Capillary Electrophoresis in DNA Analysis

*Intro to CE and ABI 310*

DNA Academy Workshop
Albany, NY
June 13-14, 2005
Dr. John M. Butler
Dr. Bruce R. McCord

Outline for Workshop

- Introductions
- STR Analysis
  - Introduction to CE and ABI 310
- Validation and Interlaboratory Studies
- Real-time qPCR and miniSTRs
- Stats and Higher Throughput Approaches
- Y-Chromosome Analysis
- Troubleshooting the ABI 310
- Review and Test

Historical Perspective

- 1928 - Griffith demonstrates DNA carries genetic information
- 1953 - Structure of DNA determined by Watson and Crick
- 1959 - Hjerten describes electrophoresis in tubes
- 1970 - Restriction enzymes begin to be used to cut DNA
- 1981 - Jorgenson performs CZE
- 1984 - Alec Jeffries utilizes restriction fragment digestion of DNA
- 1985 - Kary Mullis describes PCR

There are 23 pairs of Chromosomes

From the Inventor of PCR

"I think I might have been stupid, in some respects, if it weren't for my psychedelic experiences."

"Out of a natural laziness, I always start with the easiest possible protocol and work from there. Better yet, I suggest that someone start from there, and I come back in a month to see how things worked out."

- Kary Mullis, Ph.D., Nobel Prize Laureate, Chemistry, 1993

DNA Analysis by RFLP

http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm
Issues Being Faced in the Mid-1990s

1. RFLP techniques were time consuming and insensitive
2. PCR is rapid and highly sensitive
3. The old results don’t translate
4. Soon all sexual offenders (and other felons) would be required to submit a sample for testing
5. CODIS - Combined DNA Indexing System
6. How will all these samples be run?

Using CODIS Virginia has 788 hits with 180,000 samples analyzed – Prior conviction:

- Burglary/B&E
- Robbery/GL
- Homicide/Murder
- Kidnapping
- Wound/assault
- Misc.

Miscellaneous 19% (97)
Drug Possession 11% (58)
Drug Manufacturing & Distribution 4% (22)
Drug Possession 11% (58)

- Numbers as of October 31, 2001

The Application

Speed and detection capabilities of DNA analyses have improved since the development of the PCR

- Increase in number of complex assays necessitates automated testing procedures

Automated systems are needed to increased sample throughput

Automated systems must be robust and must demonstrate long term stability

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

Components of a Capillary Electrophoresis System

- 36 cm Capillary filled with polymer solution
- Laser
- Detection window
- Inlet Buffer
- Outlet Buffer
- 5-20 kV
- Sample tray moved automatically towards the cathode end of the capillary to allow each sample in automation

Butler, J.M. (2001) Forensic DNA Typing, Figure 9.3, ©Academic Press
Albany DNA Academy Workshop (Butler and McCord)  
June 13-14, 2005

This DNA stuff was powerful and needed to be automated

BUT HOW TO MOVE FROM GELS TO CAPILLARIES?

In the Early 90’s the real question was how to transition from a gel to a capillary

• X-linked acrylamide gel filled capillaries tried first
  – Reusable?
  – Bubble formation
  – Thermal degradation
• Alternative was to not use a gel at all
  – Entangled polymer solutions
  – Refillable
  – Resolution?

Early Work with CE and STRs

• Barry Karger’s group (1988-1990)
  – Utilized gel-filled capillaries to separate ssDNA
  – Introduced sieving polymers in the form of linear polyacrylamide to separate restriction digests
• Commercial CE systems (1992) with lasers appear (Beckman P/ACE)
• Initial STR results at FBI and AFDIL (1992-)
• First STR typing with multi-color CE (and multi-capillary) using dye-labeled primers
• ABI 310 is introduced in July 1995 as the first commercially available multi-color CE

Requirements for Reliable STR Typing

• Reliable sizing over a 75-500 bp size region
• High run-to-run precision between processed samples to permit comparison of allelic ladders to sequentially processed STR samples
• Effective color separations of different dye sets used to avoid bleed through between 4 or 5 different colors
• Resolution of at least 1 bp to >350 bp to permit reliable detection of microvariant alleles

McCord’s Comments on this:

• I want
  – 1bp resolution from 100-350 bp
    • Resolution = \( \frac{2d}{w_1 + w_2} \)
    • Resolution in bp = \( \frac{\text{difference in size}}{R} \)
    – Precision of better than 0.17 bp for any given allele
    • \( 3 \times 0.17 = 0.51 \text{bp} \) 3 standard deviations = 99.7% of data
  – Stable results from the first run to the last run
    • Calibration with one ladder reliable for 48 hours worth of runs
    • No recalibration needed

http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm
Components of ABI 310

- Chemistry
  - STR kits, fluorescent dyes, matrix samples, capillary, buffers, polymer, formamide
- Hardware
  - CCD camera, laser, electrodes, pump block, hot plate for temperature control, autosampler
- Software
  - Data collection, color separation, peak sizing & calling, genotyping, stutter removal

Process Involved in 310 Analysis

- Injection
  - electrokinetic injection process (formamide, water)
  - importance of sample stacking
- Separation
  - Capillary – 50um fused silica, 43 cm
  - POP-4 polymer – Polydimethyl acrylamide
  - Buffer - TAPS pH 8.0
  - Denaturants – urea, pyrolidinone
- Detection
  - fluorescent dyes with excitation and emission traits
  - CCD with defined virtual filters produced by assigning certain pixels

Electrokinetic Injection Process

Amount of DNA injected is inversely proportional to the ionic strength of the solution

Salty samples result in poor injections
Sample Conductivity Impacts Amount Injected

$$[\text{DNA}_{\text{inj}}] = \frac{\text{E}(\pi r^2)}{\lambda_{\text{sample}}} (\mu_{\text{ep}} + \mu_{\text{eof}})[\text{DNA}_{\text{sample}}] (\lambda_{\text{buffer}})$$

- $[\text{DNA}_{\text{inj}}]$ is the amount of sample injected
- 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


Two Major Effects of Sample Stacking

1. Sample is preconcentrated. Effect is inversely proportional to ionic strength
2. Sample is focused. Ions stop moving in low electric field
3. Mobility of sample = $\mu_{\text{ep}} = \text{velocity/l field}$

Typical Sample Preparation for ssDNA

1. Perform PCR with dye-labeled primers
2. Dilute 1 µL PCR product with 24 µL deionized formamide; add 1 µL ROX-labeled internal sizing standard
3. Denature 2 minutes at 95 °C with thermocycler
4. Cool to 4 °C in thermocycler or ice bath
5. Sample will remain denatured for at least 3 days

Injection Study

Evaluate the effects of sample injection on electrophoretic separations by CE.

- different solvents (water and formamide of varying purity);
- different concentration of the sample;
- addition of salts;
- sample stacking

Amount of Sample Injected onto ABI 310 Depends on Sample Ion Concentration

Quality of Formamide Affects Sensitivity

Measured Sample Conductance (Different Formamides)

Effect of Formamide on Peak Resolution and Sensitivity (GS500 ROX Internal Standard)

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Resolution</th>
<th>Peak Height</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>1.19+/1</td>
<td>2700 +/-</td>
</tr>
<tr>
<td>Formamide (27 µS)</td>
<td>1.15 +/-</td>
<td>2960 +/-</td>
</tr>
<tr>
<td>Formamide (360 µS)</td>
<td>1.20 +/-</td>
<td>879 +/-</td>
</tr>
<tr>
<td>Formamide (1000 µS)</td>
<td>1.20 +/-</td>
<td>290 +/-</td>
</tr>
</tbody>
</table>

Increasing the ratio of sample/formamide improves sensitivity only in poor quality formamide

Increasing salt concentration causes reannealing

Comments on Sample Preparation

- Use high quality formamide (<100 µS/cm)!
  - ABI sells Hi-Di formamide
  - regular formamide can be made more pure with ion exchange resin
- Deionized water vs. formamide
  - water works fine but samples are not stable as long as with formamide; water also evaporates over time...
- Denaturation with heating and snap cooling
  - use a thermal cycler for heating and cold aluminum block for snap cooling
  - heat/cool denaturation step is necessary only if water is substituted for formamide...

Separation
ELECTROPHORESIS THEORY

“Ok here’s my recipe idea called the electric pickle. Attach the hot lead to a screw and shove it in. The neutral lead goes in the other end. Turn out the lights and plug it in. It glows and sizzles. The juicy ones work best.”

www.voltnet.com/cook

\[ P = VI = I^2R \]  
Pickles cook

\[ \nu_{ep} = \mu_{ep}V \]  
Ions move through pickle faster at high voltage

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

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

\[ \mu_{ep} = q/6\pi\eta r \]

As size increases so does charge!

How to Sieve DNA in a Capillary?

• Cross linked gels
  – The familiar approach
  – High resolution, but not so reusable
• Entangled Linear polymers
  – Refillable, reusable
  – Lower Resolution
  – Osmotic Flow

Inside a CE Capillary

Which Factors Affect Resolution and Migration of DNA Fragments?

Variables

• pH – osmotic flow and DNA resolution
• Buffer concentration/ionic strength – osmotic flow and current/joule heating
• Polymer concentration – wall coating and DNA resolution
• Capillary diameter – resolution
• Polymer type – wall coating
• Field strength – resolution, run time, current, EOF
• Temperature- DNA conformation

Separation Mechanism

Electrophoretic flow

Capillary Wall \( \text{SiOH} \leftrightarrow \text{SiO}^- + \text{H}^+ \)

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

Albany DNA Academy Workshop (Butler and McCord)  
June 13-14, 2005
Solvated ions drag solution towards cathode in a flat flow profile.

Issues with CE separations

- Big problem – electroosmotic flow

Polyethylene oxide separation of pBR 322 HAE III digest (EOF present)

Polydimethyl Acrylamide (POP) separation of DNA (EOF not present)

Capillary Coating

- Removes effect of charged sites - eliminates EOF, sample adsorption

Dynamic coating of charged sites on fused silica capillary is accomplished with POP-4 polymer

Hydroxyethyl Cellulose (HEC)

- TBE, CAPS 7M Urea

Polydimethyl Acrylamide (POP)

- TAPS pH 8.0 8M Urea

So what are sieving buffers?

- They are gels - very similar to polyacrylamide
- They are not gels - they flow

Actually these are known as entangled linear polymers and there are many common applications

Entangled Polymer Solutions

- Polymers are not cross-linked (above entanglement threshold)
- “Gel” is not attached to the capillary wall
- Pumpable -- can be replaced after each run
- Polymer length and concentration determine the separation characteristics

- Examples:
  - 1% HEC (hydroxyethyl cellulose)
  - 4% linear polyacrylamide
  - POP-4 and POP-6
What is in POP-4 and Genetic Analyzer Buffer?

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

Running buffer contains 100 mM TAPS and 1 mM EDTA (adjusted to pH 8.0 with NaOH)

TAPS = N-Tris-(hydroxymethyl)methyl-3-aminopropane-sulfonic acid

US Patent 5,552,028 covers POP-4 synthesis

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

Transient Pores are Formed above the Entanglement Threshold

C < C*  C = C*  C > C*

Large DNA undergoing Electrophoresis in an HEC sieving buffer (confocal microscopy)

Oxazole yellow dimer labeled

Michael Morris - U Michigan

Effect of Polymer Concentration on Resolution

D1S80 725bp

HEC M_w = 150,000 amu
TBE buffer 25-35 C
ca 200 V/cm

AMG 200bp

CTTV 200bp

The electric field strength can influence the shape of the DNA molecule.

low  moderate  high
The Keys

1. Polymer strand interactions create pores
2. Average pore size~ average DNA volume
3. Viscosity should be minimized
4. Field strength optimized

Separation Issues

- Capillary wall coating — dynamic coating with polymer
  - Wall charges are masked by methyl acrylamide

- Electrophoresis buffer —
  - Urea for denaturing and viscosity
  - Buffer for consistent pH
  - 2-pyrrolidinone for denaturing
  - EDTA for stability and chelating metals

- Polymer solution — POP-4, (but others work also)

- Run temperature — 60 °C helps reduce secondary structure on DNA and improves precision.
  (Temperature control affects DNA sizing)

How to Improve Resolution?

1. Lower Field Strength
2. Increase Capillary Length
3. Increase Polymer Concentration
4. Change Polymer Length

Commercial POP-4

4% poly(dimethylacrylamide) (PDMA), 100 mM TAPS (pH 8.0),
8 M urea, and 5% 2-pyrrolidinone

GS500 ROX size standard
Synthesis of PDMA (molecular mass ~100K)

- N,N-diethylacrylamide (DMA) distilled under vacuum, middle portion of distillate collected
- 10 ml t-butanol used as a solvent
- 70% w/v monomer, 5 mmol/L 2,2’-azobisobutyronitrile (AIBN)
- N₂ bubbled through solution for 10 min, RT
- Reaction performed at 55°C for 15 min
- Final product diluted by methylene chloride, precipitated by hexane and rotovapped to dryness


Second Synthesis Procedure for PDMA (~1M)

- DMA distilled
- Added 16.3 ml of methanol to 46.3 ml dH₂O
- Added 6.3 g of DMA to mixture
- N₂ bubbled through for 1 h (covered flask to prevent excess methanol evaporation)
- Added 0.3 ml of ammonium persulfate stock solution (made by dissolving 0.2 g of APS in 1.8 ml of dH₂O) to the methanol/dH₂O mixture


DNA Separation Conclusions

- DNA molecules interact with entangled polymers – friction, sieving, entanglement
- Polymers are not cross-linked (as in slab gels)
  - “Gel” is not attached to the capillary wall – not really a gel
- Pumpable -- can be replaced after each run
- Polymer length and concentration determine the separation characteristics – too high and the capillary can't be refilled
Section II: Issues

Precision and Resolution
How to Optimize and Maintain
Stability
How to Characterize
Temperature
A Critical Parameter

Estimating Size

<table>
<thead>
<tr>
<th>STR Locus</th>
<th>Heterozygote</th>
<th>Homozygote</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>Heterozygote</td>
<td>Homozygote</td>
</tr>
</tbody>
</table>

Gel Analysis

CE Analysis

Linearity of DNA Migration Times

Plot of 20 bp ladder peak migration time vs. size from 20-1,000 bp

Migration of DNA is linear with respect to time in the region needed for STR typing (~100-450 bp)

Use of Single Color and Dual Internal Size Standards for STR Typing


DNA Size Estimation with ABI 310

1. Each sample is run with a ROX internal standard
2. An external standard is run with ROX as well
3. The unknown allele sequence is determined by comparison to the known ladder allele
4. Assumptions?
Note the exceptional linearity between 50-500 bp

 Assumptions with ABI 310 Method
1. DNA is a sphere. (It is not)
2. The conditions for unknown run are the same as the ladder run. (They are not)
3. The ROX dye migrates relatively the same as the FAM dye. (It does not)
4. A calibration for one ladder is good for an entire run (sometimes)
5. Temperature is constant (to what precision?)

Single Base? Resolution with STRs?
How is it defined?

There is a definition for resolution

\[ R = \frac{(t_2 - t_1)}{[1/2(w_1 + w_2)]} \]

- At a resolution of 1 peaks are 2 standard deviations apart
- Baseline resolution occurs at an R value of 1.5 this is 3 standard deviations apart

Why is Resolution important?

<table>
<thead>
<tr>
<th>Locus</th>
<th>Seq.</th>
<th>Repeat #</th>
<th>Variants</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF1PO</td>
<td>TAGA</td>
<td>6-15</td>
<td>10.3</td>
<td>20/1</td>
</tr>
<tr>
<td>TPOX</td>
<td>GAAT</td>
<td>6-13</td>
<td></td>
<td>9/1</td>
</tr>
<tr>
<td>THO1</td>
<td>TCAT</td>
<td>5-10</td>
<td>9.3, 5.3, 6.1, 7.17.3, 8.3, 10.3, 13.3</td>
<td>3/1</td>
</tr>
<tr>
<td>vWA</td>
<td>TCTG/TCTA</td>
<td>11-21</td>
<td>15.2, 18.2, 18.3, 19.2</td>
<td>1.4/1</td>
</tr>
</tbody>
</table>

The presence of 1 and 2 bp variants places great constraints on the analysis.!!
HEC3 mixtures
THO1 locus

Ratio = 20/1
Ratio = 9/1
Ratio = 3/1
Ratio = 1/1.3
Ratio = 1/4
Ratio = 1/1

375 V/cm  47 cm coated capillary

Simulation of Resolution
R = 1.5 bases

Blue = 1 to 1 ratio
Pink = 3 to 1 ratio

Gaussian peak shape with W = 2 σ

Simulation of Resolution
R = 1.2 bases

Blue = 1 to 1 ratio
Pink = 3 to 1 ratio

Gaussian peak shape with W = 2 σ

Simulation of Resolution
R = 0.91 bases

Blue = 1 to 1 ratio
Pink = 3 to 1 ratio

Gaussian peak shape with W = 2 σ

Results
100 samples AmpFiSTR Blue (D13S1358, vWA, FGA)
Consisting of Caucasian and Nigerian population samples

Average St. Dev. = 0.19 bases

Conditions:
3% Hydroxyethyl cellulose (MW approx. 40,000)
60C, 7.1M Urea, 100mM TBE, 40 cm 50um DB-17 capillary, 15kV, 30s @ 2.4 kV inj.

Other studies show Std Dev from 0.07 to 0.22 with typical results approx. 0.12 (polydimethyl acrylamide)

Thoughts on Resolution

• There is a trade off between resolution and run time
• Increased resolution can result in better mixture analysis
• Resolution can degrade with time due to absorption on the inner capillary wall
• Keep the column short and use thick polymers
• Or use longer capillaries (but will have slower run times)
Precision (Resolution isn’t enough!)

Microvariants can appear in the 4 base repeat motif present in these STRs

If 3 X Std. Dev. is greater than 0.5, then a certain number of 9.3 peaks will be labeled 10.

\[
\begin{align*}
\text{Precision} & \\
\text{Current values in the literature range from 0.12 to 0.24 depending on the system and type of repeat. Most papers in the forensic literature report values under 0.15.} & \\
\text{What affects precision?} & \\
\end{align*}
\]

Current values in the literature range from 0.12 to 0.24 depending on the system and type of repeat. Most papers in the forensic literature report values under 0.15.

**What affects precision?**

- PCR inhibitors
- Sample quantity
- Sample quality
- Ionic impurities

Precision

Current values in the literature range from 0.12 to 0.24 depending on the system and type of repeat. Most papers in the forensic literature report values under 0.15.

**What affects precision?**

- PCR inhibitors
- Sample quantity
- Sample quality
- Ionic impurities

**Temperature Effects and DNA Secondary Structure**

- Even under highly denaturing conditions DNA can self associate
- Differences in conformation can affect migration time
- Increase precision by limiting this effect?

- Elevate Temperature to 60°
- Add Strong Denaturants
  - 7-8M Urea
  - Pyrrolidinone
- Examine response of 250, 340 peaks in ROX ladder

**Casework vs Databasing**

Quality of sample also affects precision

- PCR inhibitors
- Sample quantity
- Sample quality
- Ionic impurities

**How to avoid 2° Structure Effects**

- Elevate Temperature to 60°
- Add Strong Denaturants
  - 7-8M Urea
  - Pyrrolidinone
- Examine response of 250, 340 peaks in ROX ladder

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


So what is the effect of Temperature on Profiler+?

Could This affect precision?

Why do some band shifts occur at only one locus?

Examine the allelic ladder at temperatures from 40-70°C

Precision and Resolution

In addition to affecting the size of the DNA, temperature can affect the precision of the results. Elevated temperatures melt out DNA 2°C structure, increasing the precision of the analysis. However, resolution is lost as a result of decreased viscosity. 100mM TBE 2% HEC, DB-17 Capillary

<table>
<thead>
<tr>
<th>Temp</th>
<th>Resolution at 200 bases</th>
<th>THO1 Allele 5</th>
<th>Std. Dev. (bases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>1.3</td>
<td>197.4</td>
<td>0.2</td>
</tr>
<tr>
<td>45</td>
<td>1.6</td>
<td>196.0</td>
<td>0.08</td>
</tr>
<tr>
<td>60</td>
<td>1.7</td>
<td>195.6</td>
<td>0.07</td>
</tr>
</tbody>
</table>

(n=7) (n=7) (n=200+)

DYS 385-14 Size vs Temperature

y = 0.1837x + 355.54

R² = 0.9972

4% pDNA with 8M urea and 5% 2-pyrrolidinone

*Rosenblum et al., Nucleic Acids Res. (1997) 25, 19, 2925
### Effect of Temperature on Allele Size

<table>
<thead>
<tr>
<th>STR</th>
<th>Allele</th>
<th>Size*</th>
<th>Slope+</th>
<th>Std. Dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>D3S1358</td>
<td>12</td>
<td>111.2</td>
<td>-0.10</td>
<td>0.01</td>
</tr>
<tr>
<td>vWA</td>
<td>21</td>
<td>194.9</td>
<td>-0.07</td>
<td>0.02</td>
</tr>
<tr>
<td>FGA</td>
<td>30</td>
<td>264.7</td>
<td>-0.14</td>
<td>0.02</td>
</tr>
<tr>
<td>Amel</td>
<td>X</td>
<td>103.5</td>
<td>-0.13</td>
<td>0.01</td>
</tr>
<tr>
<td>D8S1179</td>
<td>19</td>
<td>170.4</td>
<td>-0.16</td>
<td>0.02</td>
</tr>
<tr>
<td>D21S11</td>
<td>36</td>
<td>232.4</td>
<td>-0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>D18S51</td>
<td>26</td>
<td>341.9</td>
<td>-0.18</td>
<td>0.01</td>
</tr>
<tr>
<td>D5S818</td>
<td>7</td>
<td>131.2</td>
<td>-0.09</td>
<td>0.01</td>
</tr>
<tr>
<td>D13S317</td>
<td>8</td>
<td>205.0</td>
<td>-0.12</td>
<td>0.01</td>
</tr>
<tr>
<td>D7S820</td>
<td>15</td>
<td>292.8</td>
<td>-0.09</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*Estimated size at 61°C
+ °C/base, ave. of 4 measurements

### Effect of Operator Chosen Sizing Method

- **Global Southern Method:** Generates best-fit curve from all matched fragments in the size standard
- **Local Southern Method:** Generates best-fit curve from only nearby internal lane standard data points

### Global Southern Sizing Method

![Global Southern Sizing Method](image1)

### Local Southern Sizing Method

![Local Southern Sizing Method](image2)

### Effect of Operator Chosen Sizing Method

- **Global Southern:**
  - Similar slopes within a locus
  - Differential response in slopes between loci
- **Local Southern:**
  - Differential response between and within loci
  - Many slopes significantly larger (−0.156 vs. −0.104)

### Implications of Temperature Studies

Temperature affects precision through sample denaturation

New studies indicate there is a variable response to temperature especially between loci

The effect is far more pronounced in local southern

Temperature control is important because it affects both precision and resolution.

Band shifts are a natural consequence of differential response to temperature

---

High pH Studies

DNA analysis can proceed at elevated pH (11+)
At such pH values buffers highly denaturing
Will temperature stability improve if conditions are more denaturing?
Yes, but capillary lifetime suffers

Implications of pH & Temp. Studies

STR size and migration varies in response to
temperature and sizing method - secondary structure!

Temperature response is minimized at high pH with
fluorocarbon capillaries but capillary lifetime is limited
at this pH

What does this tell us about day to day applications?
Bottom line:
Use global southern sizing.
Keep temperature constant!

Detection Issues

- Fluorescent dyes
  - spectral emission overlap
  - relative levels on primers used to label PCR
    products
  - dye “blobs” (free dye)
- Virtual filters
  - hardware (CCD camera)
  - software (color matrix)

Filters determine which wavelengths of light are collected onto the CCD camera
**Laser Used in ABI 310**
- Argon Ion Laser
- 488 nm and 514.5 nm for excitation of dyes
- 10 mW power
- Lifetime ~5,000 hours (1 year of full-time use)
- Cost to replace ~$5,500
- Leads to highest degree of variability between instruments and is most replaced part
- Color separation matrix is specific to laser used on the instrument

**Methods for Fluorescently Labeling DNA**
- Intercalating Dyes (post-PCR)
- Dye-labeled nucleotide insertion during PCR
- Dye-labeled primer insertion during PCR

**Fluorescent Labeling of PCR Products**
- Dyes are attached to one primer in a pair used to amplify a STR marker
- Dyes are coupled to oligonucleotides (primers) through NHS-esters and amine linkages on the 5’end of the primer. Dye-(CH2)6-primer
- Dye-labeled oligonucleotides are incorporated during multiplex PCR amplification giving a specific color “tag” to each PCR product
- PCR products are distinguished using CCD imaging on the 310

**Fluorescent Dyes Used in 4-Color Detection**
- FAM (Blue)
- JOE (Green)
- TAMRA (Yellow)
- ROX (Red)

**Virtual Filters Used in ABI 310**

**Fluorescent Emission Spectra for ABI Dyes**
- NED is a brighter dye than TAMRA

http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm
Please Note!

- There are no filters in a 310
- It's just the choice of pixels in the CCD detector
- All the light from the grating is collected
- You just turn some pixels on and some off

You then make a matrix

The matrix is the solution to a problem
what's the contribution at any given wavelength (filter set) from each dye?

There are 4 dyes

- Remember algebra from high school?
- To solve a problem with 4 unknowns, you need 4 equations

For Example

I_{440} = b x_b + g y_g + y z_y + r w_r intensity of blue
I_{460} = b x_b + g y_g + y z_y + r w_r intensity of green
I_{480} = b x_b + g y_g + y z_y + r w_r intensity of yellow
I_{500} = b x_b + g y_g + y z_y + r w_r intensity of red

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 any mixture

Matrix File Table from an ABI 310

These values are used by the GeneScan® Analysis Software to separate the various dye colors from one another. The letters B, G, Y, and R represent the dye colors Blue, Green, Yellow, and Red, respectively.

http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm
Comments on Matrices (Multi-Component Analysis)

- Make sure that the right filter set and matrix are applied when collecting data
- You can always apply another matrix to a sample collected on the ABI 310 but it must be run with the right filter set (matrix must be run first with ABI 3100)
- It is important to update matrices on a regular basis (depending on use) due to differences in laser power over time
- A good indication of when to run a new matrix is the observation of pull-up between dye colors when peaks are smaller than ~4,000 RFUs

If Wrong Filter Is Used…

Y STR 10plex labeled with FAM, TET, HEX dyes

Filter A

Filter F

Filter C

Peaks change “colors”

ABI 310 Filter C

More color overlap between blue and green dyes

Matrices for Filter F with POP-4 vs. POP-6 (on the same ABI 310)

Values are very similar between the two matrices
Same Dye Set and Filter F with Different ABI 310s

Instrument lasers make a big difference

310 Data Collection Software

- Controls 310 run conditions
- Translates light on CCD camera into electropherogram (raw data)
- Sample sheets and injection lists are created

ABI manual is P/N 904958B

Injection List in Data Collection Software

- Lists samples to be analyzed (repeats can be easily performed)
- Sets virtual filter on CCD camera
- Sets electrophoresis time and voltage
- Sets injection time and voltage
- Sets run temperature
- If desired, sample analysis can be set up for automatic matrix color separation and sizing with internal standards using defined analysis parameters

Steps Performed in Standard Module

- 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; the default injection is 15 kV (kilovolts) for 5 seconds
- 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 – data collection begins; raw data is collected with re spectral deconvolution of the different dye colors; the matrix is applied during Genescan analysis

Conclusions

Current advances in DNA typing are the result of:

1) The application of short tandem repeats
2) The development of multiplex PCR
3) Multichannel laser induced fluorescence
4) Capillary electrophoresis with entangled polymer buffers
### Conclusions II

1. Systems for DNA typing must combine precision and resolution for identification of STR alleles reliably.

2. Differential response to temperature can affect precision.

3. Denaturation is important in maintaining reproducibility.

4. Separation efficiency and precision are controlled via the temperature, polymeric buffer and the injection media.

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