Advanced Topics in Forensic DNA Analysis

CE Troubleshooting

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Questions?

• What are your biggest challenges with keeping your ABI 310/3100/3130 running?

• What kind of signal intensity variation are you seeing between your different instruments?

• Have anyone seen uneven injection across a sample plate? (We believe this to be an autosampler calibration issue…e.g., position G10 or H12 does not inject properly)

Bruce McCord’s Profiles in DNA Article

Troubleshooting Capillary Electrophoresis Systems

By Bruce McCord
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INTRODUCTION
The development of capillary electrophoresis (CE) has opened a new role in bringing about the modern application of DNA forensic analysis. Forensic laboratories are the forefront of this new technology, but many analysts are not fully aware of the existing potentials of the CE system. This article attempts to address the technical issues in CE separation and detection, which are the key to producing pure DNA separation and detection of each allele.

SEPARATION
DNA analysis is performed using ntds@caltechbuffer (Page 2). These buffers can be easily obtained from a number of DNA separation and detection is the key to producing pure DNA separation and detection of each allele.
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
Non-degraded Positive Control
20 pg/µL (0.250 ng/12.5 µL)

Degraded Bone Sample
20 pg/µL (0.250 ng/12.5 µL)

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.

http://www.cstl.nist.gov/biotech/strbase/training.htm
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

Sample Problem?.
Check ROX, looks OK

http://www.cstl.nist.gov/biotech/strbase/training.htm
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.

Dye Blobs and their Removal
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
  \[ \mu_{ep} = \frac{q}{6\pi \eta r} \]

- Diffusion – band broadening
  \[ D \rightarrow \text{DNA} \rightarrow \]

- Conformation – DNA size based sieving
  \[ \text{vs} \: \mu_{ep} = \frac{q}{6\pi \eta r} \]

- Current – Power
  \[ P = VI = IR \]
  - Increased current \( \Rightarrow \) internal temperature rise \( \Rightarrow \) diffusion \( \Rightarrow \) 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**

**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*
Temperature Effects:

“OL” Alleles
"OL Alleles"

- OL allele re-injected

"OL alleles" - look at the 250 peak

http://www.cstl.nist.gov/biotech/strbase/training.htm
And the 250 peak...

True off-ladder alleles

True off-ladder allele: the 250 peak
Monitoring Room Temperature Over Time

Temperature Probes

Refrigerator and freezer monitoring

Room Monitors, # DT-23039-52 – USB Temperature-Humidity Datalogger $91.00 (Cole Parmer, Vernon Hills IL)

Temperature Monitoring of two separate instrument rooms.

Box area is a 24 hour period where temperature control is not stable.
Poor Temperature Control Causes DNA Sizing Imprecision

Ladder Overlay, 6FAM Combi-1, 3130xl

Freezer Monitoring

Refrigerator Monitoring

Sample Inventory
Use of Second Allelic Ladder to Monitor Potential Match Criteria Problems

1st Injection (standard for typing)

15th Injection (treated as a sample)

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

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
Carbon Trails
High Humidity or wet buffer vials can create other paths to ground
Keep Your System Clean!

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

Laser (488nm) → Charged coupled device (CCD) → Grating

http://www.cstl.nist.gov/biotech/strbase/training.htm
Issues with the Optical System

- Pay attention to signal to noise, not absolute peak intensity
- Argon Ion lasers outgas and eventually lose intensity; *take note of laser current and monitor it over time*
- Fluorescence expression:
  \[ I(t) = I_0 \varepsilon_b C \phi \]
  - 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
- Little spikes indicate need to change buffer... check current
- Raised baseline due to dirty window

http://www.cstl.nist.gov/biotech/strbase/training.htm
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.

Separation problems, bubbles in capillary

These spikes resulted from buffer dilution with poor water. The problem disappeared when the HPLC grade water was purchased to dilute buffer and samples.
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

- 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.

http://www.cstl.nist.gov/biotech/strbase/training.htm
Capillary Meltdowns

(A) Good resolution

(B) Poor resolution

Meltdowns can be permanent or transitory
as we have seen these may result from sample contamination effects

Does the capillary need to be replaced?

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 NiCl2 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 NiCl2 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

http://www.cstl.nist.gov/biotech/strbase/training.htm
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.
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

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

Multiplex_QA Article Published

Multiplex_QA: An exploratory quality assessment tool for multiplexed electrophoretic assays

Multiplex_QA is a data analysis tool for visualizing short- and long-term changes in the performance of multiplexed electrophoresis devices, and includes a comprehensive audit trail and report that can be saved to your local hard drive. It is useful for monitoring, analyzing, and visualizing the quality and performance of your electrophoresis devices, and includes a wide range of quality metrics that can be used to evaluate the performance of your devices over time. The tool is designed to be user-friendly, intuitive, and easy to use, and includes a comprehensive set of documentation and training materials.

Available for download from STRBase:
http://www.cstl.nist.gov/biotech/strbase/software.htm

User manual (127 pages) available for download from STRBase

Multiplex_QA Overview

- Research tool that provides quality metrics to review instrument performance over time (e.g., examines resolution and sensitivity using internal size standard peaks)
- Runs with Microsoft Excel macros. Requires STR data to be converted with NCI’s BatchExtract program into numerical form.

Available for download from STRBase:
http://www.cstl.nist.gov/biotech/strbase/software.htm

http://www.cstl.nist.gov/biotech/strbase/training.htm