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NIST National Institute of Standards and Technology

CE Troubleshooting

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Bruce McCord's *Profiles in DNA* Article

PROFILES IN DNA Volume 6 (2), Sept 2003, pp. 10-12

TECH TIPS

Troubleshooting Capillary Electrophoresis Systems

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

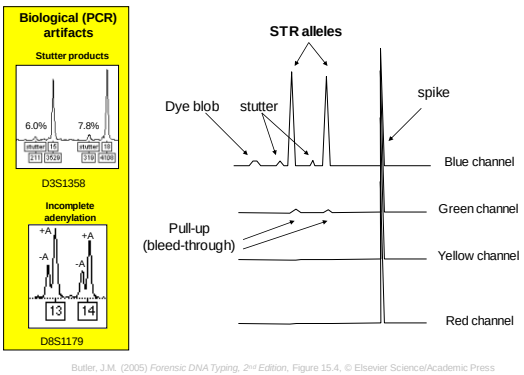
INTRODUCTION

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

Deciphering Artifacts from the True Alleles



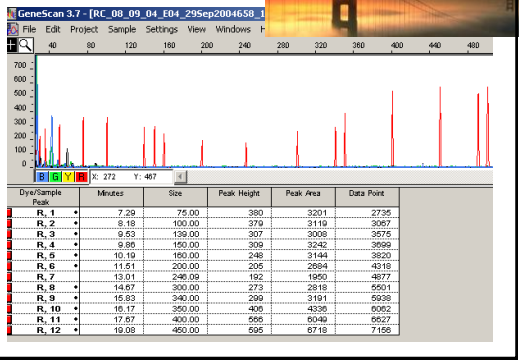
Butler, J.M. (2005) *Forensic DNA Typing, 2nd Edition*, Figure 15.4, © Elsevier Science/Academic Press

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

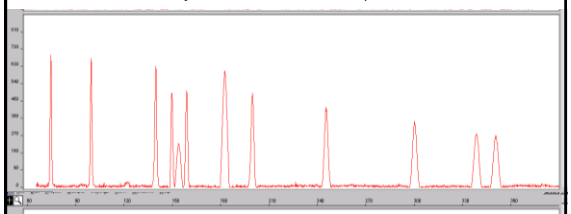
Golden Gate Effect

Attributed to poor formamide

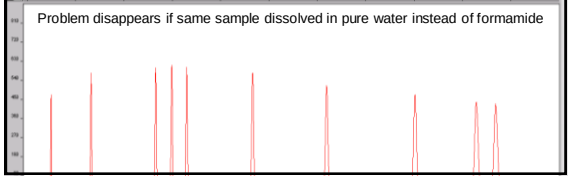


Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
R, 1	7.29	75.00	380	3201	2735
R, 2	8.18	100.00	379	3119	3057
R, 3	9.53	139.00	307	3008	3575
R, 4	9.86	150.00	309	3242	3699
R, 5	10.19	160.00	248	3144	3820
R, 6	11.51	200.00	295	2884	4318
R, 7	13.01	246.09	192	1950	4877
R, 8	14.67	300.00	273	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

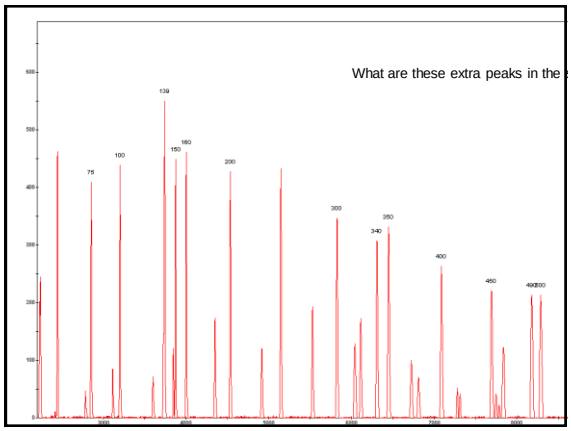
Effect of bad HI-Dye Formamide - Note broad peaks and extra bands



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



What are these extra peaks in the



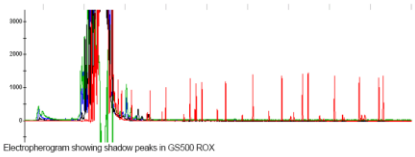
December 21, 2007

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:



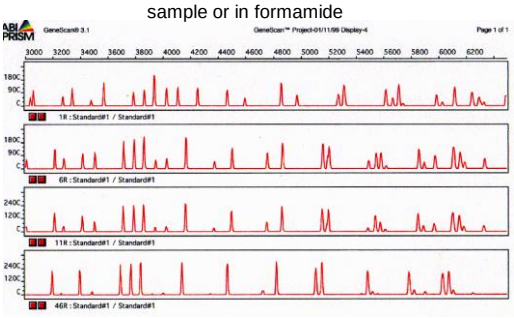
Electropherogram showing shadow peaks in GS500 ROX

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



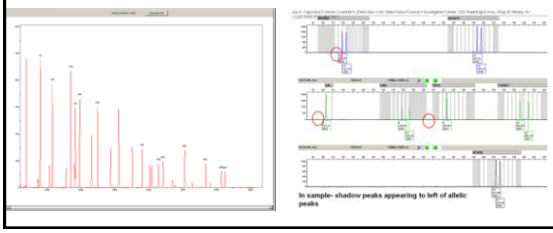
ds DNA migrates faster and over time with this set of runs
ds DNA replaced the ssDNA

Shadow peaks

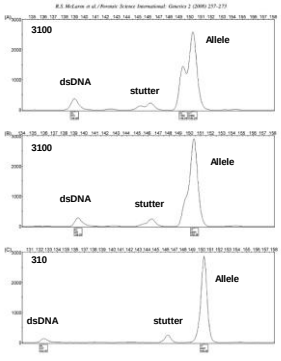
Shadow peaks result from incomplete denaturation or from rehybridization.

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



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

1. The doubled peaks and shoulders are the result of primer hybidization 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
ScienceDirect
Forensic Science International: Genetics 2 (2008) 257–273
FSI GENETICS
www.elsevier.com/locate/fsig

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

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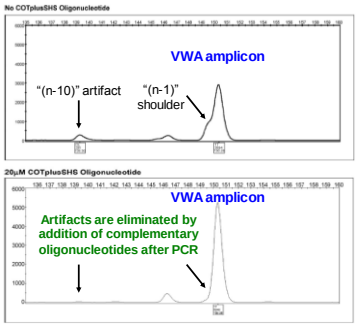
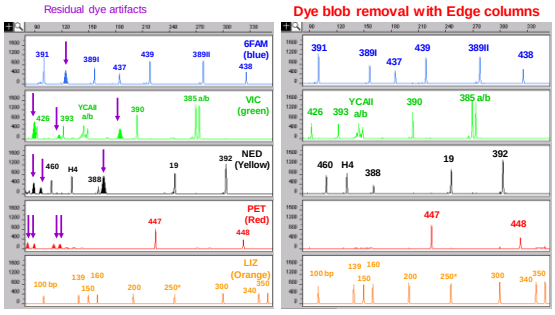
Several laboratories have reported the occurrence of a split or n -1 peak at the vWA locus in PowerPlex 16… The root cause of this artifact is post-PCR reannealing of the unlabeled, unincorporated vWA primer to the 30-end of the tetramethylrhodamine (TMR)-labeled strand of the vWA amplicon. **This reannealing 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



From Figure 5
McLaren *et al.* (2008) *Forensic Science International: Genetics* 2: 257–273

Dye Blobs and their Removal

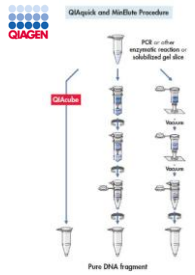


NIST Y-STR 20plex assay

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

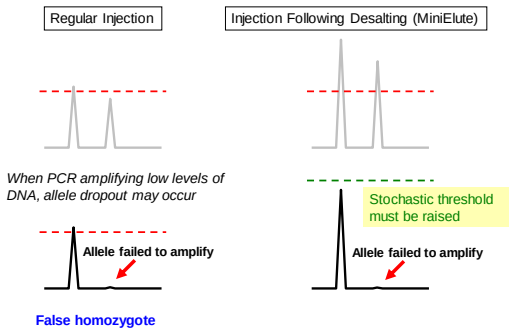
Why MiniElute increases peak heights

- QIAGEN MiniElute **reduces salt levels in samples causing more DNA to be injected**
- **Requires setting a higher stochastic threshold** to account for the increased sensitivity



Smith, P.J. and Ballantyne, J. (2007) Simplified low-copy-number DNA analysis by post-PCR purification. *J. Forensic Sci.* 52: 820-829

Stochastic Effects and Thresholds



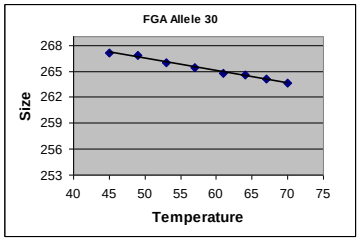
External Factors

- 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

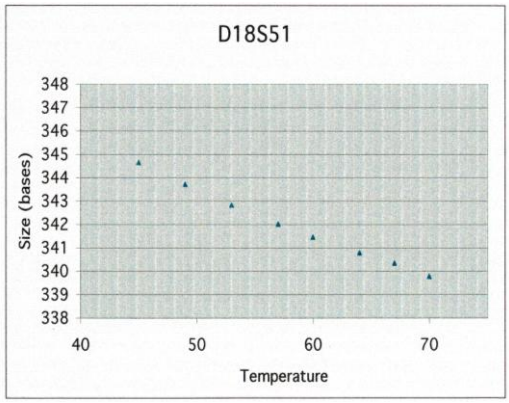
- Viscosity – mobility shift
  - $\mu_{ep} = q/6\pi\eta r$
- Diffusion – band broadening
  - ← DNA →
- Conformation – DNA size based sieving
  - vs $\mu_{ep} = q/6\pi\eta r$
- Current – Power
  - $P = VI = I^2R$
  - Increased current → internal temperature rise→ diffusion → band broadening

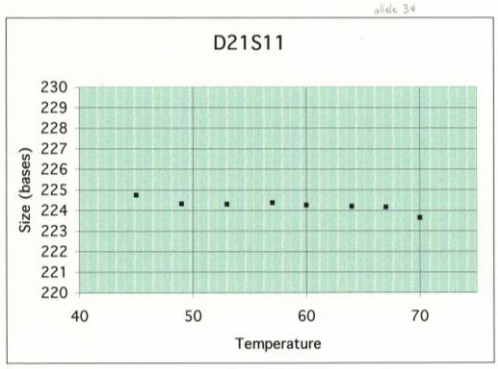
Effect of temperature on allele size



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)

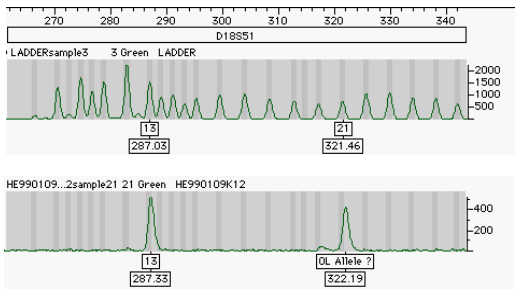
Hartzell, B., et al. (2003). Response of short tandem repeat systems to temperature and sizing methods. *Forensic Science International, 133,* 228-234.



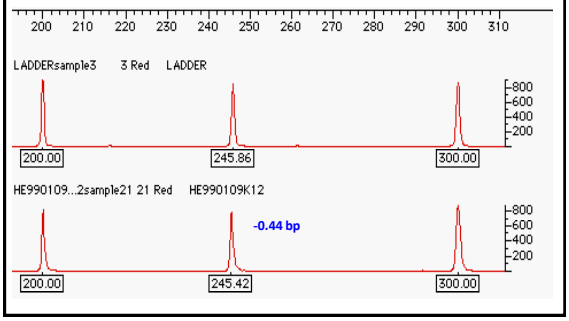


Temperature Effects

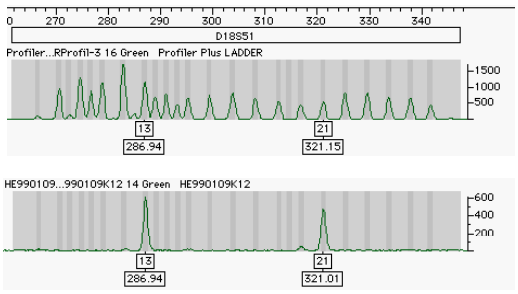
Off-Ladder "OL Alleles"



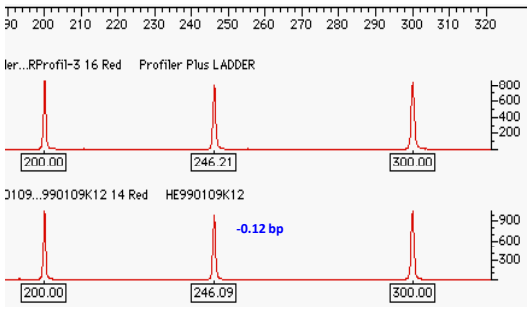
"OL alleles " - look at the 250 peak



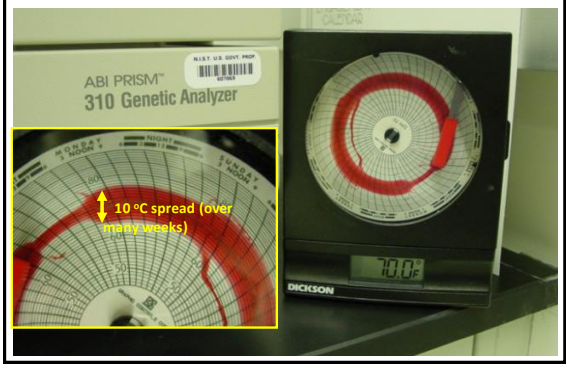
"OL allele re-injected"



And the 250 peak...



Monitoring Room Temperature Over Time



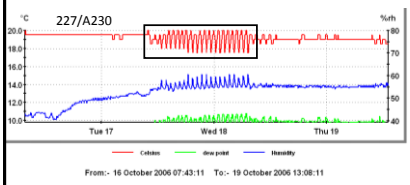
Refrigerator and freezer monitoring

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)

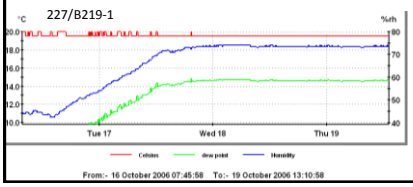
Room temperature monitoring

Monitoring Instrument Room Temperature Fluctuations

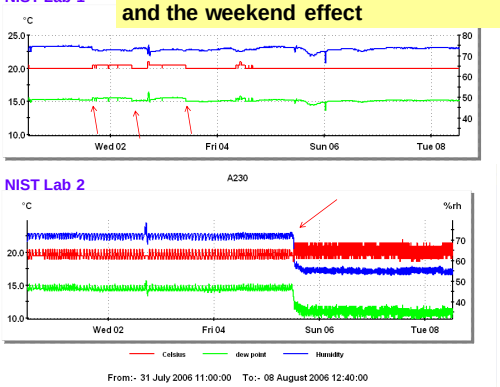


Temperature Monitoring of two separate instrument rooms.

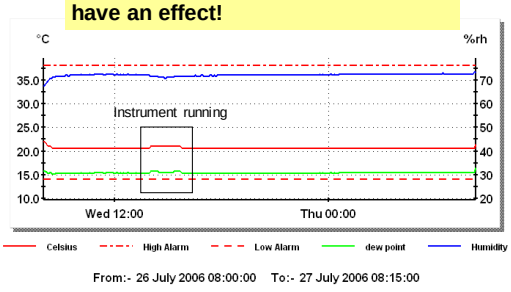
Box area is a 24 hour period where temperature control is not stable.



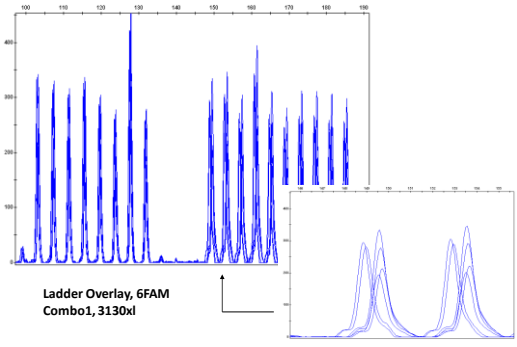
Watch out for the 5 o'clock effect and the weekend effect



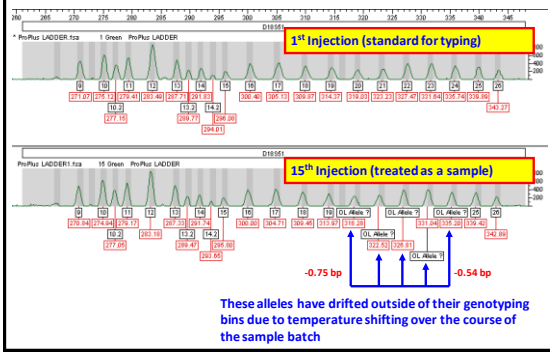
Nearby instruments – thermal cyclers, other sequencers can also have an effect!



Poor Temperature Control
Causes DNA Sizing Imprecision



Use of Second Allelic Ladder to Monitor Potential Match Criteria Problems



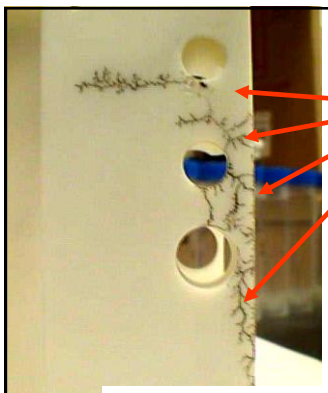
What to do if calibration is lost?

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 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



Keep Your System Clean!

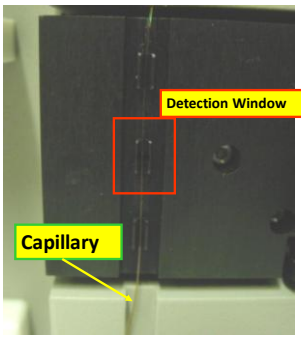
Instrumental Factors

- 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)

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:
  $I_f = I_0 k \varepsilon b C \varphi$ - 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.**

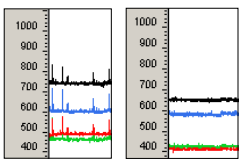
The Detection Window



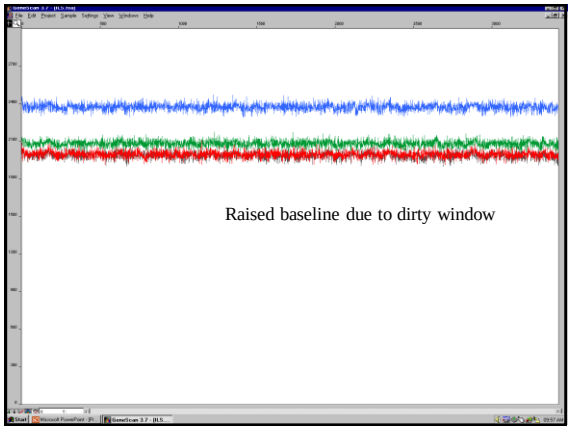
Make sure that the capillary window is lined up (if it is not, then no peaks will be seen)

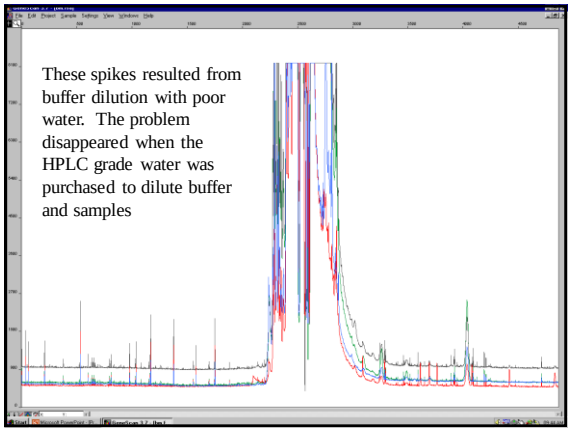
Window may need to be cleaned with ethanol or methanol

Review Start of Raw Data Collection



Little spikes indicate need to change buffer… check current

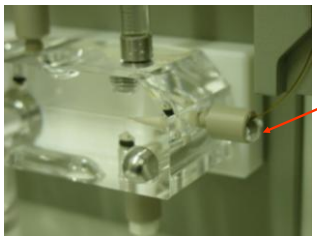




Buffer Issues

- 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

Beware of Urea Crystals



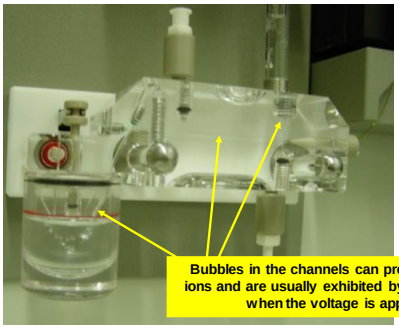
Urea crystals have formed due to a small leak where the capillary comes into the pump block

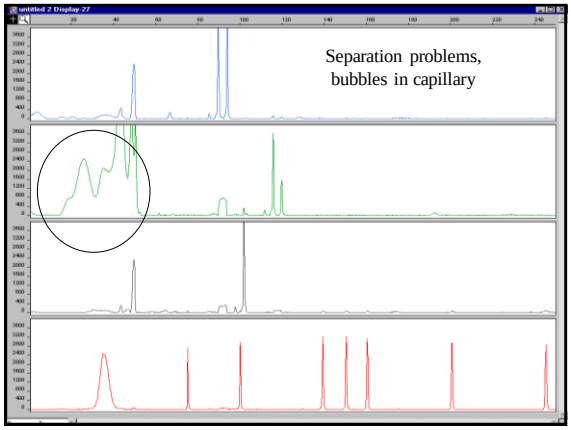
Urea sublimates and can evaporate to appear elsewhere

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

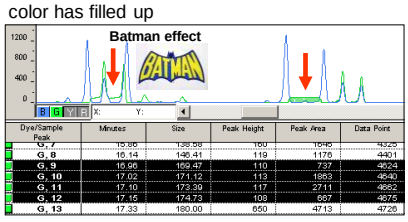
Remove all bubbles from the channels





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



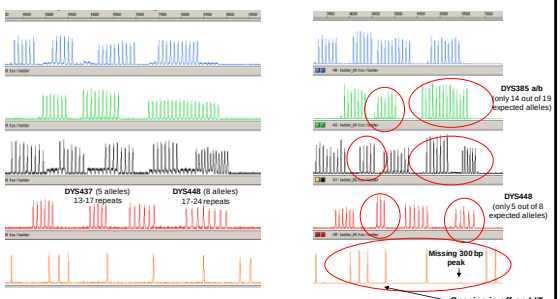
Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
G, 7	15.86	139.58	160	1646	4325
G, 8	16.14	146.41	119	1176	4401
G, 9	16.96	169.47	110	737	4624
G, 10	17.02	171.12	113	1983	4640
G, 11	17.10	173.39	117	2711	4662
G, 12	17.15	174.73	108	667	4675
G, 13	17.33	180.00	650	4713	4726

"Data gap" - phenomenon

STRs

Y-Filer Allelic Ladder (correct)

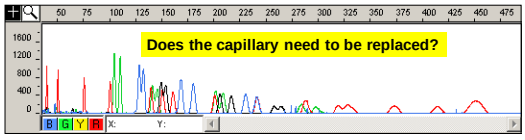
Y-Filer Allelic Ladder (with gaps)



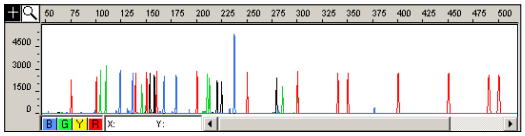
Data from Walter Parson's Lab (Innsbruck, Austria)

What we call "melt downs"…

probably due to an incompletely filled capillary

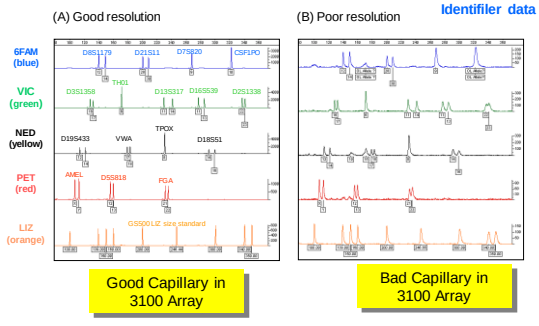


No! The next injection looks fine…



ABI 310 Data from Margaret Kline (NIST)

Capillary Meltdowns

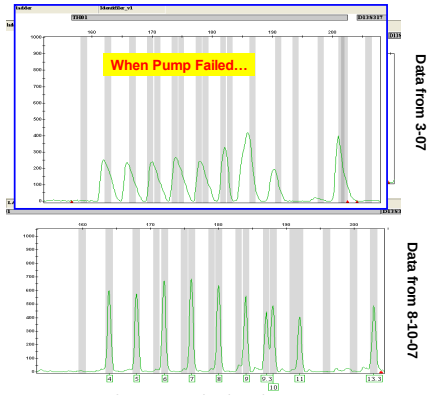


Butler, J.M., Buel, E., Crivellente, F., McCord, B.R. (2004) Forensic DNA typing by capillary electrophoresis: using the ABI Prism 310 and 3100 Genetic Analyzers for STR analysis. Electrophoresis 25:1397-1412

3130xl before pump failure

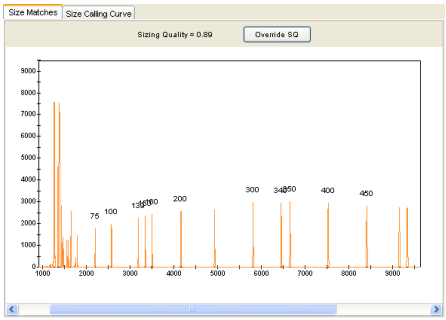
TH01 allelic ladder

3130xl after pump change

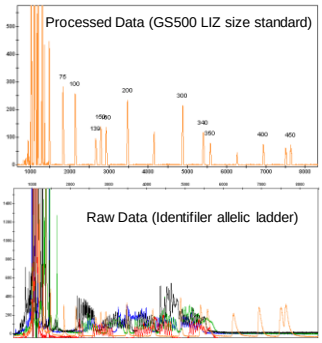


Data from Amy Decker (NIST)

The Size Standard Provides an Excellent Indicator of Performance on Every Sample



Examine the Size Standard…



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

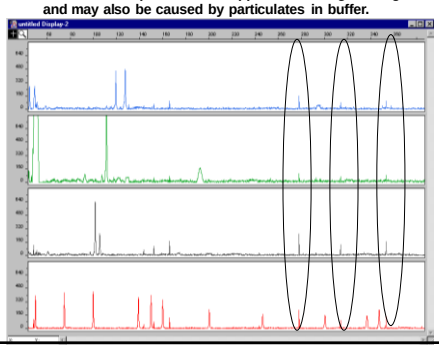
Maintenance of ABI 310/3100/3130

- 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!

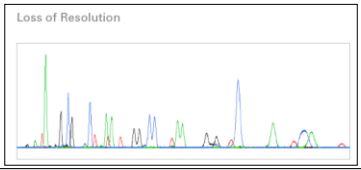
Current Spikes

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.



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



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:

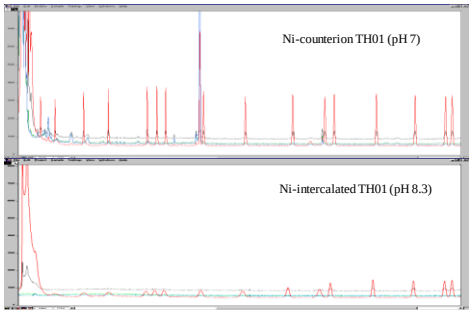
- Poor water quality
- Poor quality system reagents
- Insufficient capillary filling
  - Leak in the system fittings
- Air in the system
  - Bubbles
- Impurities
  - Protein, salts
  - Detergents
- Poor/exhausted array
- Poor instrument maintenance

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.

http://marketing.appliedbiosystems.com/images/forensic/volume11/docs/52808_FN_FAS_r3.pdf

Metal Ions in the Sample

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



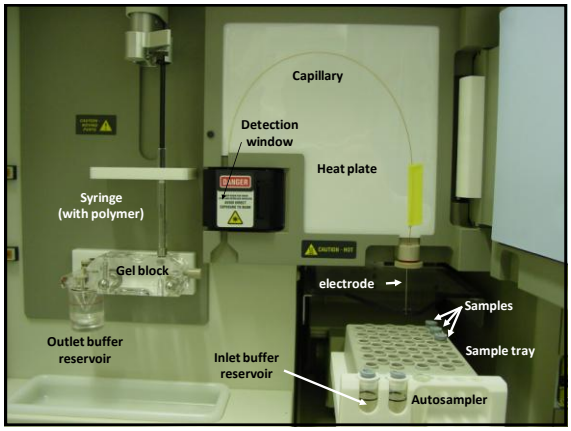
1 µl TH01 added to 10 µl of 3.0 mM $NiCl_2$ in 10 mM Tris, pH 7 or pH 8.3. Sample allowed to interact for 1 hr and then 1 µl added to ROX/formamide.

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

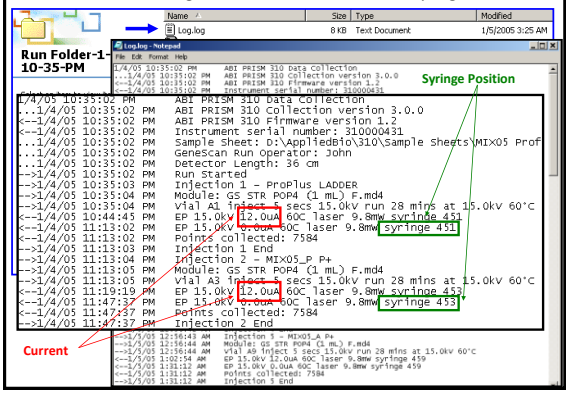
- V/I = R where R is a function of capillary diameter, [buffer], and buffer viscosity
- In a CE system the voltage is fixed, thus changes in resistance in the capillary will be reflected in the current observed
- Air bubbles, syringe leaks, alternate paths to ground, changes in temperature, changes in 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)

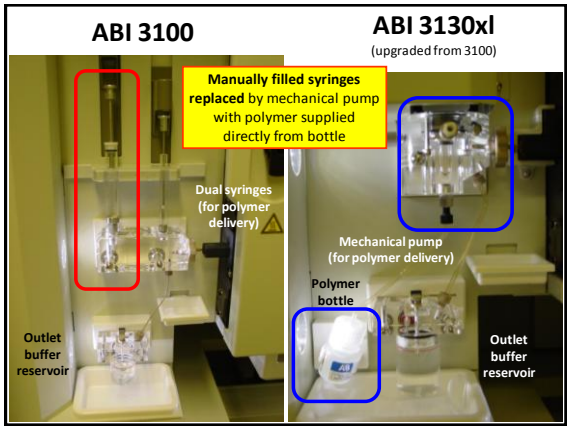


Syringe Travel

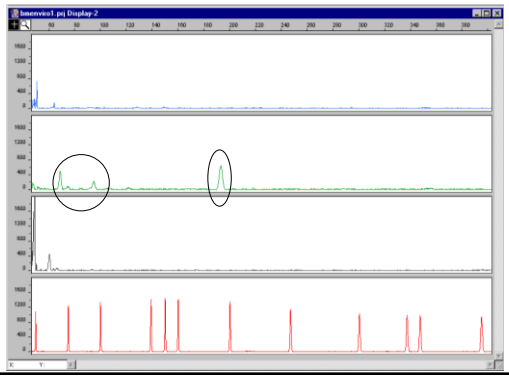
- The ABI 310 instrument also keeps track of the position of the syringe (in the log file)
- Depending on the resistance to flow, the syringe will travel different lengths
- Syringe leaks may be reflected in a longer distance traveled prior to each injection
- These leaks occur around the barrel of the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

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





Dye Blobs in the Negative Control Sample



Measuring Formamide Conductivity

(not this way)

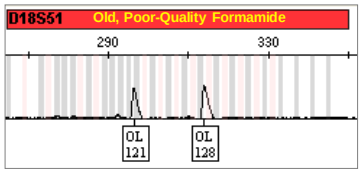
The key is to measure the bottle when it comes in or buy the good stuff and immediately pipette it out into small tubes with or without ROX already added. Then freeze the tubes.

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

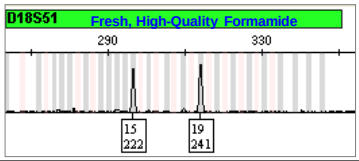
Comments on Sample Preparation

- Use high quality formamide (<100 μS/cm)
- Denaturation with heating and snap cooling is not needed (although most labs still do it…)
- **Post-PCR purification reduces salt levels** and leads to more DNA injected onto the capillary

Importance of Sample Preparation



Peak tailing and wider peaks indicative of an injection with a salty sample



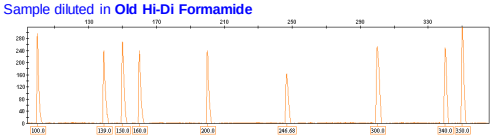
Same DNA sample prepared in fresh, high-quality formamide

Data from Amy Decker (NIST, now AFDIL)

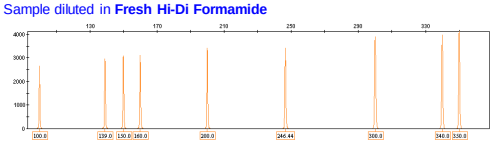
Troubleshooting Hint: **Examine Internal Size Standard**

Comparison of GS500 LIZ Size Standard

Sample diluted in **Old Hi-Di Formamide**



Sample diluted in **Fresh Hi-Di Formamide**



Data from Amy Decker (NIST, now AFDIL)

ABI Solution to Polymer Problem

The preliminary results of our investigation suggest that recent lots of 31xx POP4 polymer may be contributing to some of the reported incidents and, as such, additional efforts have focused on polymer as a potential root cause. While our root cause investigation is still on-going, a cross-functional team has been established to review all polymer testing data. The team meets regularly and is actively looking into aspects of polymer manufacturing and the relationship of polymer to other consumables that may impact data quality.

As a proactive effort, the rate of polymer production has been increased in order to help meet customer demand and all recently released POP4 polymer has been subjected to additional functional testing to ensure polymer performance. We have confirmed that all released lots of POP4 polymer have passed internal quality control testing.

We are also pleased to inform you that most reported incidents have been successfully resolved through the efforts of the local support teams utilizing the following procedure:

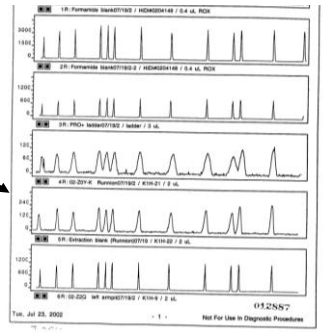
- Flush the system 10 to 15 times with warm (40°C) deionized system water wash prior to array/polymer replacement. Using a high purity bottled water source may help to eliminate water as a potential contributing factor.
- The warm water wash should be followed immediately by replacement of the capillary array and consumables lots (e.g. polymer, buffer and water) as advised by your Applied Biosystems Field Applications Specialist.
- In extreme cases, replacement of the lower block or front end may also be required to recover performance.

The most recent reports are specific to low quality data on the 3100 instrument platform. Internal testing indicates that packaging is contributing to this low quality data. We are focusing our efforts on variation in packaging between our different bottle configurations. To help minimize packaging variation we are evaluating an alternative bottle plastic for 3100 POP4, which will be more similar to the 3130 POP4 bottle configuration. We are also closely monitoring shipping conditions to determine any potential impacts from shipping.

Effect of contaminant in reference sample

Contamination results in problems in subsequent analyses

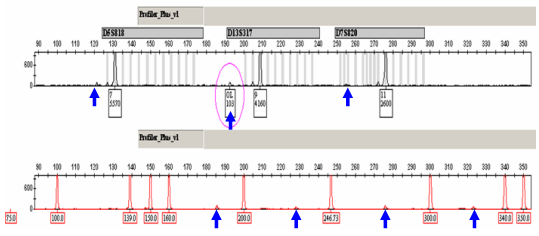
Effect is transitory



Data from Bruce McCord (Florida International University)

Sample Renaturation (minor dsDNA peaks running in front of primary ssDNA STR alleles)

ROX Artifacts
Comparison Casework Blood Sample



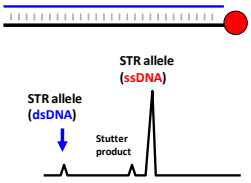
Data from Peggy Philion (RCMP)

Why dsDNA migrates through CE capillary faster than ssDNA…

- DNA molecule separation depends on interactions with the polymer
  - Higher polymer concentration (or longer polymer molecules) permits more polymer interactions and provides better resolution (i.e., POP-6 vs POP-4)
- **Single-stranded DNA (ssDNA) is more flexible than double-stranded DNA (dsDNA)** and therefore moves more slowly through the capillary because it is interacting with polymer strands more

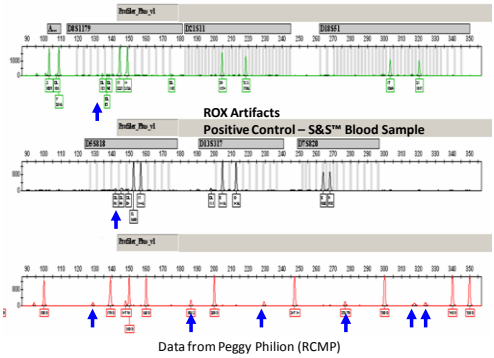
dsDNA vs ssDNA CE Migration

- If a small amount of the complementary strand re-hybridizes to the labeled STR allele strand, then a little peak will be seen in-front of each internal lane standard peak and



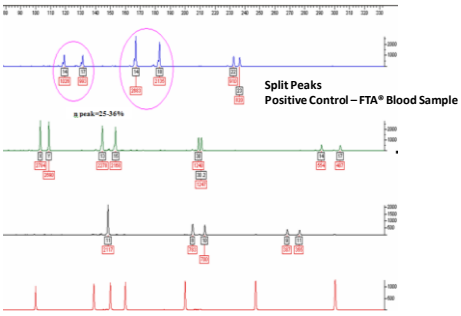
- Height of dsDNA peak will depend on amount of re-hybridization between the two strands (**some loci will re-hybridize more readily giving rise to larger dsDNA peaks**)
- Local temperature environment of capillary impacts amount of re-hybridization (may change over time)

More Sample Renaturation

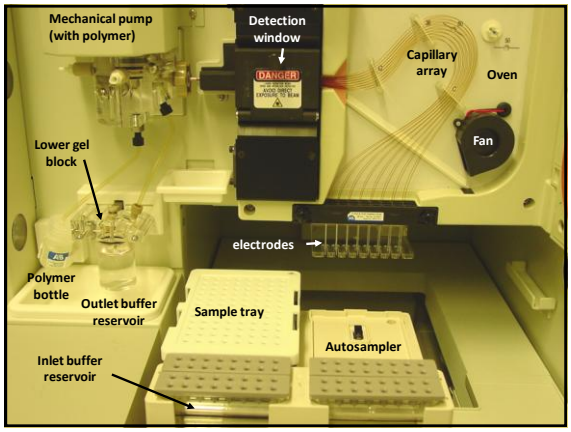


Data from Peggy Philion (RCMP)

Split Peaks (amplification reagents starting to go bad – dNTPs, polymerase, etc.)



Data from Peggy Philion (RCMP)



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

Thank you for your attention

Acknowledgments: NIJ & FBI Funding

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Our team publications and presentations are available at:
http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm