Fundamentals of Capillary Electrophoresis

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Presentation Outline

- History and background on CE
- Fundamentals of CE
  - sample prep, injection, separation, detection

BREAK

- ABI 3500
- Troubleshooting strategies and solutions
- Questions

My Goal:
To help you understand the basic chemistry behind DNA separations and to help make CE instruments less of a "black box"

Steps in DNA Analysis

Usually 1-2 day process (a minimum of ~5 hours)

Collection
Specimen Storage
Extraction
Quantitation
Multiplex PCR
STR Typing
Interpretation of Results
Database Storage & Searching
Calculation of Match Probability

Biology
DNA Extraction Quantitation
Multiplex PCR Amplification
DNA Database Search
DNA separation and sizing

Technology
STR Typing

Steps Involved

In 2003 at age 75
With first fully automated capillary free zone electrophoresis apparatus in 1967

Stellan Hjertén
Uppsala University (Sweden)

1967
First high voltage CE system (with rotating 3 mm i.d. capillaries)

1981
First "modern" CE experiments (with 75 µm i.d. capillaries)

1988/90
First DNA separations in a capillary (gel-filled sieving polymer)

James Jorgenson
University of North Carolina

Barry Karger
Northeastern University

Stellan Hjertén
Received his PhD (1967) under Professor Arne Tiselius who had developed moving boundary zone electrophoresis in 1937 (Noble Prize in 1948)

http://www.rsc.org/delivery/_ArticleLinking/DisplayArticleForFree.cfm?doi=b307798p&JournalCode=AN

http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm
A Brief History of Capillary Electrophoresis

- 1937 – Tiselius develops moving boundary electrophoresis
- 1967 – Hjertén uses rotating 3 mm i.d. tubes for CE
- 1981 – Jorgenson and Lukacs demonstrate first high performance CE separations with 75 µm i.d. capillary
- 1988 – Karger’s group shows DNA separations of single stranded oligonucleotides with gel-filled capillaries
- 1990 – Karger’s group shows DNA separations with sieving polymers on DNA restriction fragments
- 1991 – Grossman expands work with sieving polymers
- 1992 – Bruce McCord starts working on PCR product separations with STR allele ladders

My Experience with CE, STRs, etc.

- May 1993 – began working in Bruce McCord’s lab at Quantico
- Sept 1993 – developed mtDNA amplicon quantitation method (used in FBI casework from 1996 to present)
- Nov 1993 – first demonstration of STR typing by CE (using dual internal standards and TH01 ladder)
- July 1995 – defended Ph.D. dissertation entitled “Sizing and Quantitation of Polymerase Chain Reaction Products by Capillary Electrophoresis for Use in DNA Typing”
- July 1995 – ABI 310 Genetic Analyzer was released

First Rapid STR Typing with Capillary Electrophoresis

Single color detection with dual internal size standards
Butler et al. (1994) BioTechniques 17: 1062-1070

Technology Implementation Takes Time – the FBI did not start running casework samples using STRs and CE until January 1999

Why Use CE for DNA Analysis?

1. Injection, separation, and detection are automated.
2. Rapid separations are possible
3. Excellent sensitivity and resolution
4. The time at which any band elutes is precisely determined
5. Peak information is automatically stored for easy retrieval

http://www.cstl.nist.gov/strbase/NISTpub.htm
Important Differences Between CE and Gels

- **Room temperature control** is essential for run-to-run precision
  - CE uses sequential rather than simultaneous separations
  - Usually need < 2.0°C (must inject allelic ladder regularly)

- **Lower amount of DNA loaded** (injection = nL vs µL) and thus detection sensitivity must be better

- Electrokinetic injection enables **dye artifacts** (blobs) to enter the capillary or microchip CE channel and thus possibly interfere with STR allele interpretation

More Differences between CE and Gels...

- Filling the capillary (or microchip CE channel) is analogous to pouring a gel into a tiny tube...

- Must be more clean around a CE system
  - Because the capillaries (µCE channels) are small, particles of dust or urea crystals can easily plug them
  - Tips of capillary cannot dry out (once buffer solutions have been run through them) for the same reasons

- Bubbles are a BIG problem in CE as they can easily block current flow in the capillary...

Capillary Electrophoresis (CE)

<table>
<thead>
<tr>
<th>ABI Genetic Analyzer</th>
<th>Years Released for Human ID</th>
<th>Number of Capillaries</th>
<th>Laser</th>
<th>Polymer delivery</th>
<th>Other features</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABI 310</td>
<td>1995–1996 Mac A230, now B233</td>
<td>1</td>
<td>50 mW Ar+ (488/514 nm) syringe</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>ABI 3100</td>
<td>2001-2002 as ABI 3100</td>
<td>8</td>
<td>50 mW Ar+ (488/514 nm) syringe</td>
<td>-</td>
<td></td>
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<tr>
<td>ABI 3500</td>
<td>2010-2012</td>
<td>8</td>
<td>100 mW Ar+ (488/514 nm) syringe</td>
<td>-</td>
<td></td>
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<tr>
<td>ABI 3500xl</td>
<td>2010-2012</td>
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<td>100 mW Ar+ (488/514 nm) syringe</td>
<td>-</td>
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</tr>
<tr>
<td>ABI 3700</td>
<td>2002-2003</td>
<td>8</td>
<td>50 mW Ar+ (488/514 nm) syringe</td>
<td>-</td>
<td>Split laser technology</td>
</tr>
</tbody>
</table>

Information courtesy of Michelle S. Shepherd, Applied Biosystems, LIFE Technologies.

Typical Instruments Used for STR Typing

<table>
<thead>
<tr>
<th>ABI 310</th>
<th>GeneAmp 9700</th>
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<tbody>
<tr>
<td>Thermal Cycler for PCR Amplification</td>
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Capillary electrophoresis instruments for separating and sizing PCR products

- **Single capillary**
- **16-capillary array**
DNA Samples Run at NIST

we have processed >100,000 samples (from 1996-present)

- **STR kits**
  - Identifiler, PP16, PP16HS, Identifiler Plus, Identifiler Direct, Profiler Plus, Cotfler, SGM Plus, ESI/ESX 17, SE33 monoplex

- **Research & development on new assays**
  - **STRs**: Y-STR 20plex, MeowPlex, miniSTRs, 26plex
  - **SNPs**: SNaPshot assays: mtDNA (one 10plex), Y-SNPs (four 6plexes), Orchid SNPs (twelve 6plexes), ancestry SNPs (two 12plexes), SNPforID (one 29plex), SNPplex (one 48plex)

- **DNA sequencing**
  - Variant allele sequencing

We have a unique breadth and depth of experience with these instruments...

### Analytical Requirements for STR Typing


- Fluorescent dyes must be **spectrally resolved** in order to distinguish different dye labels on PCR products
- PCR products must be **spatially resolved** – desirable to have single base resolution out to >350 bp in order to distinguish variant alleles
- High **run-to-run precision** – an internal sizing standard is used to calibrate each run in order to compare data over time

### Steps in STR Typing with ABI 310/3100

- **Sample Preparation**
  - ABI Prism spectrograph
  - Fixed laser, moving capillaries
  - Mixture of dye-labeled PCR products from multiplex PCR reaction

- **Sample Injection**
  - Capillary filled with polymer solution
  - Sample stacking

- **Detection**
  - Fluorescent dyes with excitation and emission traits
  - CCD with defined virtual filters produced by assigning certain pixels

### Process Involved in 310/3100 Analysis

- **Separation**
  - Capillary – 50μm fused silica, 43 cm length (36 cm to detector)
  - POP-4 polymer – Polydimethyl acrylamide
  - Buffer - TAPS pH 8.0
  - Denaturants – urea, pyrrolidinone

- **Injection**
  - Electrophoretic injection process (formamide, water)
  - Importance of sample stacking

- **Detection**
  - Fluorescent dyes with excitation and emission traits
  - CCD with defined virtual filters produced by assigning certain pixels

### Detection with Multiple Capillaries (Irradiation for Capillary Arrays)

- ABI 3100, 3130, 3100 Avant
- Sheath flow detection
- Fixed laser, moving capillaries

### Review Article on STRs and CE

Ohm’s Law

- \( V = IR \) (where \( V \) is voltage, \( I \) is current, and \( R \) is resistance)
- Current, or the flow of ions, is what matters most in electrophoresis
- CE currents are much lower than gels because of a higher resistance in the narrow capillary
- CE can run a higher voltage because the capillary offers a higher surface area-to-volume ratio and can thus dissipate heat better from the ion flow (current)

DNA and Electrophoresis

“From a practical point of view it is disappointing that electrophoresis cannot be used to fractionate or analyze DNA’s on the basis of size” Olivera, Biopolymers 1964, 2, 245

\[ \mu_{ep} = \frac{q}{6\pi \eta r} \]  
small ions with high charge move fastest

As size increases so does charge!

Separation Issues

- Electrophoresis buffer --
  - Urea for denaturing and viscosity
  - Buffer for consistent pH
  - Pyrrolidone for denaturing DNA
  - EDTA for stability and chelating metals
- Polymer solution -- POP-4 (but others work also)
- Capillary wall coating -- dynamic coating with polymer
  - Wall charges are masked by methyl acrylamide
- Run temperature -- 60 °C helps reduce secondary structure on DNA and improves precision. (Temperature control affects DNA sizing)

What is in POP-4 and Genetic Analyzer Buffer?

**Improved single-strand DNA sizing accuracy in capillary electrophoresis**

See also Wenz et al. (1998) Genome Research 8: 69-80

**POP-4** (4% poly-dimethylacrylamide, 8 M urea, 5% 2-pyrrolidinone)

POP-4 buffer contains 100 mM TAPS and 1 mM EDTA (adjusted to pH 8.0 with NaOH). TAPS = N-Tрис(hydroxymethyl)methyl-3-aminopropane sulfonic acid
Impact of Capillary Length and Polymer Concentration on DNA Sequencing Resolution

How to Improve Resolution?
1. Lower Field Strength
2. Increase Capillary Length
3. Increase Polymer Concentration
4. Increase Polymer Length

All of these come at a cost of longer separation run times

Injection

Electrokinetic Injection Process

Capillary and Electrode Configurations

Salty samples result in poor injections
Sample Conductivity Impacts Amount Injected

\[
\left[\frac{\text{DNA}_{\text{inj}}}{\text{DNA}_{\text{sample}}}\right] = \frac{E(t^2) (\mu_{\text{ep}} + \mu_{\text{eof}}) [\text{DNA}_{\text{sample}}]}{\lambda_{\text{sample}}} \left[\text{DNA}_{\text{sample}}\right] \left[\lambda_{\text{buffer}}\right]
\]

- \(E\) is the electric field applied
- \(t\) is the injection time
- \(r\) is the radius of the capillary
- \(\mu_{\text{ep}}\) is the mobility of the sample molecules
- \(\mu_{\text{eof}}\) is the electroosmotic mobility

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

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

Steps Performed in Standard Module

See J.M. Butler (2005) Forensic DNA Typing, 2nd Edition; Chapter 14

- Capillary fill – polymer solution is forced into the capillary by applying a force to the syringe
- Pre-electrophoresis – the separation voltage is raised to 10,000 volts and run for 5 minutes
- Water wash of capillary – capillary is dipped several times in deionized water to remove buffer salts that would interfere with the injection process
- Sample injection – the autosampler moves to position A1 (or the next sample in the sample set) and is moved up onto the capillary to perform the injection; a voltage is applied to the sample and a few nanoliters of sample are pulled onto the end of the capillary
- Water wash of capillary – capillary is dipped several times in waste water to remove any contaminating solution adhering to the outside of the capillary
- Water dip – capillary is dipped in clean water (position 2) several times
- Electrophoresis – autosampler moves to inlet buffer vial (position 1) and separation voltage is applied across the capillary; the injected DNA molecules begin separating through the POP-4 polymer solution
- Detection – raw data is collected with no spectral deconvolution of the different dye colors; the matrix is applied during Genescan analysis

Removal of Dye Artifacts Following PCR Amplification

- TH01
- TPOX
- CSF1PO
- FGA
- D21S11
- D7S820

No Filtering (Straight from PCR)

Note higher RFU values due to salt reduction with spin columns

Filtered with Edge columns

Removal of Cl- ions and other buffer ions present in PCR reaction contribute to the sample conductivity and thus will compete with DNA for injection onto the capillary.

http://www.cstl.nist.gov/strbase/NISTpub.htm
Stochastic Effects and Thresholds

When PCR amplifying low levels of DNA, allele dropout may occur.

False homozygote

Detection

Optics for ABI 310

Methods for Fluorescently Labeling DNA

The polymerase chain reaction (PCR) is used to amplify STR regions and label the amplicons with fluorescent dyes using locus-specific primers.
**ABI Fluorescent Dyes Used in Four-Color Detection**

- **FAM** (blue)
- **JOE** (green)
- **TAMRA** (yellow)
- **ROX** (red)

**Fluorescent Emission Spectra for ABI Dyes**

- Laser excitation (488, 514.5 nm)
- NED is a brighter dye than TAMRA

**Importance of Spectral Calibration**

*Before Color Separation*

*After Color Separation*

**Matrix with 4 Dyes on ABI 310**

\[
egin{align*}
I_{520} &= bx + gy + yz + rw, \quad \text{intensity of blue} \\
I_{540} &= bx + gy + yz + rw, \quad \text{intensity of green} \\
I_{560} &= bx + gy + yz + rw, \quad \text{intensity of yellow} \\
I_{580} &= bx + gy + yz + rw, \quad \text{intensity of red}
\end{align*}
\]

Where
- \( b \) is the %blue labeled DNA
- \( g \) is the %green labeled DNA, etc.
- \( x, y, z, w \) are the numbers in the matrix (sensitivity to each color)

If you solve \( xyzw \) for each dye individually

Then you can determine dye contribution for any mixture

http://www.cstl.nist.gov/strbase/NISTpub.htm
Virtual Filters Used in ABI 310

Variable Binning Increases Red Peaks

Comparison of Data Collection Versions

Deciphering Artifacts from the True Alleles

NIST ABI 3100 Analysis Using POP-6 Polymer

Protocols Used for STR Typing

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!
 ABI 3500 Genetic Analyzer

New Features of the ABI 3500 CE
- an improved polymer delivery pump design,
- ready-to-use consumables and containers,
- Radio Frequency Identification (RFID) consumable tracking,
- quality control software features for rapid identification and re-injection of failed samples,
- increased throughput,
- new laser technology,
- reduced power requirements,
- peak height normalization,
- intuitive user software, and integrated primary analysis software,
- improved peak height uniformity across capillaries, runs and instruments
- 6-dye channel capability

• 3500 (8 capillary)
• 3500xl (24 capillary)

Details of the new ABI 3500
No lower pump block (less polymer waste)
Improved sealing for better temperature control (improved precision?)

 ABI 3500 "Dash Board" Data Collection

Tracks the numbers of samples for 'QC purposes'

Primary Differences Between 31xx and 3500

31xx Instruments
- Argon ion (Ar+) lasers with 488/514 nm wavelengths for fluorescence excitation
- 220V power requirement
- Optimal signal intensity 1500-3000 RFU
- Currently validated and operational in most forensic laboratories

3500 Instruments
- Single-line 505 nm, solid-state long-life laser
- Smaller footprint
- 110V power requirement
- Optimal signal intensity can approach 20,000-30,000 RFU
- Normalization of instrument-to-instrument signal variability
  - Ability to increase or decrease overall signal
- Requires the use of GeneMapper IDX v1.2

ABI 3500 Generates Excellent Data

STR typing with a 1:7 mixture using 36 cm array and POP4

DNA sequencing of an SE33 allele using 50 cm array and POP7

http://www.cstl.nist.gov/strbase/NISTpub.htm
Troubleshooting: Strategies and Solutions

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.
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)


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

10 °C spread (over many weeks)

Temperature Probes

Temperature Monitoring of two separate instrument rooms.
Box area is a 24 hour period where temperature control is not stable.

Monitoring Instrument Room Temperature Fluctuations

Poor Temperature Control Causes DNA Sizing Imprecision

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

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)

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

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

Keep Your System Clean!
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

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

Trouble-shooting 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

**ABI 3100**

- Manually filled syringes replaced by mechanical pump with polymer supplied directly from bottle.
- Dual syringes (for polymer delivery).
- Mechanical pump (for polymer delivery).

**ABI 3130xI** (upgraded from 3100)

- Mechanical pump (for polymer delivery).
- Polymer bottle.

Dye Blobs in the Negative Control Sample

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.
Example Problems Seen and Provided by Others

Effect of contaminant in reference sample

Contamination results in problems in subsequent analyses

Effect is transitory

Data from Bruce McCord (Florida International University)

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 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.

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

http://www.cstl.nist.gov/strbase/NISTpub.htm
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)

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