

Advanced Topics in STR DNA Analysis

Capillary Electrophoresis Instrumentation: Theory and Application

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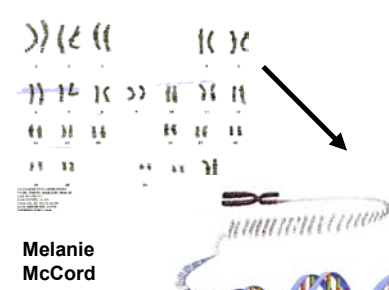
Capillary Electrophoresis Instrumentation: Theory and Application

Outline for This Section

- Historical perspective
- Advantages and disadvantages of CE
- ABI 310 and 3100 instrument components
- Injection
- Separation
- Detection

There are 23 pairs of Chromosomes

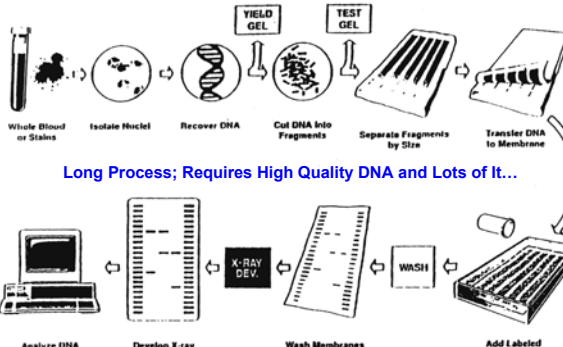
3 billion base pairs means $1/(1/4)^{3,000,000,000}$ possible combinations



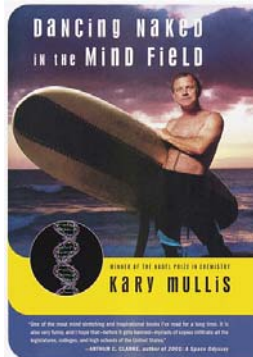
 **Melanie McCord**

Where is the important information for identification
Vs
The part that tells us how to play the saxophone?

DNA Analysis by RFLP



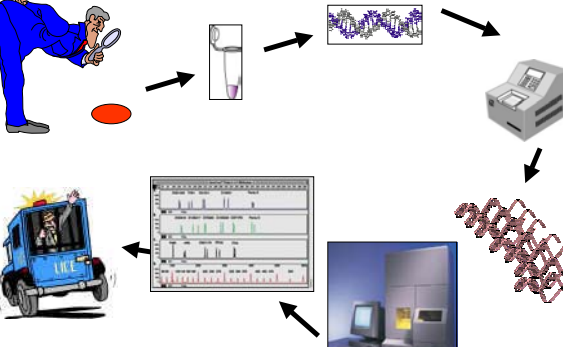
Long Process; Requires High Quality DNA and Lots of It...

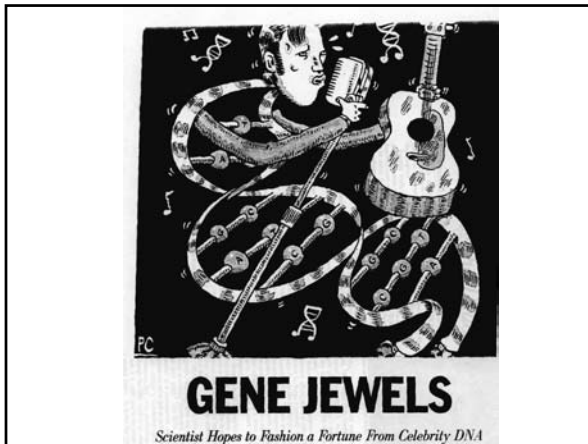


Inventor of the PCR

"I think I might have been stupid, in some respects, if it weren't for my psychedelic experiences."
-Kary Mullis, Ph.D., Nobel Prize Laureate, Chemistry, 1993

The Process of DNA Typing via PCR





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

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
- Precision under 0.17 bp for size separation
- Stability over time and insensitivity to matrix effects
- Relative accuracy (not absolute)

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

How do the various methods add up at present?

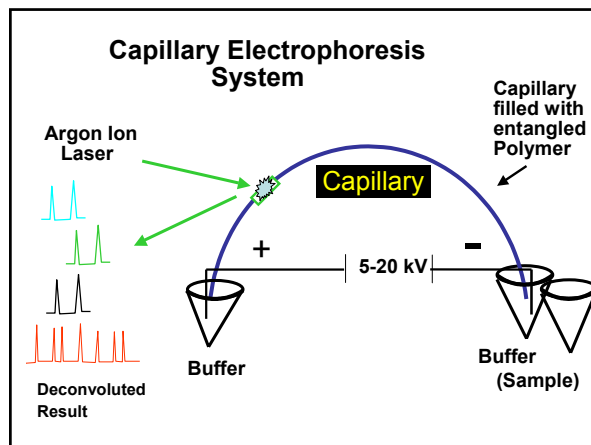
- Probe based methods-
 - can be difficult to detect length variations
- HPLC-
 - lacks resolution
- MS –
 - has trouble with sizes above 90bp
- Conformational polymorphisms-
 - will not always vary sufficiently
- Electrophoresis-
 - currently best option- but can have trouble with precision and resolution

The Issues

1. Although the PCR is rapid and efficient, sample loads keep increasing
2. Soon all sexual offenders (and other felons) will be required to submit a sample for testing. Current estimated backlog is 540,000 samples.
3. The number of untested rape kits nationwide is estimated to be 180,000 to 500,000.
4. What technique could be used to automate the analysis of so many samples?

Why Use Capillary Electrophoresis for DNA Analysis?

1. Injection, separation, and detection are automated.
2. Rapid separations are possible
3. Peak information is automatically stored for easy retrieval.



Process Involved in 310/3100 Analysis

- **Injection**
 - electrokinetic injection process (formamide, water)
 - importance of sample stacking
- **Separation**
 - Capillary – 50µm fused silica, 47 cm (36 cm to detector)
 - POP-4 polymer – Polydimethyl acrylamide
 - Buffer – TAPS pH 8.0
 - Denaturants – urea, pyridinone
- **Detection**
 - fluorescent dyes with excitation and emission traits
 - CCD with defined virtual filters produced by assigning certain pixels

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



$$P = VI = I^2R$$

Pickle cooks

$$v_{ep} = \mu_{ep} V$$

Ions move through pickle faster at high voltage

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

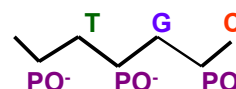
Small ions with high charge move fastest

DNA and Electrophoresis

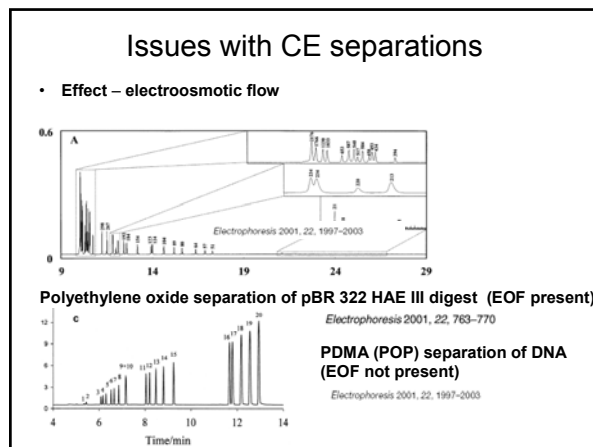
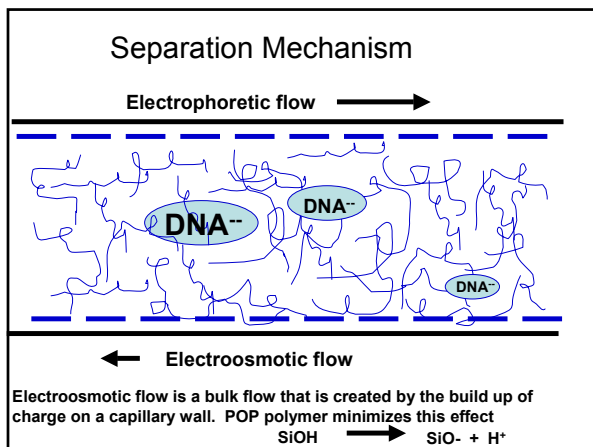
"From a practical point of view it is disappointing that electrophoresis cannot be used to fractionate or analyze DNA on the basis of size" Olivera, Biopolymers 1964, 2, 245

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

small ions with high charge move fastest



As size increases so does charge!



- 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?

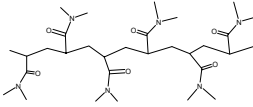
They are gels - very similar to polyacrylamide

They are not gels - they flow

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

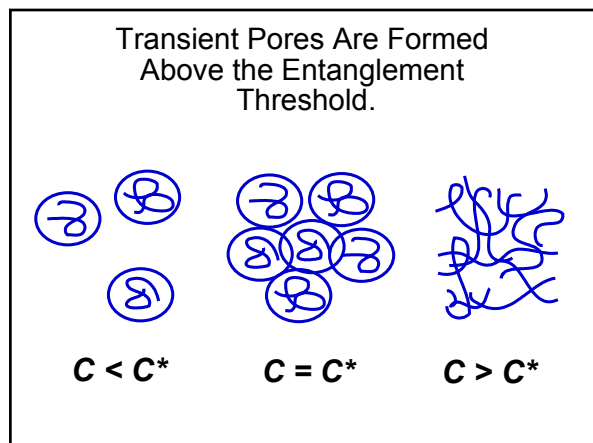
Entangled Polymer Solutions

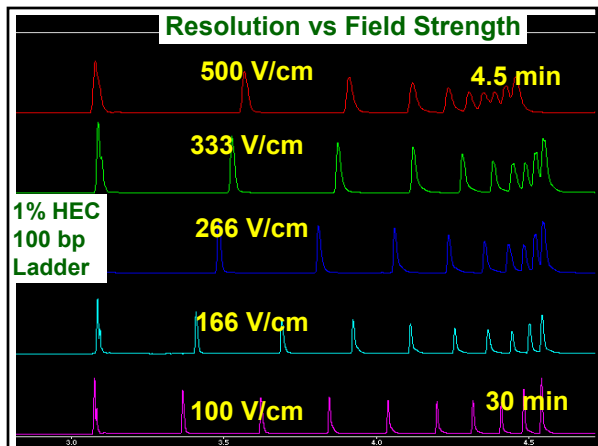
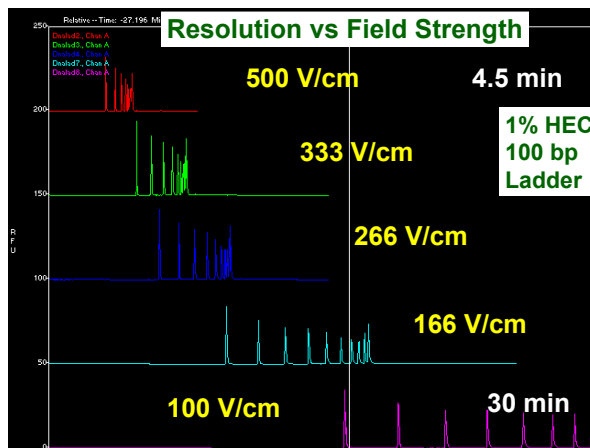
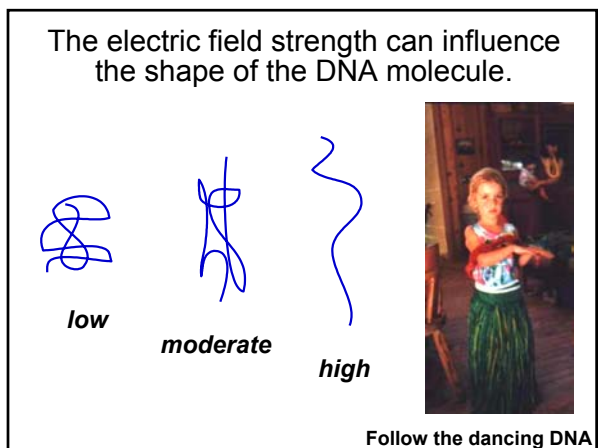
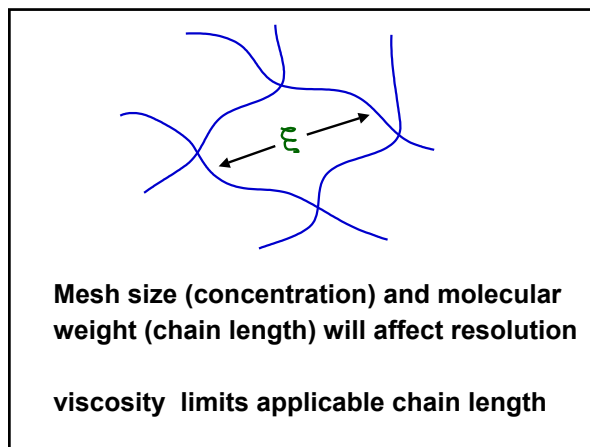
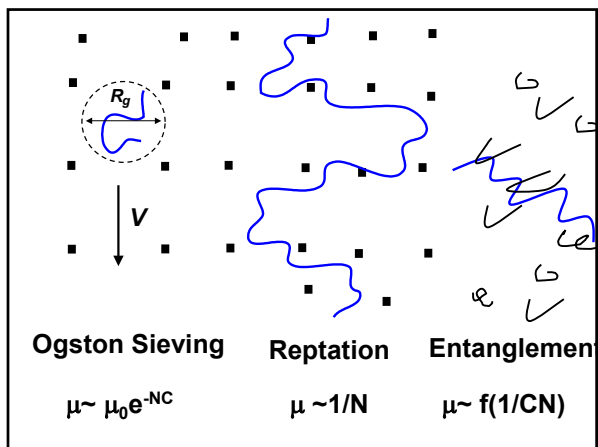
- Polymers are not cross-linked (above entanglement threshold)
- "Gel" is not attached to the capillary wall
- Pumpable -- can be replaced after each run
- Polymer length and concentration determine the separation characteristics
- Examples:
 - 1% HEC (hydroxyethyl cellulose)
 - 4% polyvinyl pyrrolidone
 - POP-4 and POP-6



POP4 Polymer

Polydimethyl acrylamide





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

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

How Are Separations Performed in a 310?

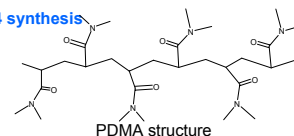


We use POP-4

(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)



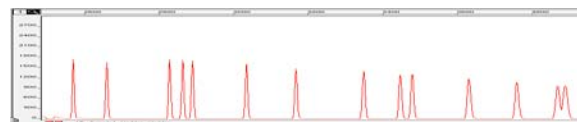
Synthesis Procedure for PDMA (Molecular Wt = 1 Million amu)

- 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

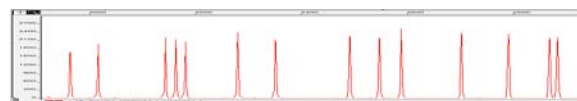
Madabhushi, R.S. DNA Sequencing in Noncovalently Coated Capillaries Using Low Viscosity Polymer Solutions. In *Methods in Molecular Biology* 2001. Vol. 163.

Synthesis Results

Effect of Concentration and Molecular Weight on resolution



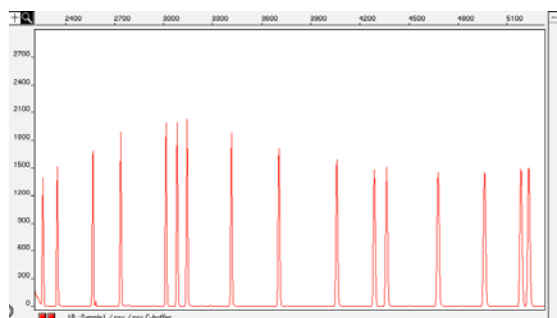
4% PDMA (100K), Taps buffer



7.3% PDMA (1M), Taps buffer

Commercial POP-4

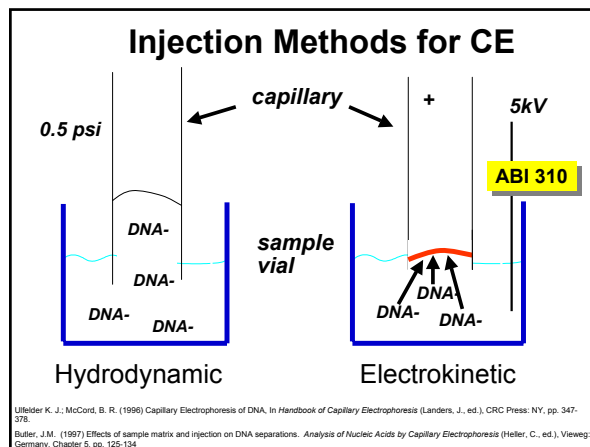
- 4% poly(dimethylacrylamide) (PDMA),
- 100 mM TAPS (pH 8.0), 8 M urea, 5% 2-pyrrolidinone



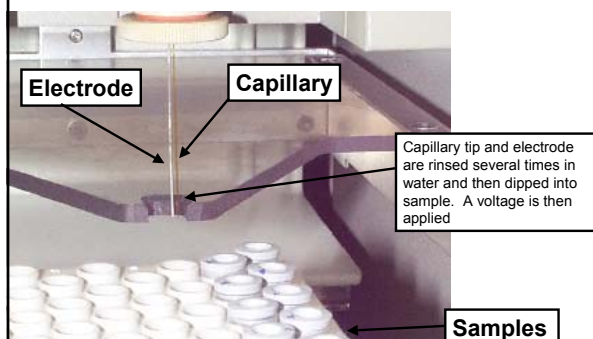
Separation Issues

- **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

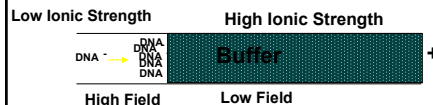


How is injection accomplished on a 310

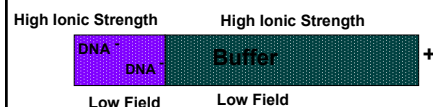


Stacking Effects

(a) Stacking with Low Ionic Strength



(b) Regular EK Injection



Ion Mobility Effects

The Injection of DNA by voltage is described by

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

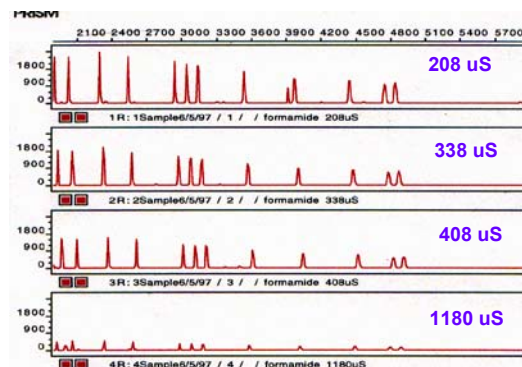
However this equation assumes no interfering ions are present.

Cl⁻ ions and other interferents will compete with DNA

$$\{DNA_{inj}\} = [DNA_{inj}]/[other\ ions_{inj}]$$

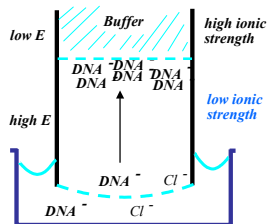
Ions such as Cl⁻ have a higher charge/mass ratio and μ_{ep} is greater

Effect of Formamide Conductance on DNA Concentration



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 = $\mu_{ep} = \text{velocity} / \text{electric field}$



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 $^{\circ}\text{C}$ with thermocycler
4. Cool to 4 $^{\circ}\text{C}$ in thermocycler or ice bath
5. Sample will remain denatured for at least 3 days

Comments on Sample Preparation

- Use high quality formamide (<100 $\mu\text{S}/\text{cm}$)!
 - ABI sells Hi-Di formamide
 - regular formamide can be made more pure with ion exchange resin (or less! You better measure it, aliquot it out, and freeze it!)
- Deionized water vs. formamide
 - Biega and Ducean (1999) *J. Forensic Sci.* 44: 1029-1031
 - Crivellente, *Journal of Capillary Electrophoresis* 2002, 7 (3-4), 73-80.
 - 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

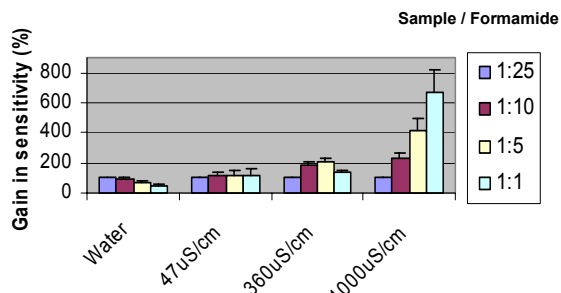
Electrokinetic injection has some unusual properties!

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

Solvent	Resolution	Peak Height
Water	1.19+/- 0.01	2700+/- 300
Formamide (27 μS)	1.15+/- 0.05	2960+/- 30
Formamide (360 μS)	1.20+/- 0.08	879 +/- 4
Formamide 1000 μS)	1.20+/- 0.06	290 +/- 14

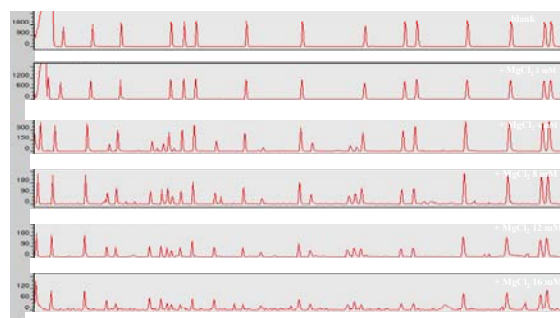
Effect of increasing relative sample volume

Sensitivity improves only in poor quality formamide!



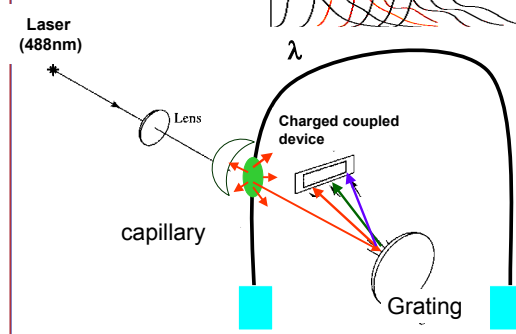
As [DNA] increases so does ionic strength, making injection almost independent of μL DNA injected

Effect of Increasing salt concentration (Renannealed ds DNA moves faster)



Detection

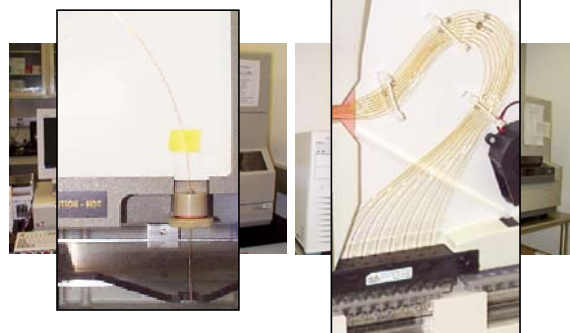
OPTICS



Capillary Electrophoresis Instrumentation

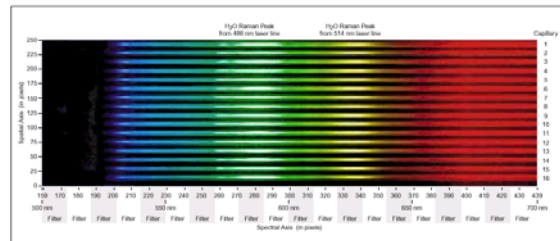
ABI 310
single capillary

ABI 3100
16-capillary array



CCD Image of 16-Capillary Array

CCD CAMERA IMAGE



Full frame CCD camera image of capillary array #30A228-09 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.

Detection Issues

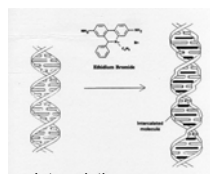
- Fluorescent dyes
 - spectral emission overlap
 - relative levels on primers used to label PCR products
 - dye “blobs” (free dye)
- Virtual filters (determine which pixels are used)
 - hardware (CCD camera)
 - software (color matrix)

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



3'TGCATCTACGATGTAATCG5'

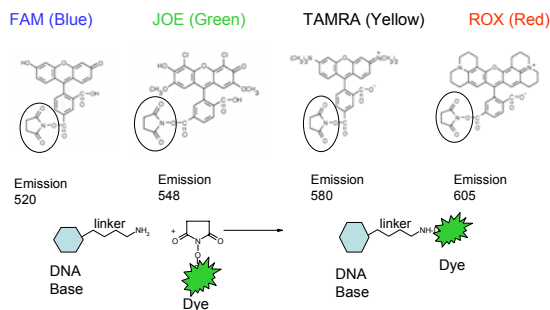
CGTAGCTG3'

ENZYME

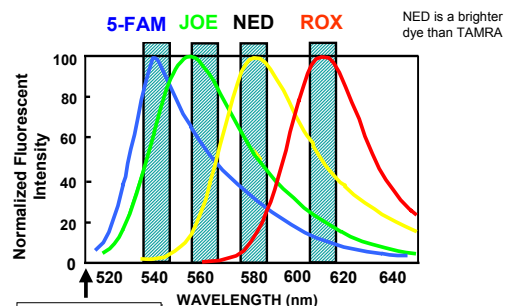
Linkers, dyes, etc. can be added to the 5' end of the primer without disturbing the reaction. Individual bases can also be tagged

Covalent labeling process

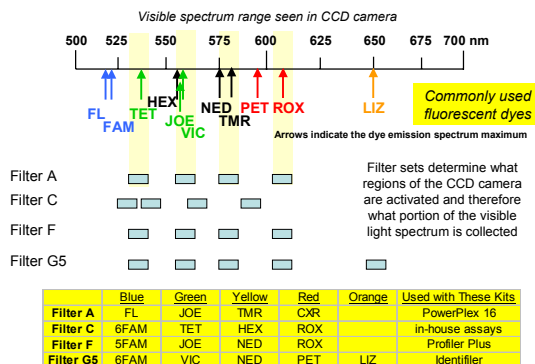
Amine Reactive Dyes used in Labeling DNA



Fluorescent Emission Spectra for ABI Dyes

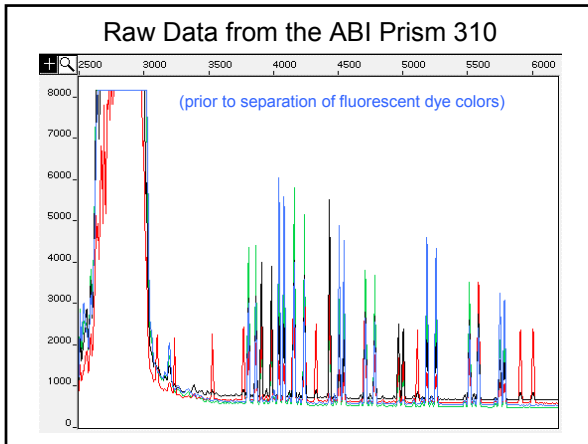


Virtual Filters Used in ABI 310



Please Note!

- There are no filters in a 310
- Its 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

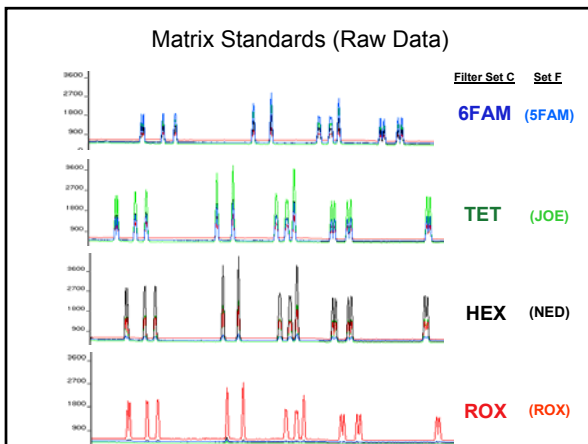


Why Make a Matrix?

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



For Example

$$I_{540} = bx_b + gy_b + yz_b + rw_b \text{ intensity of blue}$$

$$I_{560} = bx_g + gy_g + yz_g + rw_g \text{ intensity of green}$$

$$I_{580} = bx_y + gy_y + yz_y + rw_y \text{ intensity of yellow}$$

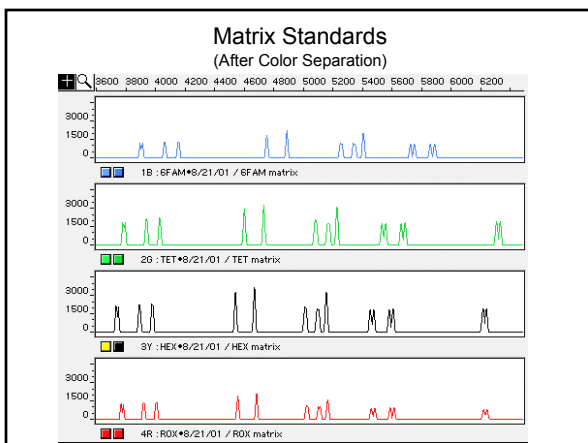
$$I_{610} = bx_r + gy_r + yz_r + rw_r \text{ intensity of red}$$

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)

POP-4STR-MODF				
Reactions				
	B	G	Y	R
B	1.0000	0.8502	0.1380	0.0009
G	0.8300	1.0000	0.7622	0.0051
Y	0.6416	0.8324	1.0000	0.1102
R	0.4493	0.6484	0.7851	1.0000

If you solve xyzw for each dye individually
Then you can determine dye contribution for any mixture

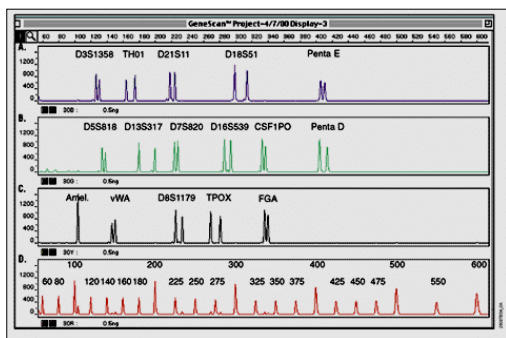


DNA Size Estimation with ABI 310

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



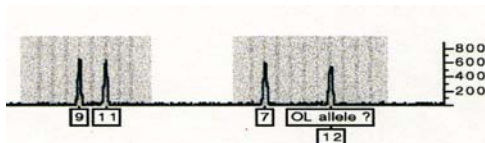
The red internal lane standard establishes the allele size.
This size is compared to an allelic ladder run earlier.

Assumptions with ABI 310 Method affecting precision

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
- That will be discussed in future sections.

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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- Florida International University
- Kerry Opel
- Rayna Hebard
- Sarah Hughes
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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