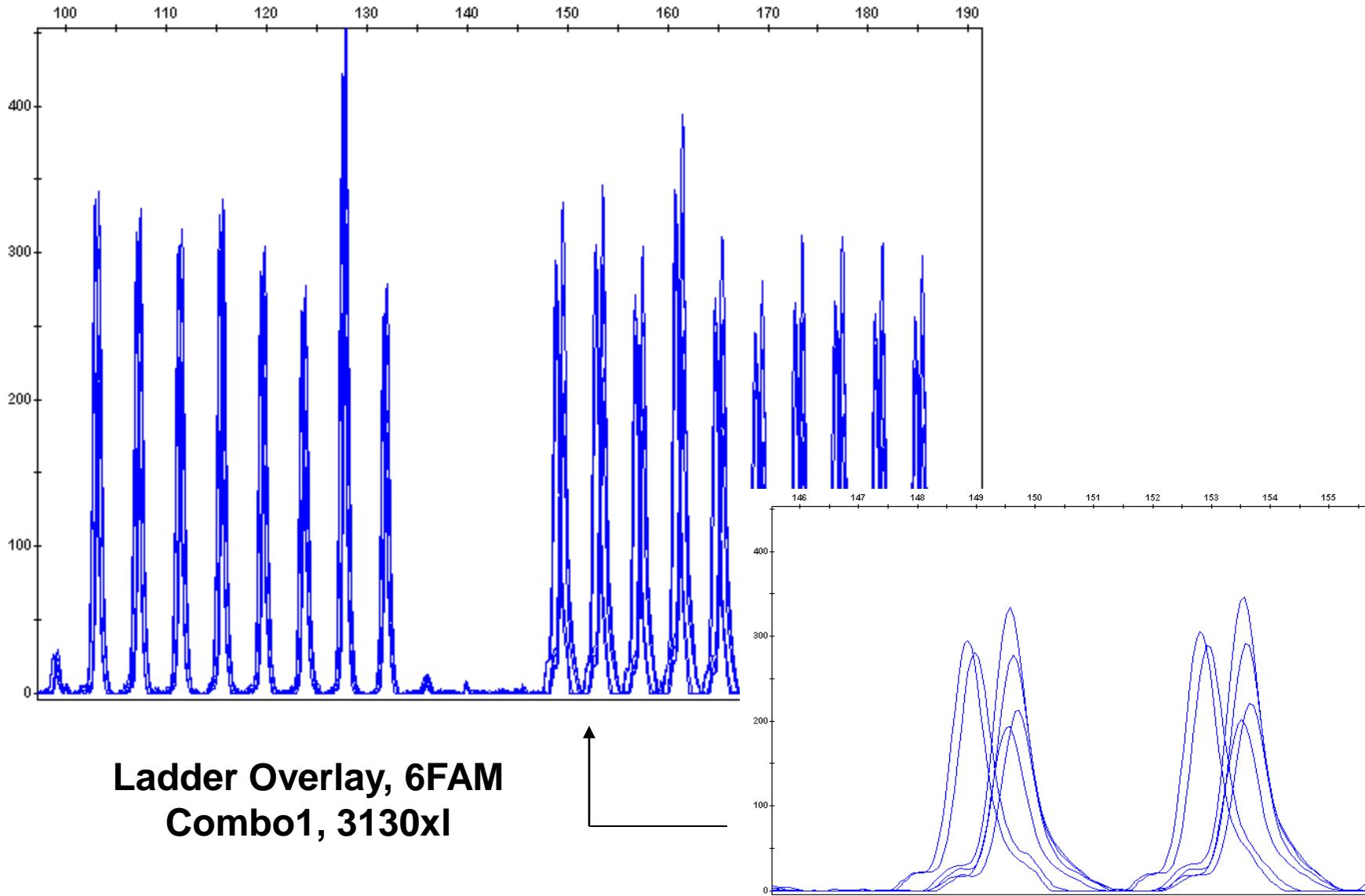
## NIST Lab 2

A230



From:- 31 July 2006 11:00:00 To:- 08 August 2006 12:40:00

# Poor Temperature Control Causes DNA Sizing Imprecision

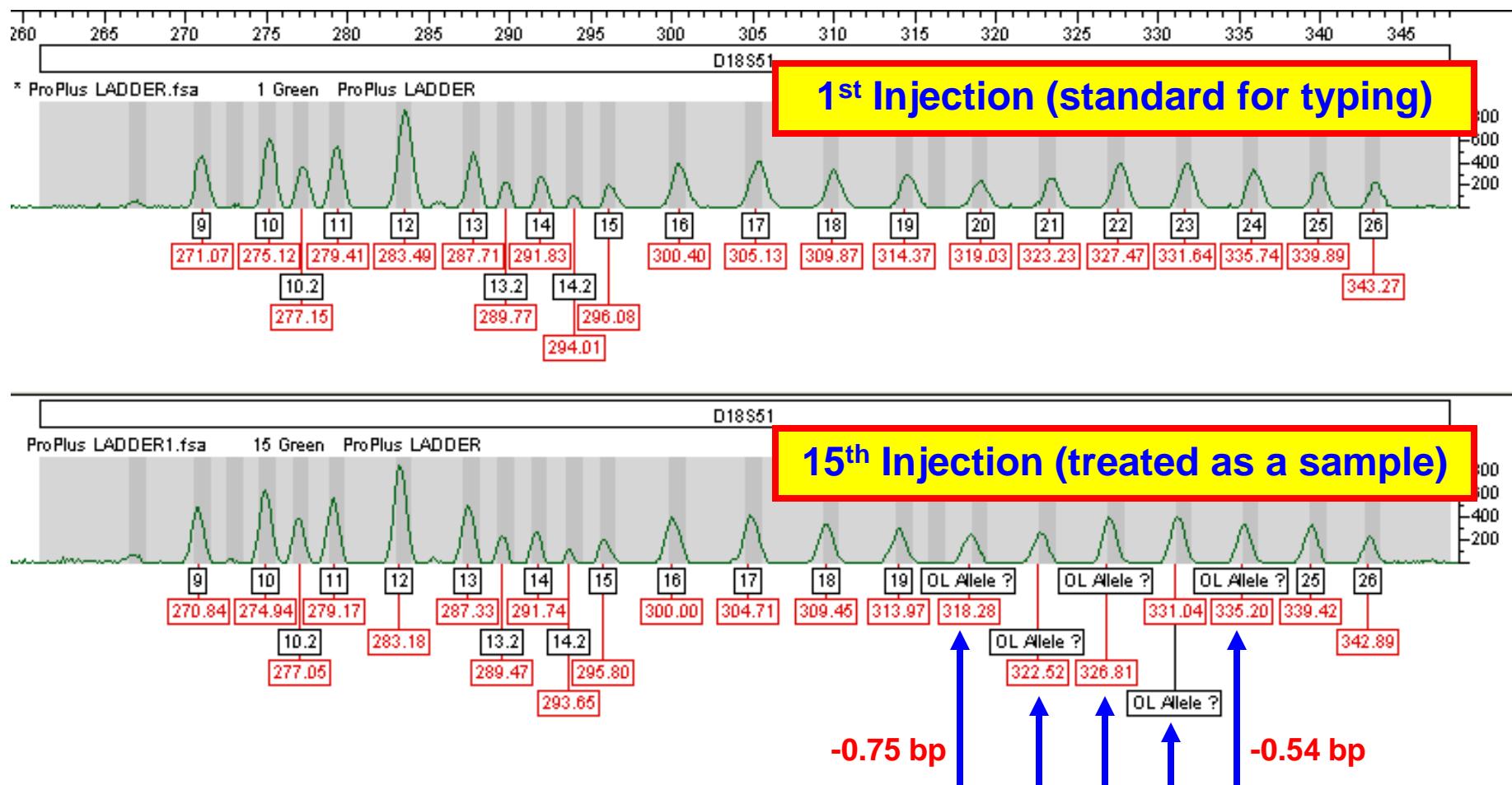


# What to do if calibration is lost?

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

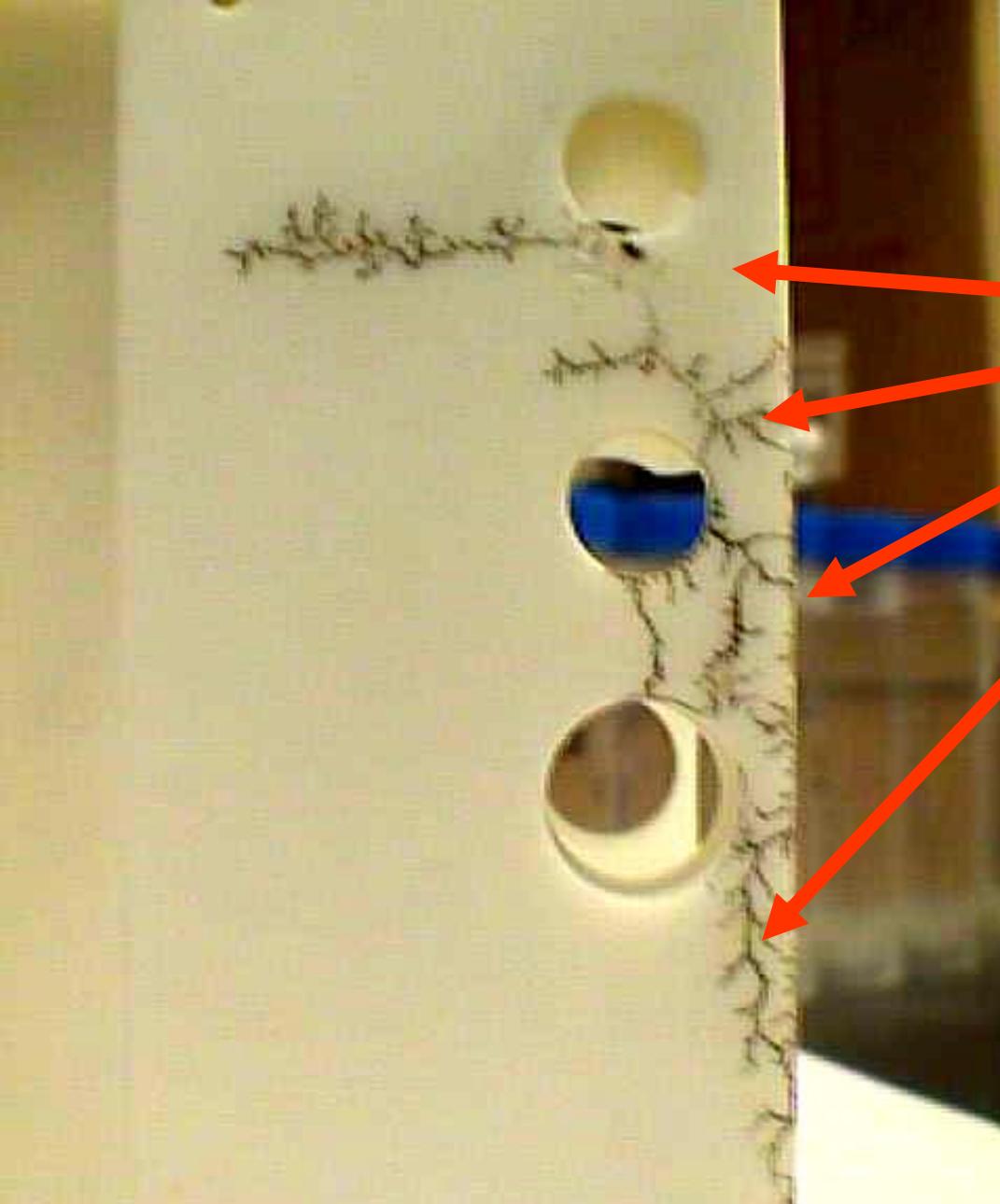
# Use of Second Allelic Ladder to Monitor Potential Match Criteria Problems



These alleles have drifted outside of their genotyping bins due to temperature shifting over the course of the sample batch

# 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



**Carbon Trails**

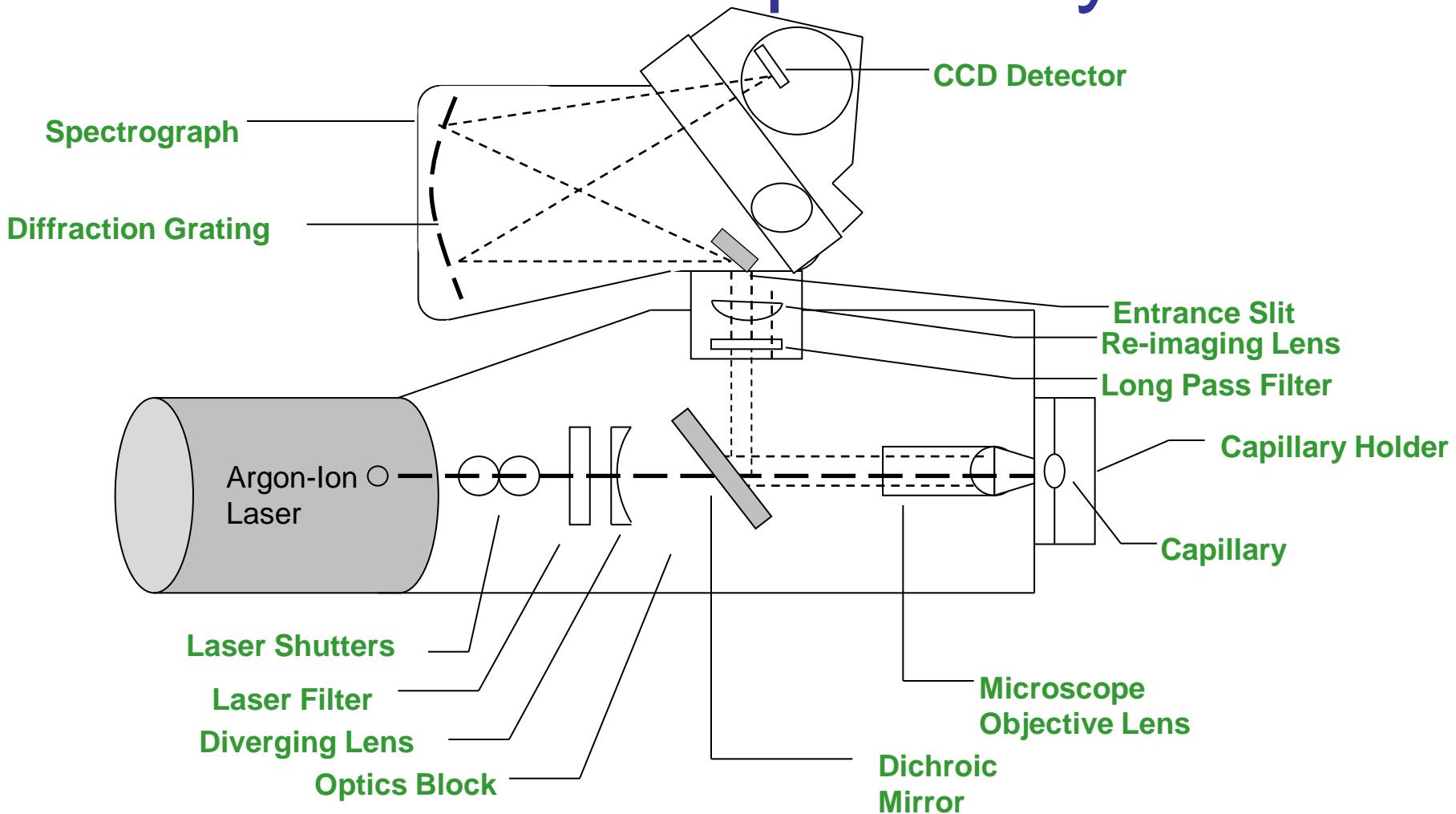
**High Humidity  
or wet buffer vials  
can create other  
paths to ground**

**Keep Your System Clean and Dry!**

# 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 band broadening and inconsistent resolution (meltdowns)

# Consider the Optical System

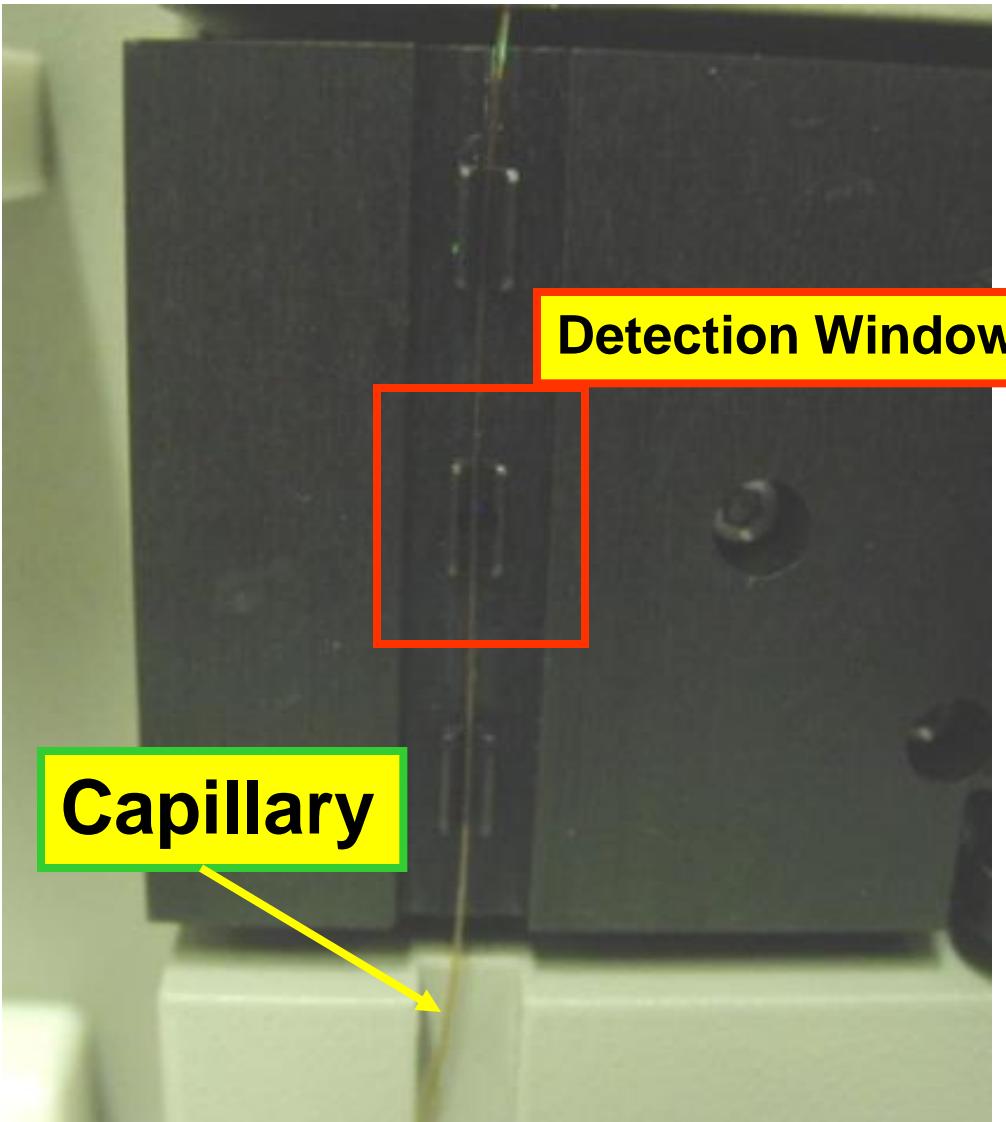


Watts, D. Genotyping STR Loci using an automated DNA Sequencer. In *Forensic DNA Profiling Protocols*; Lincoln, P.J.; Thomson, J. Eds.; Humana Press Inc.: Totowa, NJ, 1998; Vol. 48, pp 193-208.

# Issues with the Optical System

- Argon Ion lasers outgas and eventually loose intensity; **take note of laser current and monitor it over time**
- Fluorescence expression:  
 $I_f = I_0 k \epsilon b C \varphi$  - changes in input intensity:  $I_0$ 
  - changes in capillary diameter:  $b$
  - cleanliness of capillary, optics:  $k$
  - 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.**

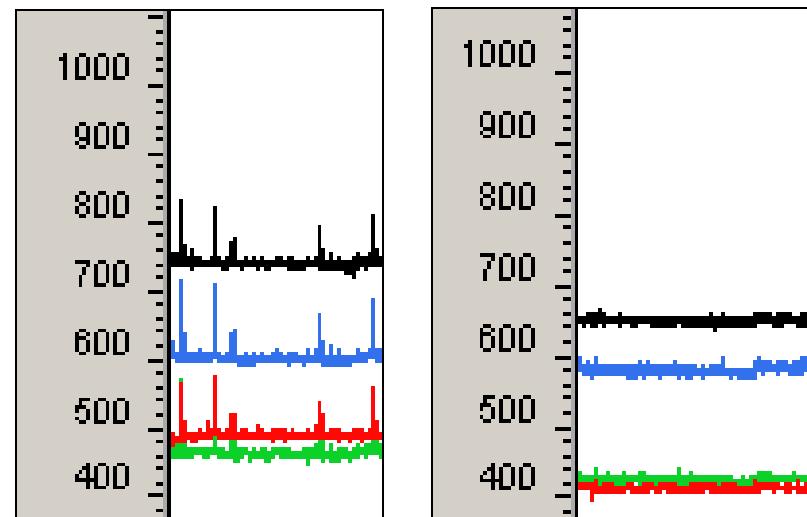
# The Detection Window



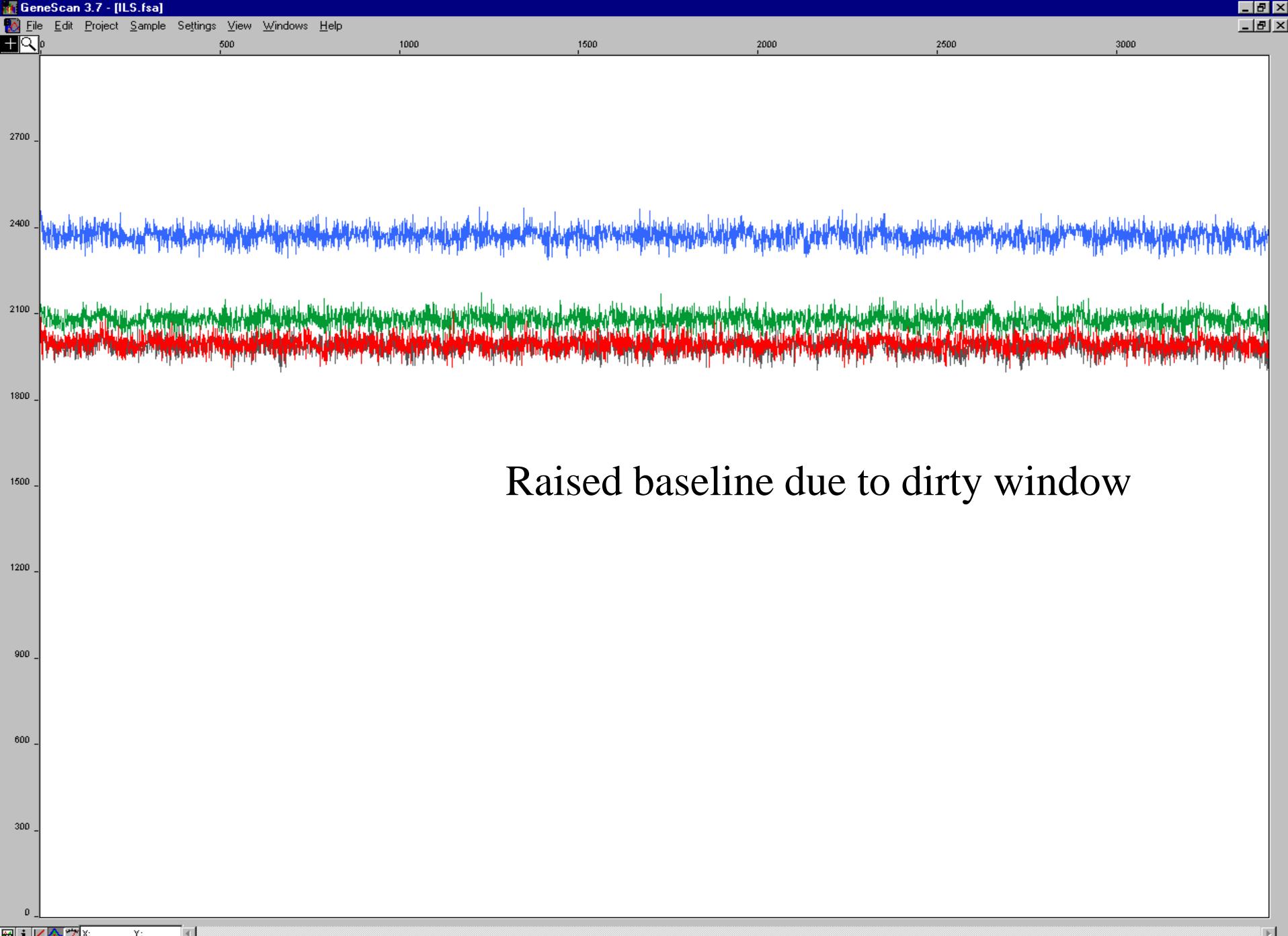
**Make sure that the capillary window is lined up (if it is not, then no peaks will be seen)**

**Window may need to be cleaned with ethanol or methanol**

## Review Start of Raw Data Collection



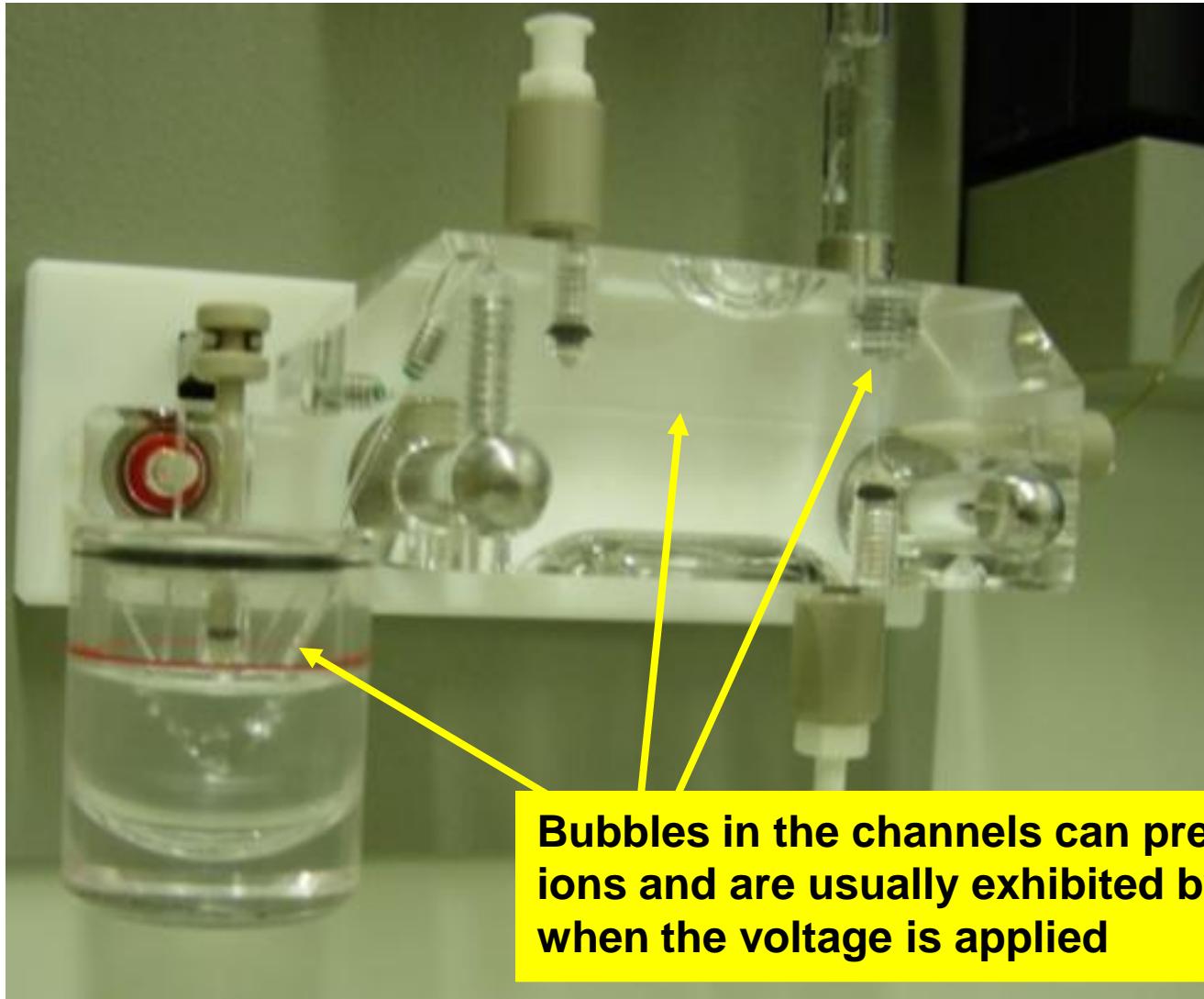
**Little spikes indicate need to change buffer... check current**

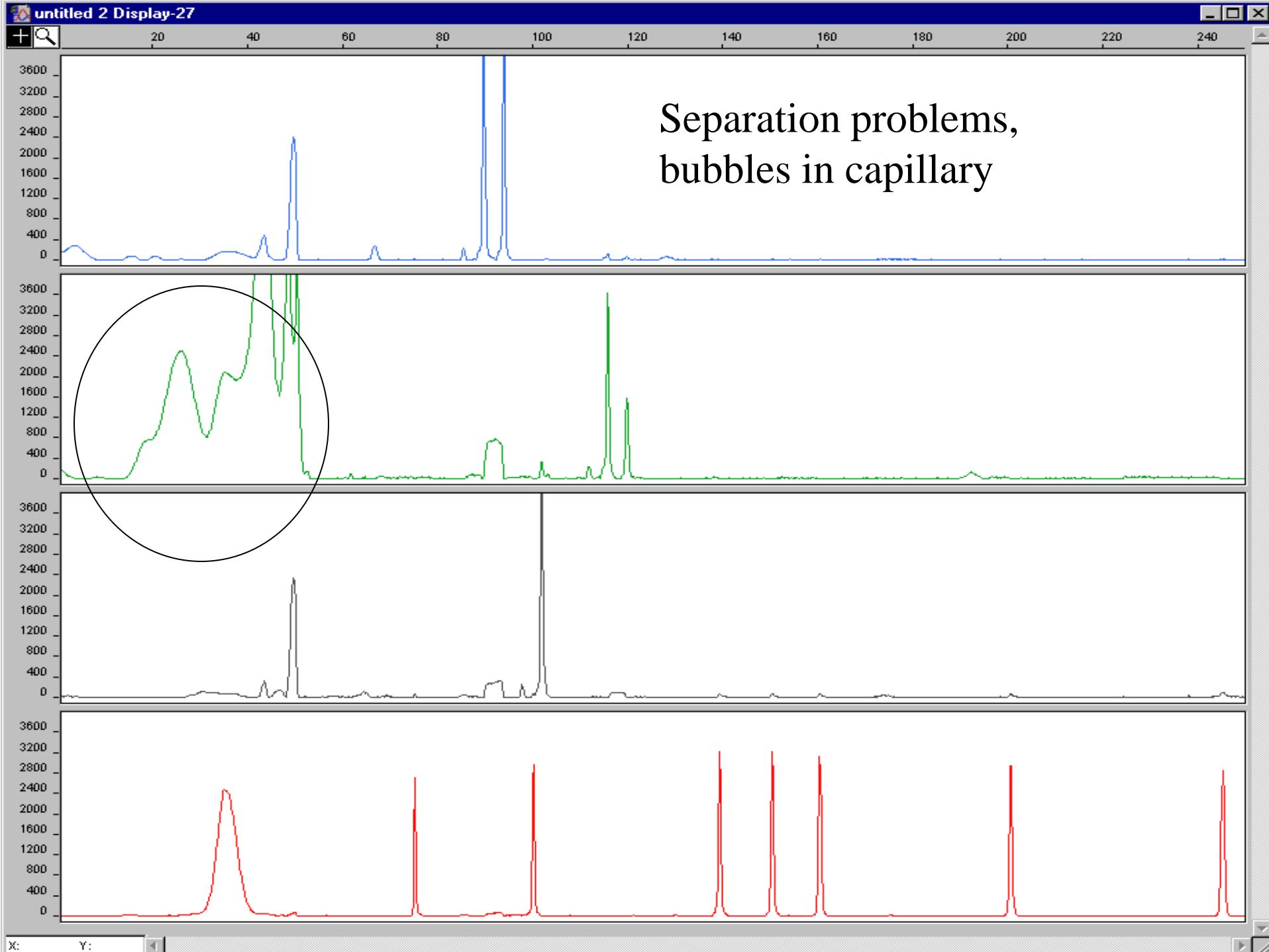


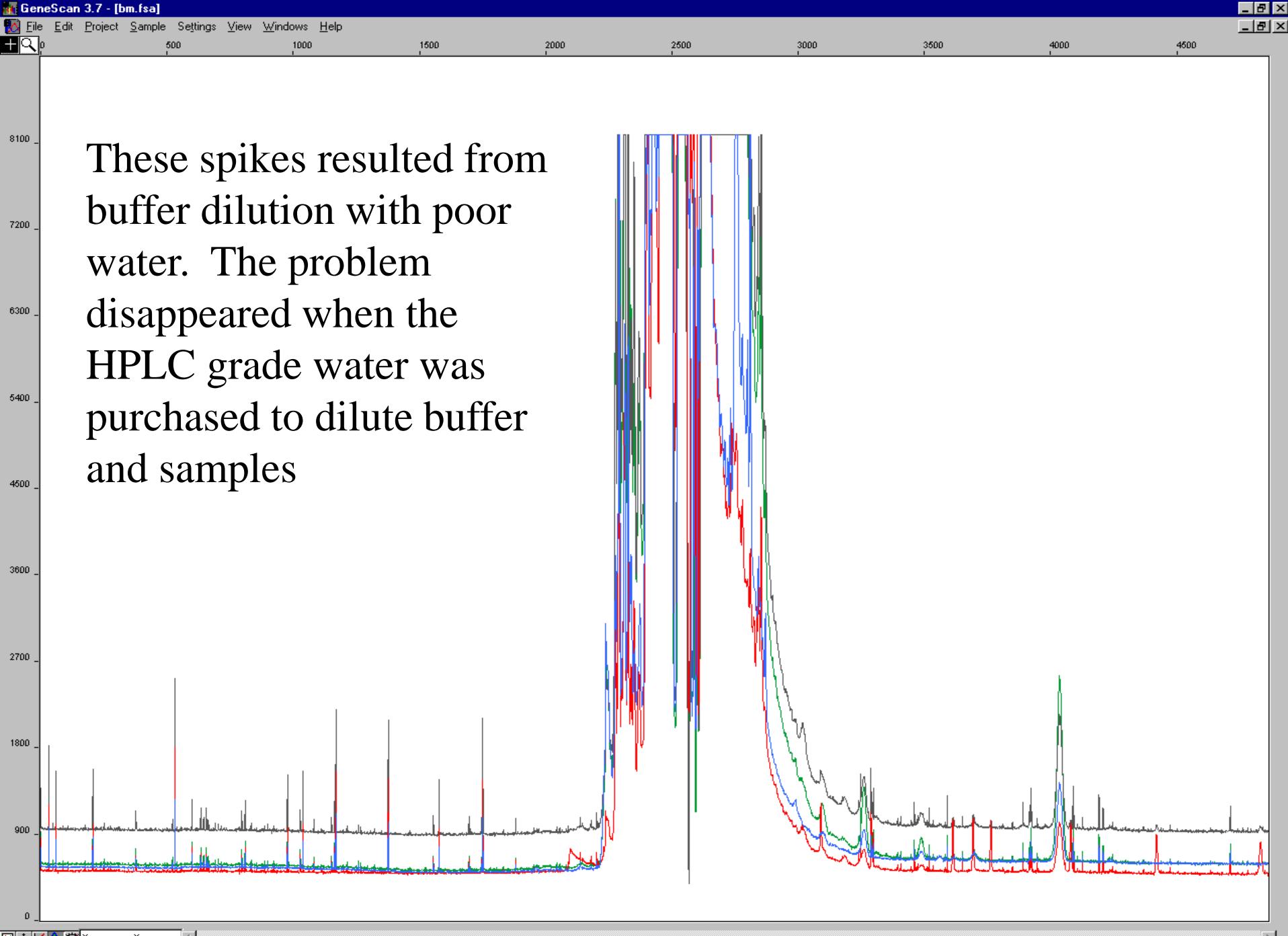
# Buffer Issues

- 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

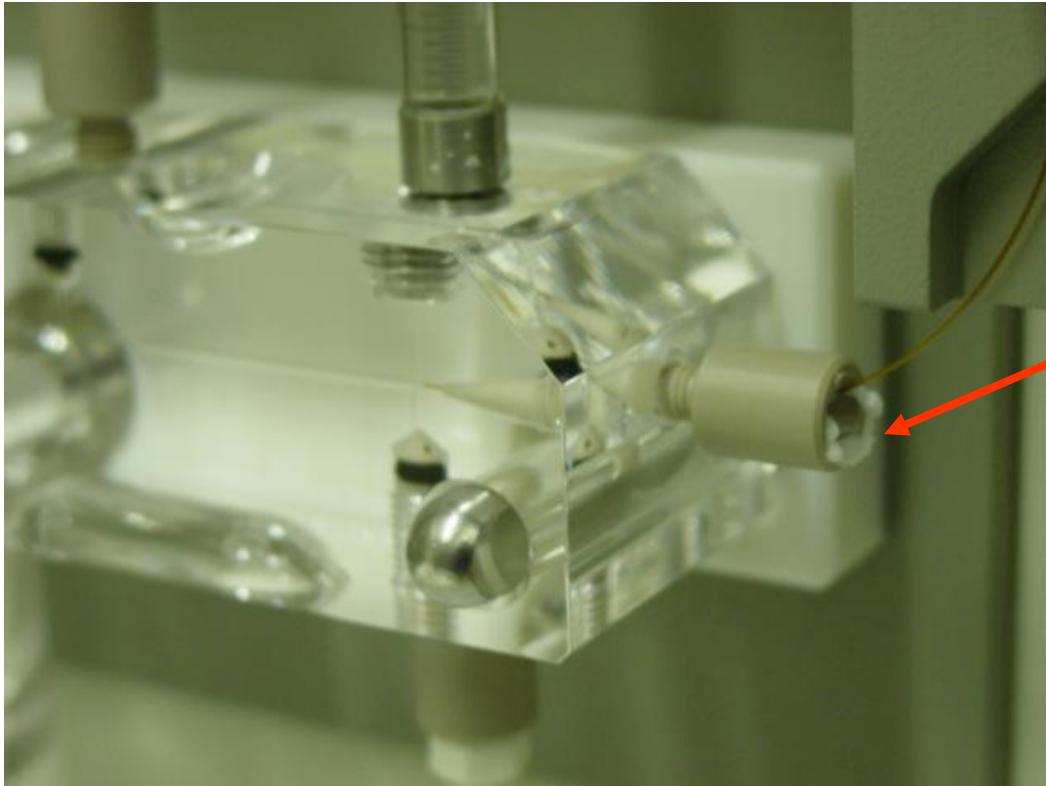
# Remove all bubbles from the channels







# Beware of Urea Crystals



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

Urea sublimes 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

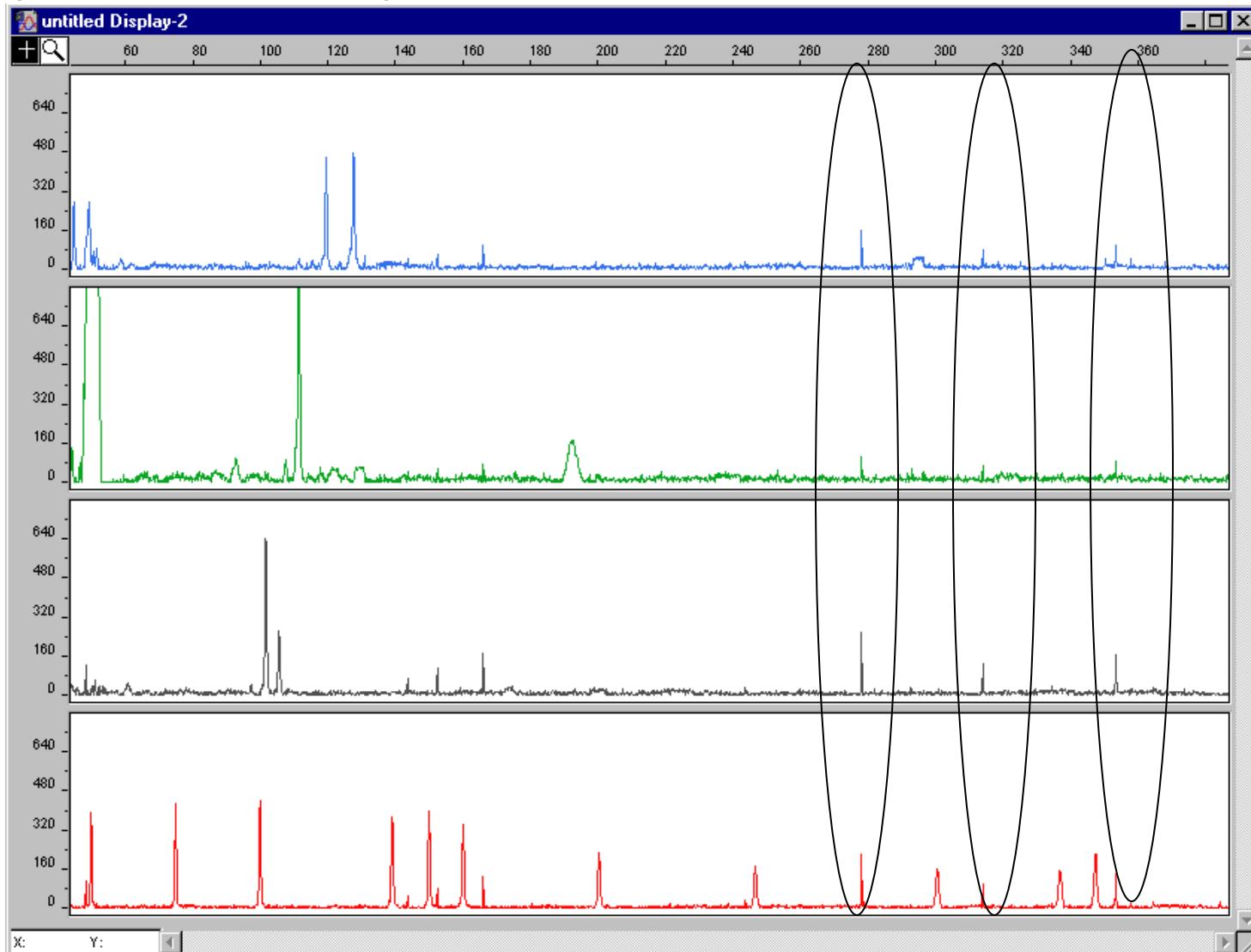
# Maintenance of ABI 310/3100/3130

- Syringe – leaks cause capillary to not fill properly
- Capillary storage & wash – **it dries, it dies!**
- Pump block – cleaning helps insure good fill
- Change the running buffer regularly

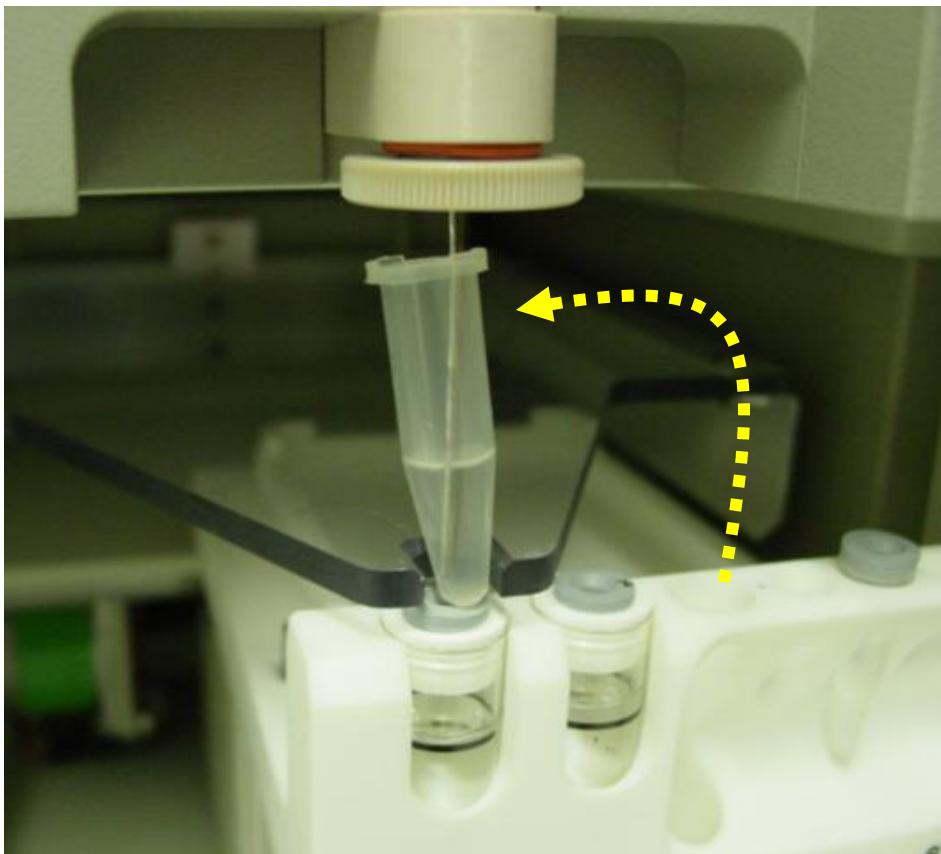
**YOU MUST BE CLEAN AROUND A CE!**

# 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  
and may also be caused by particulates in buffer.**



# Storage when ABI 310 is not in use

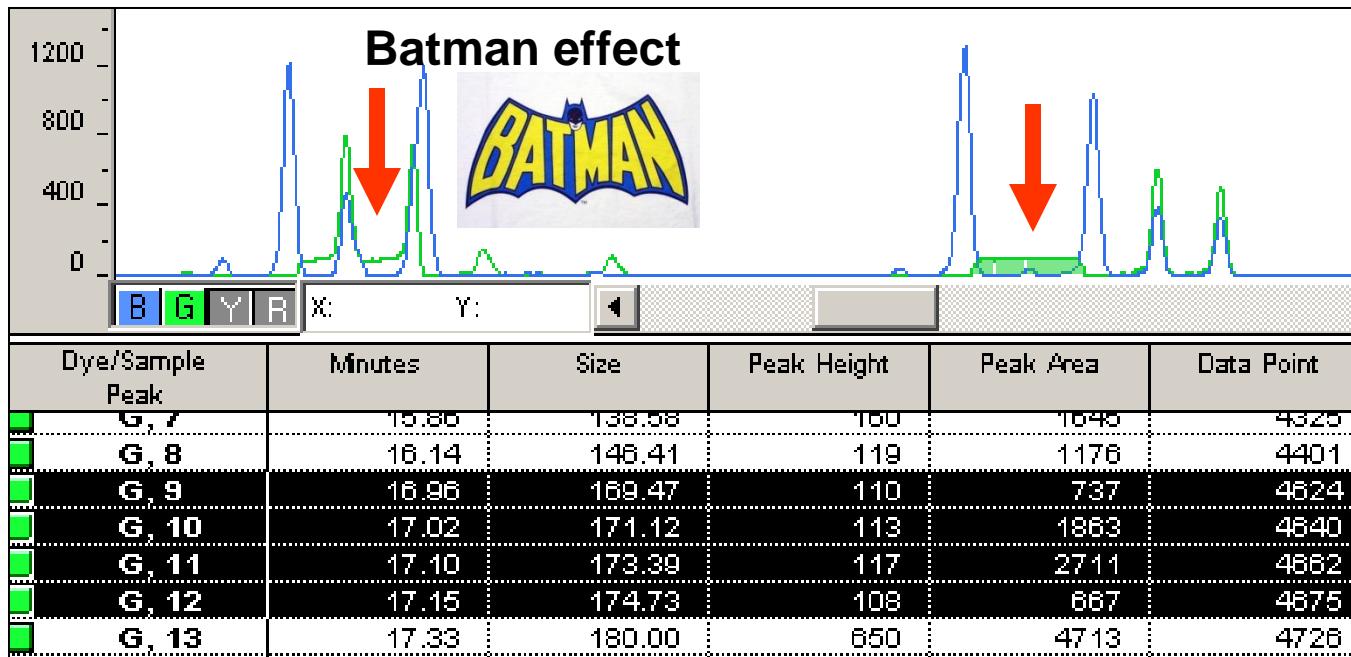


- Keep inlet of capillary in water...if it dries out then urea crystals from the polymer will clog the opening
- The waste vial (normally in position 3) can be moved into position
- A special device can be purchased from Suppelco to rinse the capillary off-line
- Store in distilled water
- Note that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

Remember that the water in the open tube will evaporate over time...  
Also this will destroy the electrode if turned on without removing the tube

# Matrix Problems

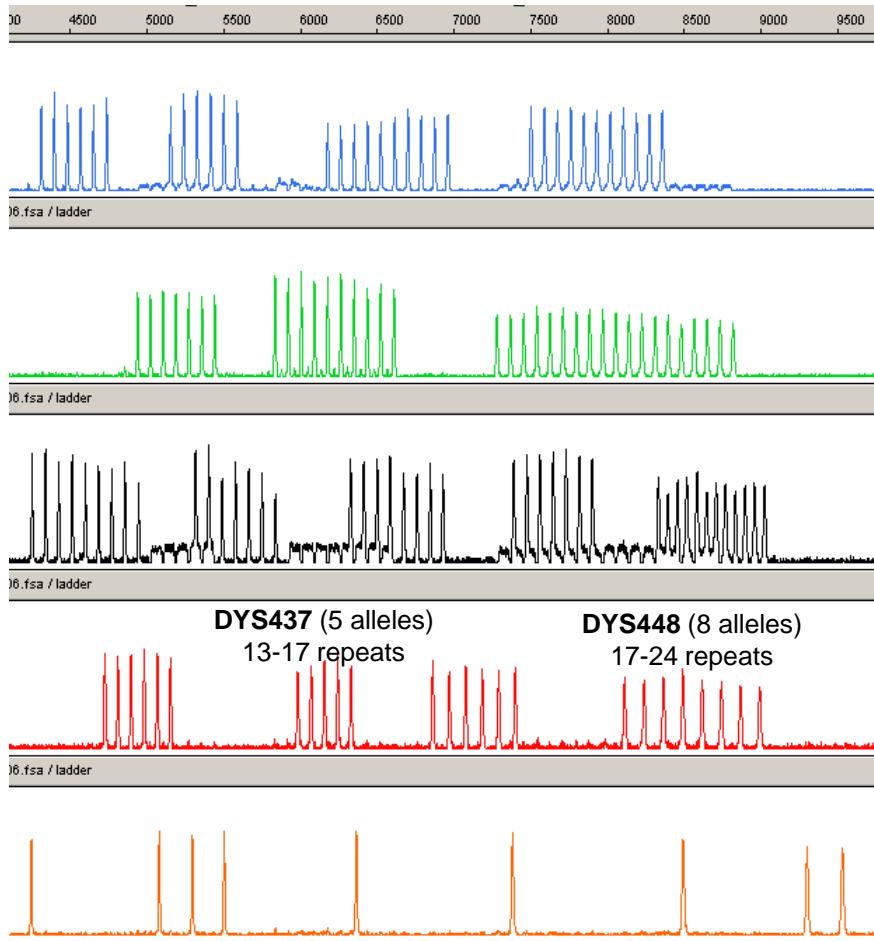
- A poor matrix can lead to raised baseline and therefore calling of too many peaks
- Larger sized alleles will not be identified as peaks because the GeneScan table for a particular dye color has filled up



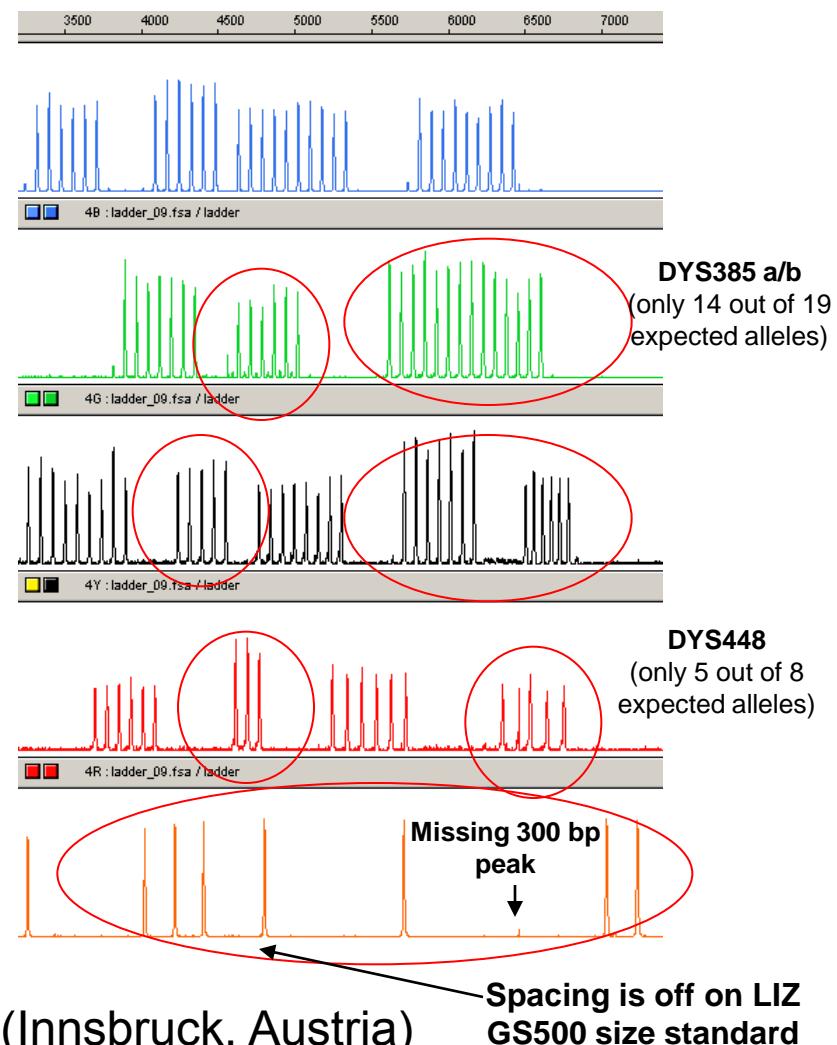
# “Data gap” - phenomenon

## STRs

Y-Filer Allelic Ladder (correct)



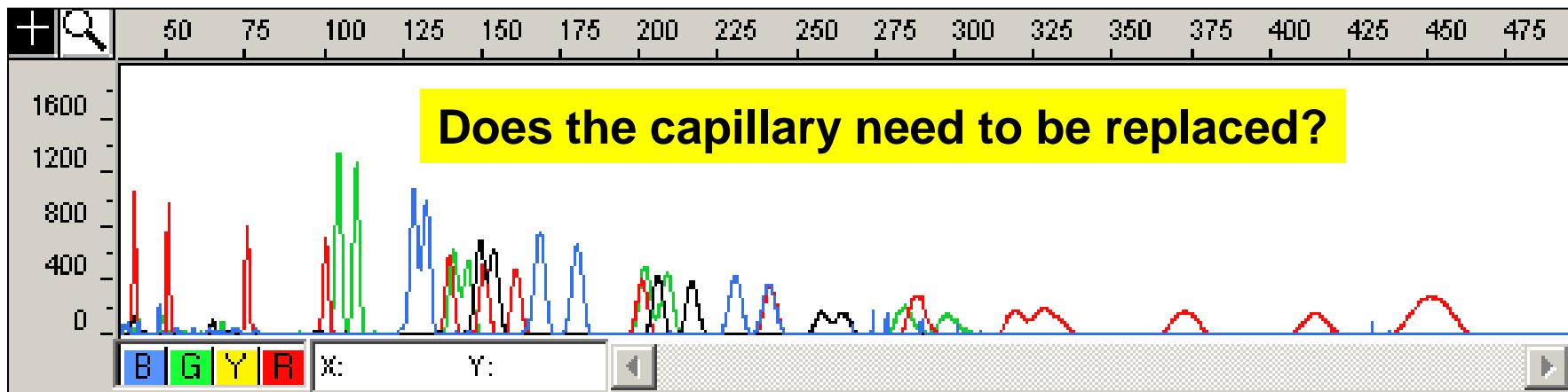
Y-Filer Allelic Ladder (with gaps)



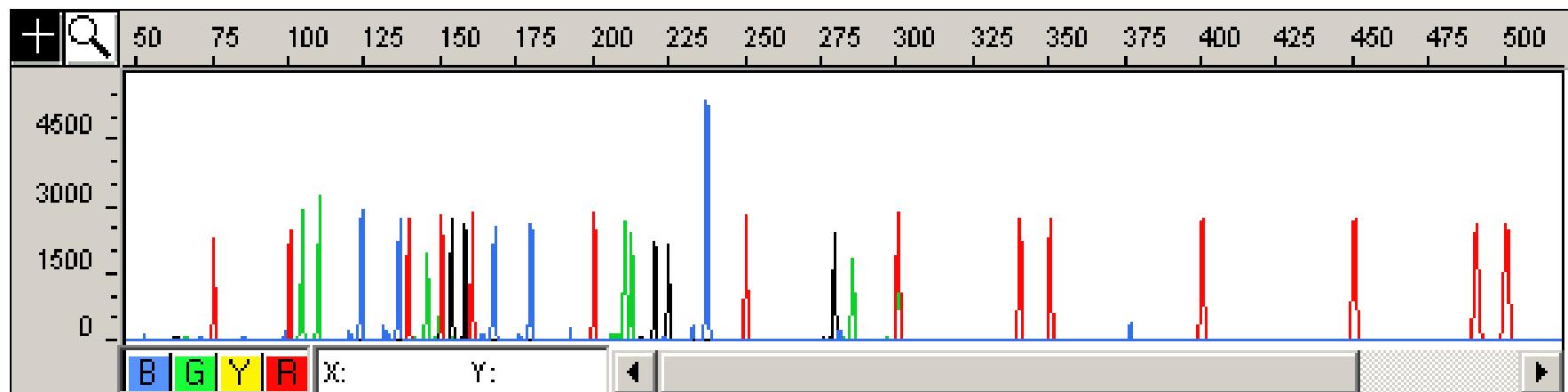
Data from Walter Parson's Lab (Innsbruck, Austria)

Missing 300 bp peak  
↓  
Spacing is off on LIZ GS500 size standard

What we call “melt downs”...  
probably due to an incompletely filled capillary



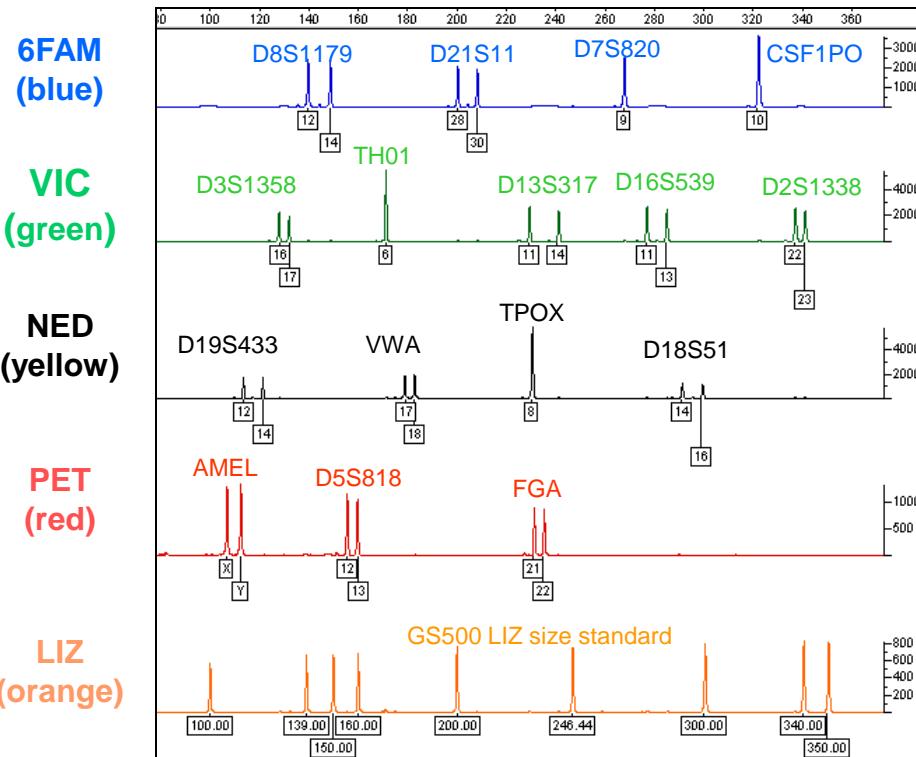
No! The next injection looks fine...



ABI 310 Data from Margaret Kline (NIST)

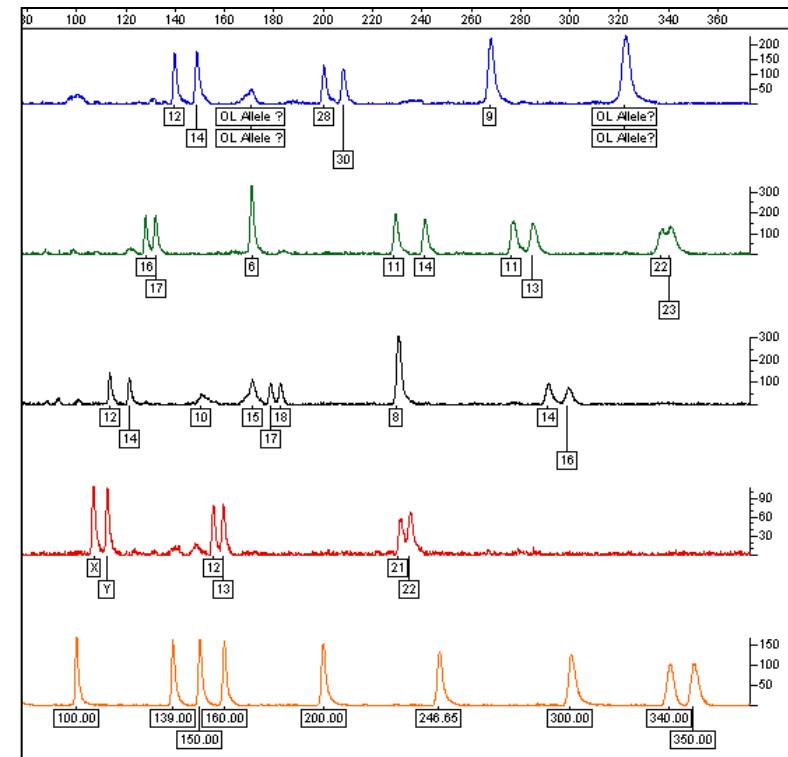
# Capillary Meltdowns

(A) Good resolution



Good Capillary in  
3100 Array

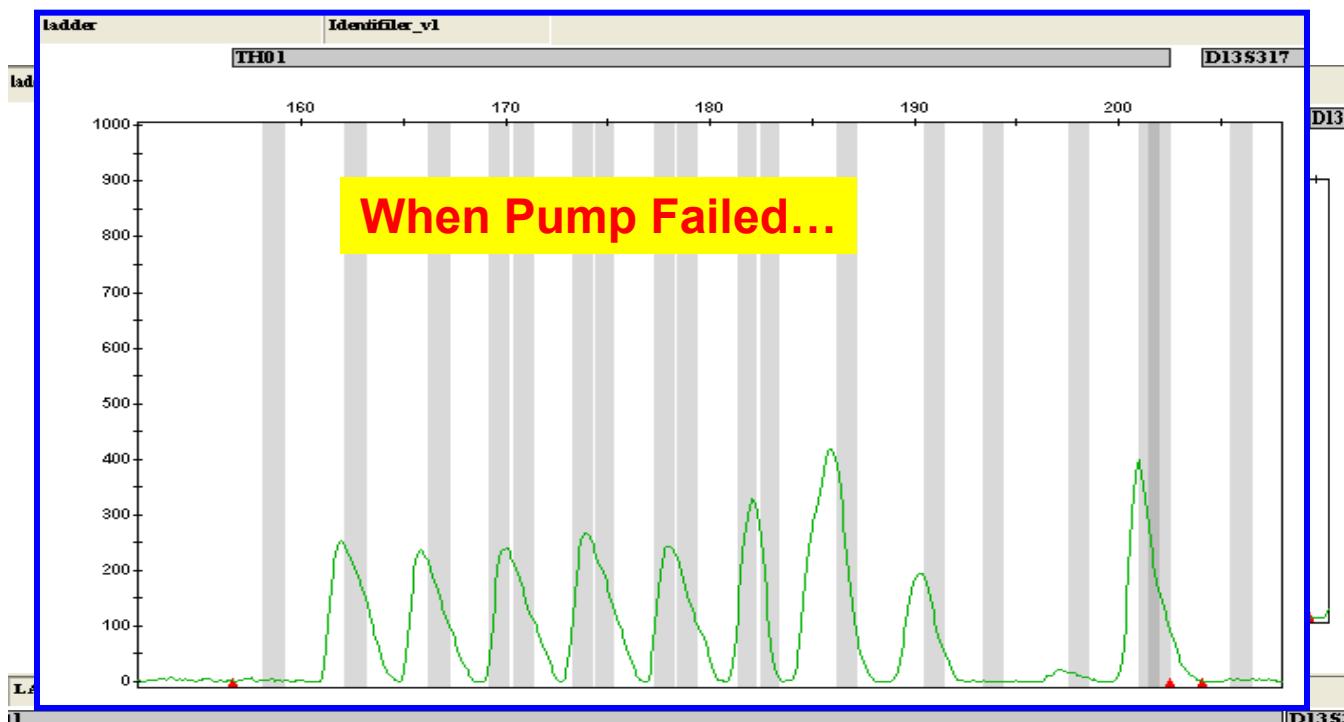
(B) Poor resolution



Bad Capillary in  
3100 Array

Identifier data

Data from 3-07

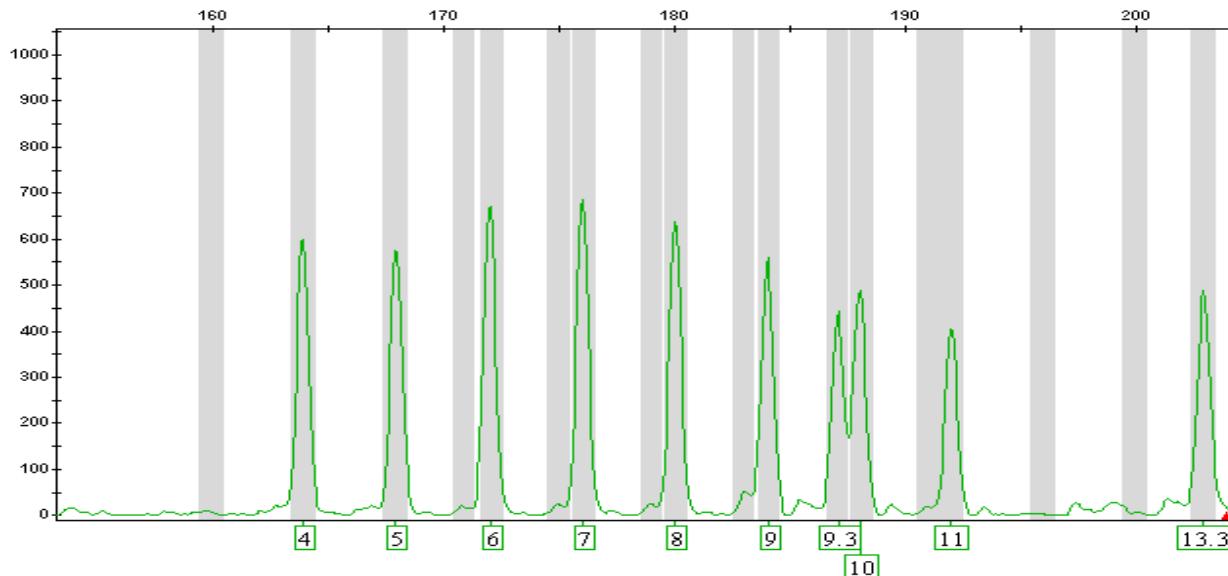


3130xl  
before  
pump  
failure

TH01  
allelic  
ladder

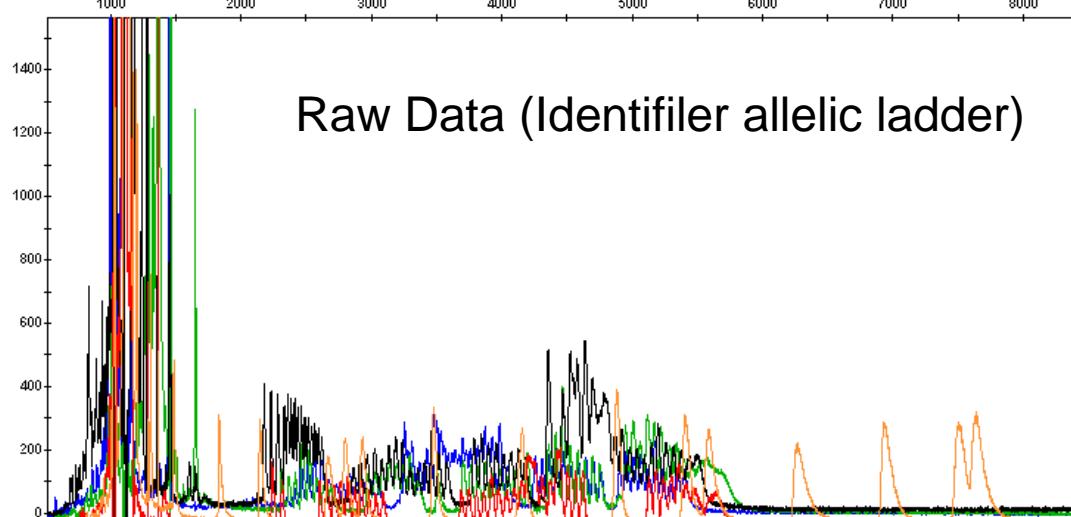
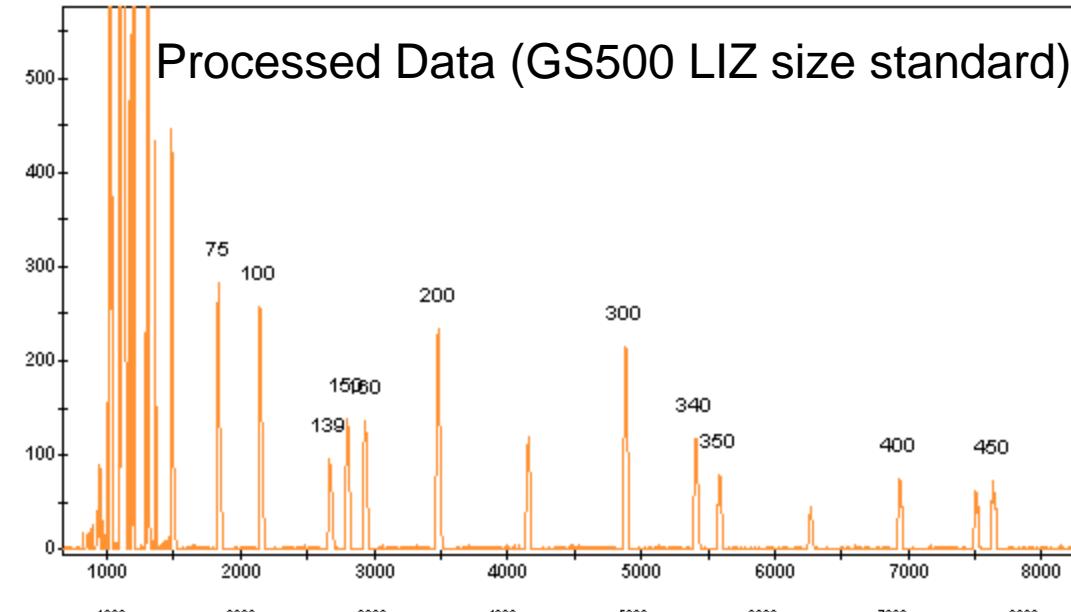
3130xl  
after  
pump  
change

Data from 8-10-07



Data from Amy Decker (NIST)

# Examine the Size Standard...



Data from Becky Hill (NIST)

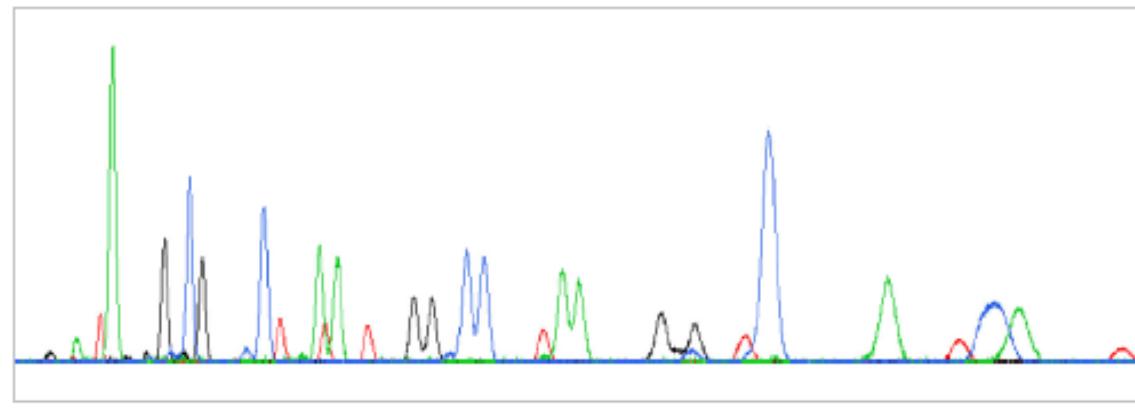
# Meltdowns can be the result of

- Bad formamide
- Bubbles in the sample vial
- 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 in sample

# A permanent loss of resolution may mean

- Adsorptive sites on a capillary
- Initiation of electroosmotic flow
- Conductivity changes in buffer/polymer
- Wrong buffer formulation
- Bad formamide or internal lane standard
- Contaminated syringe

## Loss of Resolution



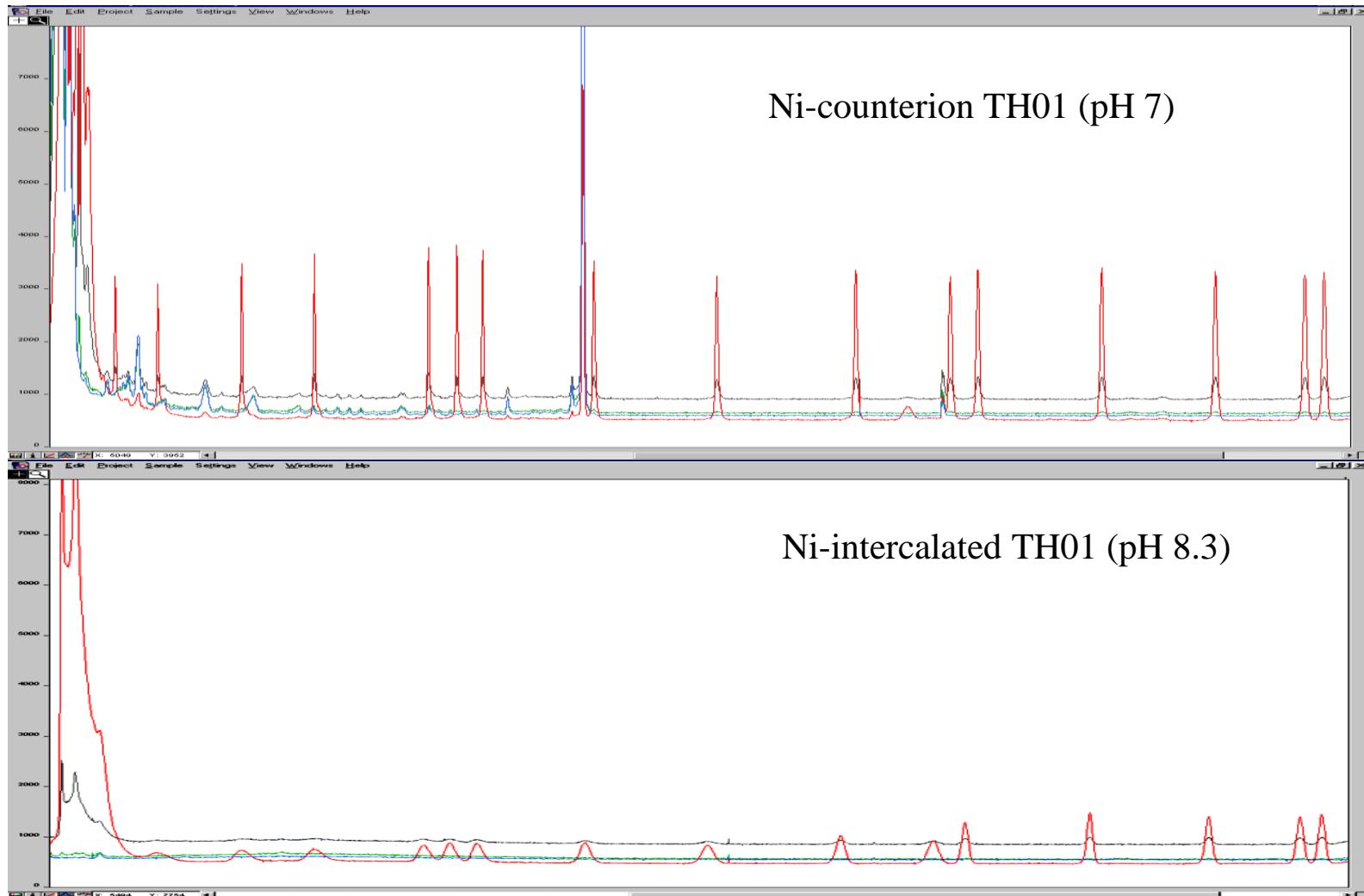
Gradual broadening of peaks as the molecular weight of the data increases results in a sample that fails to genotype and can be caused by the following:

- Poor water quality
- Poor quality system reagents
- Insufficient capillary filling
  - Leak in the system fittings
- Air in the system
  - Bubbles
- Impurities
  - Protein, salts
  - Detergents
- Poor/exhausted array
- Poor instrument maintenance

Attention to detail with regard to instrument maintenance and remaining aware of when an array may need to be replaced will help to avoid such issues.

# Metal Ions in the Sample

DNA clumps and injects poorly. Effect is pH and EDTA dependent



1  $\mu$ l TH01 added to 10  $\mu$ l of 3.0 mM NiCl<sub>2</sub> in 10 mM Tris, pH 7 or pH 8.3. Sample allowed to interact for 1 hr and then 1  $\mu$ l added to ROX/formamide.

# 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  $\mu$ A (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

Run Folder-1-  
10-35-PM

→ Log.log

Name	Size	Type	Modified
Log.log	8 KB	Text Document	1/5/2005 3:25 AM

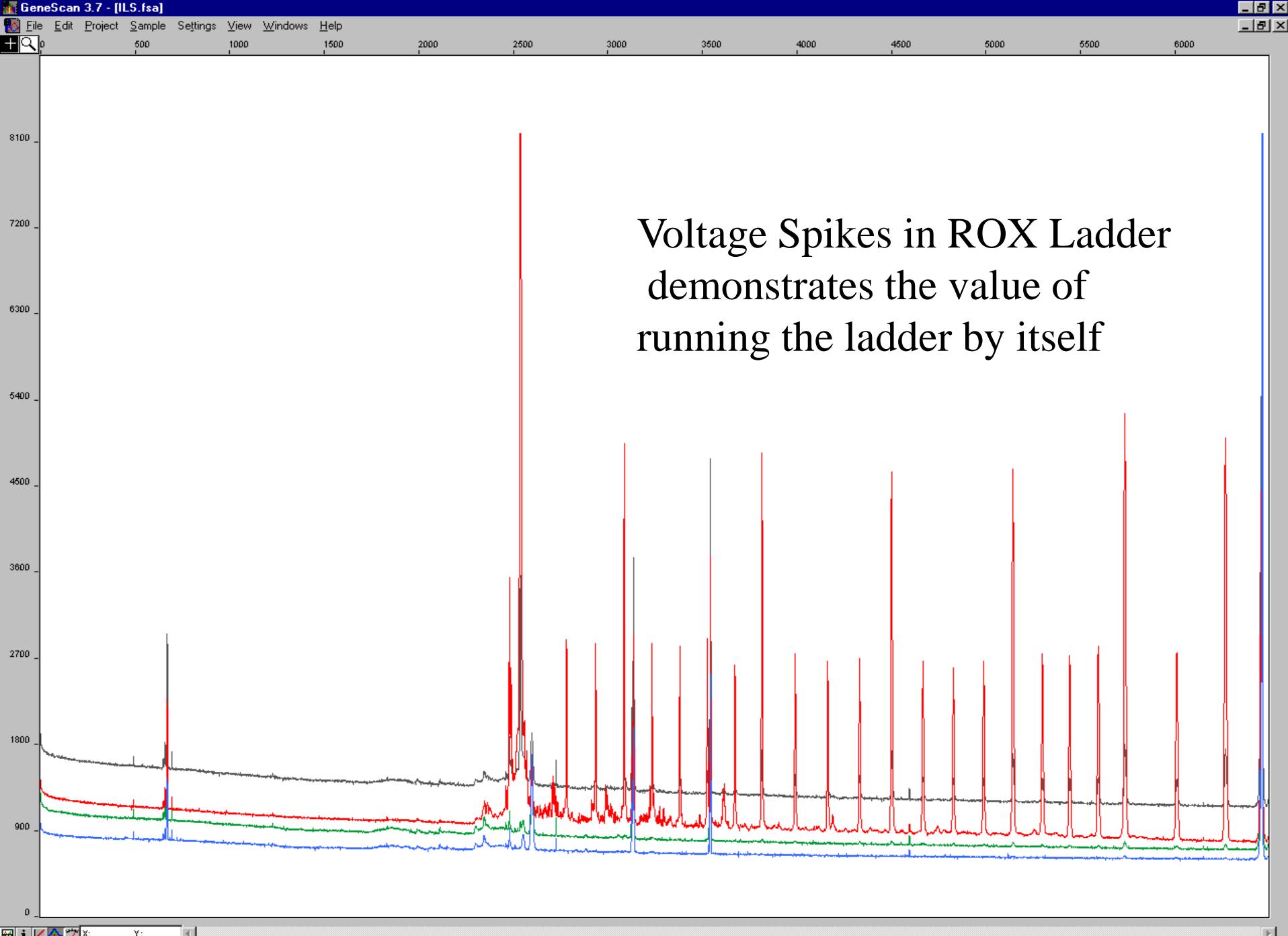
**Syringe Position**

```
1/4/05 10:35:02 PM    ABI PRISM 310 Data Collection
...1/4/05 10:35:02 PM    ABI PRISM 310 Collection version 3.0.0
<--1/4/05 10:35:02 PM    ABI PRISM 310 Firmware version 1.2
<--1/4/05 10:35:02 PM    Instrument serial number: 310000431
1/4/05 10:35:02 PM    ABI PRISM 310 Data Collection
...1/4/05 10:35:02 PM    ABI PRISM 310 Collection version 3.0.0
<--1/4/05 10:35:02 PM    ABI PRISM 310 Firmware version 1.2
<--1/4/05 10:35:02 PM    Instrument serial number: 310000431
...1/4/05 10:35:02 PM    Sample sheet: D:\AppliedBio\310\Sample sheets\MIX05 Prof
...1/4/05 10:35:02 PM    Genescan Run Operator: John
...1/4/05 10:35:02 PM    Detector Length: 36 cm
-->1/4/05 10:35:02 PM    Run Started
-->1/4/05 10:35:03 PM    Injection 1 - ProPlus LADDER
-->1/4/05 10:35:04 PM    Module: GS STR POP4 (1 mL) F.md4
-->1/4/05 10:35:04 PM    Vial A1 inject 5 secs 15.0kv run 28 mins at 15.0kv 60°C
<--1/4/05 10:44:45 PM    EP 15.0kv 12.0uA 60C laser 9.8mw syringe 451
<--1/4/05 11:13:02 PM    EP 15.0kv 0.0uA 60C laser 9.8mw syringe 451
<--1/4/05 11:13:02 PM    Points collected: 7584
-->1/4/05 11:13:03 PM    Injection 1 End
-->1/4/05 11:13:04 PM    Injection 2 - MIX05_P P+
-->1/4/05 11:13:05 PM    Module: GS STR POP4 (1 mL) F.md4
-->1/4/05 11:13:05 PM    Vial A3 inject 5 secs 15.0kv run 28 mins at 15.0kv 60°C
<--1/4/05 11:19:19 PM    EP 15.0kv 12.0uA 60C laser 9.8mw syringe 453
<--1/4/05 11:47:37 PM    EP 15.0kv 0.0uA 60C laser 9.8mw syringe 453
<--1/4/05 11:47:37 PM    Points collected: 7584
-->1/4/05 11:47:37 PM    Injection 2 End
-->1/5/05 12:56:43 AM    Injection 5 - MIX05_A P+
-->1/5/05 12:56:44 AM    Module: GS STR POP4 (1 mL) F.md4
-->1/5/05 12:56:44 AM    Vial A9 inject 5 secs 15.0kv run 28 mins at 15.0kv 60°C
<--1/5/05 1:02:54 AM    EP 15.0kv 12.0uA 60C laser 9.8mw syringe 459
<--1/5/05 1:31:12 AM    EP 15.0kv 0.0uA 60C laser 9.8mw syringe 459
<--1/5/05 1:31:12 AM    Points collected: 7584
-->1/5/05 1:31:12 AM    Injection 5 End
```

Current

# ROX Ladder QC procedures

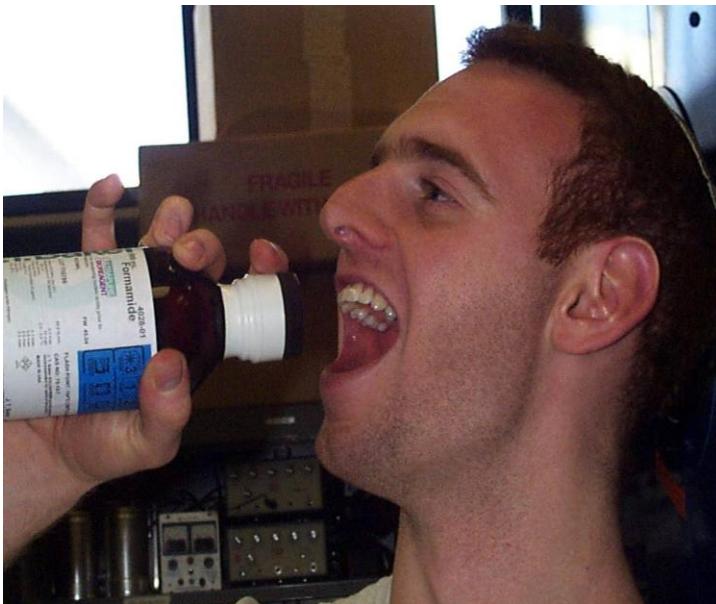
- A recommended sequence for initial operation of the 310
  - Rox ladder – initial injection - throwaway
  - Rox ladder- QC to test peak intensity and look for problems in blank
  - Allelic ladder- to determine resolution and to provide standard
  - 10-15 samples
  - Allelic ladder
  - 10-15 samples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

# 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

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# JAMES CARVILLE

A Political Novel by James Carville and Jeff Koons

**It's the  
Current,  
Stupid!**

