Capillary Electrophoresis
Instrumentation:
Theory and Application
The Issues

1. Although the PCR is rapid and efficient, sample loads keep increasing.

2. Current estimated backlog is 540,000 samples.

3. The number of untested rape kits nationwide is estimated to be 180,000 to 500,000.
But How To Process All This Data?

Hundreds of thousands of samples?

Silver Stained Slab Gel?

Lab Floors like a Darkroom!
Methods of determination of genetic variability

- Probe hybridization
- Charge based mobility and separation – gel and capillary electrophoresis
- Partitioning and ion exchange – HPLC
- Conformation – SSCP, heteroduplex polymorphism
- Size measurement – Mass Spectrometry

- All of these have been used one time or another for STR/VNTR analysis
STR Typing Technology Review

Gels

Capillary Electrophoresis

Capillary Arrays

Microchip CE

Mass Spectrometry

Hybridization Arrays

Data courtesy of Bill Hudlow


PNAS (1997) 94: 10273-10278

Int. J. Legal Med (1998) 112: 45-49


http://www.cstl.nist.gov/biotech/strbase/tech.htm
What are the keys to a useful measure of genetic variability, esp. with STRs?

- Reproducible results from day to day
- Resolution of a single base over the range of analysis 60-450 bp
- Precision under 0.17 bp for size separation
- Stability over time and insensitivity to matrix effects
- Relative accuracy (not absolute)
Requirements for Accurate STR Typing

• High precision (0.15 bp or less) to permit comparison of allelic ladders to sequentially processed STR samples
• Resolution of at least 1 bp to >300 bp (to detect microvariants)
• Wide range of analysis (60-450bp)
• Reproducibility over a long time frame
• Insensitivity to matrix interferences
How do the various methods add up at present?

- **Probe based methods** - can be difficult to detect length variations
- **HPLC** - lacks resolution
- **MS** - has trouble with sizes above 90bp
- **Conformational polymorphisms** - will not always vary sufficiently
- **Electrophoresis** - currently best option - but can have trouble with precision and resolution
The Application

• Speed and detection capabilities of DNA analyses have improved since the development of 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
Capillary Electrophoresis
The alternative

1. Injection, separation, and detection are automated.

2. Rapid separations are possible

3. Peak information is automatically stored for easy retrieval.
Capillary Electrophoresis and Daubert: Time for Admission
Capillary Electrophoresis System

Argon Ion Laser

Deconvoluted Result

Capillary filled with entangled Polymer

Buffer (Sample)

Capillary

5-20 kV

Buffer

[Diagram showing a capillary filled with entangled polymer, an argon ion laser, and deconvoluted result.]
Process Involved in 310/3100 Analysis

- **Injection**
  - electrokinetic injection process (formamide, water)
  - importance of sample stacking

- **Separation**
  - Capillary – 50um fused silica, 47 cm (36 cm to detector)
  - 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
Separation
“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

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

Ions move through pickle faster at high voltage

\[ v_{ep} = \mu_{ep}V \]

Small ions with high charge move fastest

\[ \mu_{ep} = \frac{q}{6\pi\eta r} \]
DNA and Electrophoresis

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

μ_{ep} = q/6πηr

small ions with high charge move fastest

As size increases so does charge!
Electroosmotic flow is a bulk flow that is created by the build up of charge on a capillary wall. POP polymer minimizes this effect.

\[
\text{SiOH} \rightarrow \text{SiO}^- + \text{H}^+ 
\]
Issues with CE separations

- Effect – electroosmotic flow

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

PDMA (POP) separation of DNA (EOF not present)
In the early 1990s the real question was how to transition from a gel to a capillary

- Cross-linked acrylamide gel filled capillaries were tried first
  - Reusable?
  - Bubble formation
  - Thermal degradation

- Alternative was to not use a gel at all
  - **Refillable sieving polymers**
  - However, resolution initially was poor with these polymers
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% polyvinyl pyrrolidinone
  - POP-4 and POP-6

POP4 Polymer
Polydimethyl acrylamide
Transient Pores Are Formed Above the Entanglement Threshold.

\[ C < C^* \]

\[ C = C^* \]

\[ C > C^* \]
Ogston Sieving

$\mu \sim \mu_0 e^{-NC}$

Reptation

$\mu \sim 1/N$

Entanglement

$\mu \sim f(1/CN)$
Large DNA undergoing Electrophoresis in an HEC sieving buffer (confocal microscopy)
Oxazole yellow dimer labeled
Mesh size (concentration) and molecular weight (chain length) will affect resolution viscosity limits applicable chain length
The electric field strength and gel can influence the shape of the DNA molecule.

low

moderate

high

Follow the dancing DNA
Resolution vs Field Strength

500 V/cm  4.5 min
333 V/cm
266 V/cm
166 V/cm
100 V/cm

1% HEC
100 bp Ladder
30 min
Resolution vs Field Strength

- 500 V/cm: 4.5 min
- 333 V/cm
- 266 V/cm
- 166 V/cm
- 100 V/cm: 30 min

1% HEC
100 bp Ladder
100bp DNA ladder

Note compression as DNA fragment length increases
This compression defines the practical limits to STR sizes in multiplex PCR
The Keys

1. Polymer strand interactions create pores

2. Average pore size~ average DNA volume

3. Viscosity should be minimized

4. Field strength optimized
What is in POP4?

Improved single-strand DNA sizing accuracy in capillary electrophoresis

Barnett B. Rosenblum*, Frank Oaks, Steve Menchen and Ben Johnson

PE Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404, USA

Received May 29, 1997; Revised and Accepted August 6, 1997

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

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

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

Running buffer contains 100 mM TAPS and 1 mM EDTA (adjusted to pH 8.0 with NaOH)
Best Synthesis Procedure for PDMA
(Molecular Wt = 1 Million amu)

- Distill dimethyl acrylamide to remove stabilizers
- Add 16.3 ml of methanol to 46.3 ml dH₂O
- Added 6.3 g of dimethyl acrylamide to mixture
- N₂ bubbled through for 1 h (covered flask to prevent excess methanol evaporation)
- Add 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/ H₂O mixture
- Remove solvents and dry to powder

Synthesis Results

Effect of Concentration and Molecular Weight on resolution

4% PDMA (100K), Taps buffer

7.3% PDMA (1M), Taps buffer
Commercial POP-4

- 4% poly(dimethylacrylamide) (PDMA),
- 100 mM TAPS (pH 8.0), 8 M urea, 5% 2-pyrrolidinone
How to Improve Resolution?

1. Lower Field Strength

2. Increase Polymer Concentration

3. Increase Polymer Length

4. Use a longer capillary

All of these come at a cost of longer separation run times
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
  – Pyrrolidinone for denaturing DNA
  – EDTA for stability and chelating metals

• **Polymer solution** –
  – Entangled to separate DNA
  – High molecular weight for good resolution
  – Minimum concentration/viscosity for easy refilling (POP4, POP6)

• **Run temperature** -- 60 °C helps reduce secondary structure on DNA and improves precision. (Temperature control affects DNA sizing)
Injection
Injection Methods for CE

Hydrodynamic

Electrokinetic


ABI Prism 310 Genetic Analyzer

- Syringe with polymer solution
- Capillary
- Autosampler tray
- Outlet buffer
- Injection electrode
- Inlet buffer
End of capillary should be near end of electrode (and autosampler position should be calibrated to these tips)
Stacking Effects

(a) Stacking with Low Ionic Strength

Low Ionic Strength

High Field

DNA

Low Field

DNA

Buffer

High Ionic Strength

High Field

DNA

DNA

DNA

DNA

(b) Regular EK Injection

High Ionic Strength

Low Field

DNA

DNA

Buffer

High Ionic Strength

Low Field

DNA

DNA

+
Ion Mobility Effects

The Injection of DNA by voltage is described by

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

However this equation assumes no interfering ions are present.

Cl\(^-\) ions and other interferents will compete with DNA

$$\{\text{DNA}_{\text{inj}}\} = [\text{DNA}_{\text{inj}}]/[\text{other ions}_{\text{inj}}]$$

Ions such as Cl\(^-\) have a higher charge/mass ratio and \(\mu_{\text{ep}}\) is greater
Effect of Formamide Conductance on DNA Concentration

PRISM

208 uS

338 uS

408 uS

1180 uS
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 $= \mu_{ep} = \text{velocity/ electric field}$
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
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…
Injection Study

Evaluate of 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
### Effect of Formamide on Peak Resolution and Sensitivity (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 0.01</td>
<td>2700+/- 300</td>
</tr>
<tr>
<td>Formamide (27µS)</td>
<td>1.15+/- 0.05</td>
<td>2960+/- 30</td>
</tr>
<tr>
<td>Formamide (360µS)</td>
<td>1.20+/- 0.08</td>
<td>879 +/- 4</td>
</tr>
<tr>
<td>Formamide 1000µS)</td>
<td>1.20+/- 0.06</td>
<td>290 +/- 14</td>
</tr>
</tbody>
</table>
Effect of increasing sample concentration

Sensitivity improves only in poor quality formamide!

- Water
- 47 uS/cm
- 360 uS/cm
- 1000 uS/cm

Sample / Formamide

Gain in sensitivity (%)

- 1:25
- 1:10
- 1:5
- 1:1
Effect of Increasing salt concentration
(Reannealed ds DNA moves faster)
Detection
Capillary Electrophoresis Instrumentation

ABI 310
single capillary

ABI 3100
16-capillary array
Laser (488nm)

OPTICS

Laser (488nm)

Lensed light

Grating

Charged coupled device

Capillary
Detection Issues

• Fluorescent dyes
  – spectral emission overlap
  – relative levels on primers used to label PCR products
  – dye “blobs” (free dye)

• Virtual filters (determine which pixels are used)
  – hardware (CCD camera)
  – software (color matrix)
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

- **Laser is on unless instrument is off!**
Methods for Fluorescently Labeling DNA

- Intercalating Dyes (post-PCR)
- Dye-labeled nucleotide insertion during PCR
- Dye-labeled primer insertion during PCR

Linkers, dyes, etc. can be added to the 5’ end of the primer without disturbing the reaction. Individual bases can also be tagged.

Covalent labeling process
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
The succinimimidyl ester reacts rapidly with amine linkers on DNA bases
Virtual Filters Used in ABI 310

Visible spectrum range seen in CCD camera

Filter sets determine what regions of the CCD camera are activated and therefore what portion of the visible light spectrum is collected.

<table>
<thead>
<tr>
<th>Filter</th>
<th>Blue</th>
<th>Green</th>
<th>Yellow</th>
<th>Red</th>
<th>Orange</th>
<th>Used with These Kits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filter A</td>
<td>FL</td>
<td>JOE</td>
<td>TMR</td>
<td>CXR</td>
<td></td>
<td>PowerPlex 16</td>
</tr>
<tr>
<td>Filter C</td>
<td>6FAM</td>
<td>TET</td>
<td>HEX</td>
<td>ROX</td>
<td></td>
<td>in-house assays</td>
</tr>
<tr>
<td>Filter F</td>
<td>5FAM</td>
<td>JOE</td>
<td>NED</td>
<td>ROX</td>
<td></td>
<td>Profiler Plus</td>
</tr>
<tr>
<td>Filter G5</td>
<td>6FAM</td>
<td>VIC</td>
<td>NED</td>
<td>PET</td>
<td>LIZ</td>
<td>Identifiler</td>
</tr>
</tbody>
</table>

Commonly used fluorescent dyes

Arrows indicate the dye emission spectrum maximum.
Fluorescent Emission Spectra for ABI Dyes

5-FAM  JOE  NED  ROX

NED is a brighter dye than TAMRA

Laser excitation (488, 514.5 nm)

Butler, J.M. (2001) *Forensic DNA Typing*, Figure 10.4, ©Academic Press
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
Raw Data from the ABI Prism 310

(prior to separation of fluorescent dye colors)
Uncorrected Raw Data
(AFLP of a Marijuana Sample)
AFLP Analysis of Plant DNA

Corrected Result
Why 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
Matrix Standards (Raw Data)

Filter Set C

Set F

6FAM (5FAM)

TET (JOE)

HEX (NED)

ROX (ROX)
For Example

\[ I_{540} = bx_b + gy_b + yz_b + rw_b \] intensity of blue
\[ I_{560} = bx_g + gy_g + yz_g + rw_g \] intensity of green
\[ I_{580} = bx_y + gy_y + yz_y + rw_y \] intensity of yellow
\[ I_{610} = bx_r + gy_r + yz_r + yw_r \] 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 dye contribution for any mixture
The results of the calculation are in a matrix

(remember linear algebra?)

The values represent the percent spectral overlap from each dye

Values outside this range represent mixtures
5 x 5 matrix for 5-dye analysis on ABI 310

From Identifier User's Manual

Raw Data for Matrix Standards

Processed Data (matrix applied with baselining)
Liz is likely an energy transfer dye

Energy absorbed at 488 nm is transferred to a nearby second dye which fluoresces at a longer wavelength
Capillary Arrays and Higher Throughput STR Typing
Ways to Increase Sample Throughput

- Run more gels (FMBIO approach)
- Increase speed of single sample analysis (microchip CE systems)
- Multiplex fluorescent dyes of different colors (higher level PCR multiplexes)
- Parallel separations using capillary arrays
- Microfluidic channel arrays
- New Detection Technologies (MALDI-TOF mass spectrometry)
Methods for Parallel Sample Processing

Multiplex by Size

Multiplex by Dye Color

Multiplex by Number of Capillaries

ABI 3100/3130: 16 capillaries
ABI 3700/3730: 96 capillaries
ABI 3100 Avant: 4 capillaries
Capillary Array Electrophoresis

• Higher sample throughput
• Commercial 96 capillary systems were used to sequence the human genome
  – ABI 3700
  – MegaBACE
• Engineering and hardware challenges
• Software challenges
Multicapillary Arrays

- By proper design of the laser excitation and computerized analysis multicapillary systems are possible.

These systems played a huge part in the Human genome project.

96 capillary systems
Have tremendous throughput
16 Capillary 3100

6 foot Table on wheels
Capillary Electrophoresis Instrumentation

ABI 310
single capillary

ABI 3100
16-capillary array
Inside the 3100

- Oven fan
- Capillary array
- Oven
- Seal
- Better temp control
- Autosampler
- Detection window
- Buffer reservoir
- 5 mL syringe: Polymer reservoir
- 1 mL syringe: Loads polymer
Capillaries in buffer tank
Running and storage position
At 45-60 minutes per run two plates represent 12 runs or ~10-12 hours for 192 samples.
ABI 3100 Array Detection

16 Capillary Array detection cell
CCD Image of 16-Capillary Array

Full frame CCD camera image of capillary array #30A028-09 filled with POP-4™ polymer, colorized to show wavelength dispersion and cropped to show collection range. This image shows that the system is sensitive enough to detect the Raman scattering of water in the polymer associated with the 488 nm and 514 nm laser lines.
Mechanisms for Capillary Array Detection

**Capillary Array**
- Side irradiation (on-capillary)
- ABI 3100, 3130, 3100Avant

**Sheath flow detection**
- ABI 3700

**Fixed laser, moving capillaries**
- MegaBACE

**LASER Excitation**
- (488 nm)
Improvements in Information Throughput with Multiplexed Markers and Multiple Capillaries

**Time required to obtain each genotype…**

<table>
<thead>
<tr>
<th>#Loci in Multiplex</th>
<th>Single capillary (ABI 310)</th>
<th>16 capillary array (ABI 3100)</th>
<th>96 capillary array (ABI 3700)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Each run: 30 min</td>
<td>Each run: 45 min</td>
<td>Run: 2 h 46 min</td>
</tr>
<tr>
<td>1</td>
<td>1800 s (per capillary)</td>
<td>2700 s (per capillary)</td>
<td>9960 s (per capillary)</td>
</tr>
<tr>
<td>8</td>
<td>1800 s (30 min)</td>
<td>169 s (2.8 min)</td>
<td>104 s (1.7 min)</td>
</tr>
<tr>
<td>16</td>
<td>225 s</td>
<td>21 s</td>
<td>13 s</td>
</tr>
<tr>
<td></td>
<td>113 s</td>
<td>10.5 s</td>
<td><strong>6.5 s</strong></td>
</tr>
</tbody>
</table>
Microchip CE Systems

What is under development for STR typing?
Attorney General John D. Ashcroft, holding a slide for DNA, hailed the technology as a tool in solving crimes. With him is Kellie Greene, whose attacker was found by DNA testing.
CE Microchips

- Channels are etched in glass microscope slides to make miniature CE columns
- More rapid separations are possible due to the shorter separation length

Channels etched into glass microchip  Electrodes placed in each well allow injection and analysis

Confocal fluorescence detection  Permits sensitive and specific detection
Injection onto a Microchip CE Channel

- A sample injection bias can also exist with µCE systems (smaller fragments inject preferentially).
- A narrow injection plug means equivalent resolution can be achieved in shorter distances (faster speeds).
- Detection must be more sensitive due to lower amounts of material being loaded.
- Geometry of the channels defines plug size (typically ~100 pL).
Rapid Microchip CE Separation of STR Alleles

Dan Ehrlich’s group
Whitehead Institute

270: 148-152

PowerPlex™ 1.1
Allelic ladders mixed with samples for genotyping purposes

Can miniSTRs solve the problem of microfluidic resolution to permit portable DNA analyzers at a crime scene?

**Agilent Bioanalyzer**
A disposable analytical platform
That works right out of the box. Fieldable for mass disaster?
but resolution is too low for 4base STRs
How to improve resolution?
1. Use mini 5 base penta STRs
2. Switch to denaturing polymer
Agilent Bioanalyzer
(size of a postage stamp)

Kit-on-a-Chip
Speeds Cell Assays

Micro-channels are filled with a sieving polymer and fluorescence dye

Sample Components
injection starts

Analysis Fluorescence
The separation of pentameric STRs on a 2 cm beta Agilent chip.
The evolution of Microfluidic arrays

Slide from Rich Mathies (UC-Berkeley)
Berkeley Rotary Confocal Fluorescence Scanner

**Scanning Fluorescence Microscopy Instrumentation**

- Fiber Optic
- Radial Microchip
- Rhomb Prism
- Stepper Motor
- Laser 488 nm
- Dichroic Beamsplitter
- PMT
  - 4 colors
  - FAM
  - JOE
  - TAMRA
  - ROX

Microfluidic devices are also being developed to integrate all operations.

Figure 2. An Integrated DNA chip. This system, currently under development by James Landers at the University of Virginia, has the potential to integrate all current processes in the forensic laboratory in one device.
Time-of-Flight Mass Spectrometry
TH01 Alleles: CE vs. Mass Spec

ABI 310 Result
- 9.3 allele: 1071 sec
- 10 allele: 1073 sec

Mass Spec Result
- 9.3 allele: 203.3 μsec
- 10 allele: 204.8 μsec
### Timing for Data Collection

<table>
<thead>
<tr>
<th>Laser pulse (10 nsec)</th>
<th>Wait (500 nsec)</th>
<th>Turn on voltages for ion optics (+20 kV)</th>
<th>Collect spectrum for ~300,000 nsec</th>
</tr>
</thead>
</table>

**REPEAT process 100+ times**

- All this occurs in less than 5 seconds per sample

**Sum multiple spectra into final sample spectrum**

**Data processing and genotype determination**
Time-of-Flight Mass Spectrometry (TOF-MS)

DNA Reaction Products (Size separated and drifting to the detector)

Pulsed Laser Beam

High-Density Sample Array

Ion Extractor

Acceleration Region (20 kV)

Drift Region Electric-Field Free

Detector

X-Y sample control
384 samples processed routinely in ~45 min (best was 96 samples in 2 min)

~4,000 samples in 11 hours on single mass spec and 3 robots

averaged around 2,000 samples daily at GTS per instrument (Jan-Aug 1999)

most samples run as singleplex reactions but demonstrated 10-plex SNP assay and 3-plex STR assay
Where is the Future Going?…

Currently being released

1. More robust STR kits – less sensitive to inhibition
2. MiniSTRs- less sensitive to degradation
3. Y STR kits- better databases and more loci
4. Advanced real time PCR kits
5. High speed PCR amplification

On the drawing board

1. RNA based tissue typing
2. LCN based procedures based on laser microdissection
3. SNP based phenotyping
4. Rapid DNA sequencing by next generation systems
5. Point of entry chip based DNA typing
DNA Size Estimation

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?
Estimating Size

- **STR Locus**
  - Heterozygote
  - Homozygote

- **Gel Analysis**
  - Standard
  - Heterozygote
  - Homozygote

- **CE Analysis**
  - Standard
  - Heterozygote
  - Homozygote
Comparison of An Individual’s Genotype to HUMTH01 Allelic Ladder Using Dual Internal Standards

TH01
179-203 bp
Alleles
6, 9
300 bp
Sizing DNA Fragments using dye labeled DNA

Standard

139 150 160 200

Unknown

*
Sizing Ladder
ROX Standard
75-400

Note the exceptional linearity
Resolution

- Chromatographic resolution is defined as

$$R = (t_2 - t_1)/\left[0.5(w_2 + w_1)\right] \quad \text{OR}$$
$$R = (2\ln2)^{1/2} \frac{(t_2 - t_1)}{0.5(whh_2 + whh_1)}$$

Divide the # bases between the peaks by the resolution to get resolution in basepairs.

For a quick and dirty procedure:
Measure peak height divided by area or

Use the percent valley between the Tho1 9.3 and 10 peak.
3 individual samples amplified and typed

AmpFlSTR Green     Genotyper Software
POP4 mixtures
THO1 locus

320 V/cm  47 cm uncoated capillary
POP4 Polymer
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 $W = 2 \sigma$
Simulation of Resolution

R = 1.2 bases

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

Gaussian peak shape with $W = 2 \sigma$
Simulation of Resolution
\[ R = 0.91 \text{ bases} \]

<table>
<thead>
<tr>
<th>sigma</th>
<th>mean</th>
<th>x</th>
<th>f(x)</th>
<th>resolution</th>
<th>sigma</th>
</tr>
</thead>
<tbody>
<tr>
<td>199</td>
<td>1</td>
<td>0.25</td>
<td>200</td>
<td>199</td>
<td>1.595769</td>
</tr>
<tr>
<td>199.1</td>
<td>1</td>
<td>0.25</td>
<td>200</td>
<td>199.1</td>
<td>1.595769</td>
</tr>
</tbody>
</table>

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

Gaussian peak shape with \( W = 2 \sigma \)
Thoughts on Resolution

There is a trade off between resolution and run time

Increased resolution results in better mixture analysis

Resolution can degrade with time

Keep the column short and use thick polymers

Or use longer capillaries
Resolution can be important in mixture analysis.
Precision and size estimates

The red internal lane standard establishes the allele size. This size is compared to an allelic ladder run earlier.
Assumptions with ABI 310 Method affecting precision

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 degree?)
There is a size range for calling an allele

These bins define the precision of the system

They are commonly defined as +/- 0.5 bp

However they can also be defined by the precision of an allele call (+/- 3 standard deviations)
Once you can label DNA you must next determine its size

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?
Estimating size

The red internal lane standard establishes the allele size. This size is compared to an allelic ladder run earlier.
Assumptions with ABI 310 Method affecting precision and resolution

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 degree?)*
There is a size range for calling an allele

These bins define the precision of the system

They are commonly defined as +/- 0.5 bp

However they can also be defined by the precision of an allele call (+/- 3 standard deviations)
What affects precision?

Lots of things:

- Temperature
- Sequence of Rox standard vs sample
- Sequence of allele vs ladder
- Conformation of DNA
- Polymer matrix
- Capillary condition
- Buffer concentration
- pH

- **Showing that you can control these factors is the goal of laboratory validation**
Conclusions

DNA typing by capillary electrophoresis involves:

1) The use of entangled polymer buffers

2) Injection by sample stacking

3) Multichannel laser induced fluorescence

4) Internal and external calibration
McCord Research Group

Ohio

Miami
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Points of view expressed in this presentation are those of the authors and do not necessarily represent the official view of the US department of Justice