Capillary Electrophoresis

Fundamentals and Troubleshooting

John M. Butler, PhD
National Institute of Standards and Technology (NIST)

john.butler@nist.gov
001-301-975-4049
http://www.cstl.nist.gov/biotech/strbase/training.htm

ISFG Pre-Conference Workshop
Copenhagen, Denmark
August 21, 2007

NIST and NIJ Disclaimer

Funding: Interagency Agreement 2003-IJ-R-029 between the National Institute of Justice and NIST Office of Law Enforcement Standards

Points of view are mine and do not necessarily represent the official position or policies of the US Department of Justice or the National Institute of Standards and Technology.

Certain commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose.

Our publications and presentations are made available at:
http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm

Steps in DNA Analysis

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

Steps Involved
- Collection
- Specimen Storage
- Extraction
- Quantitation
- Multiplex PCR
- STR Typing
- Interpretation of Results
- Database (Storage & Searching)
- Calculation of Match Probability

Blood Stain Buccal swab
Sample Collection & Storage

DNA Extraction

Slot Blot
1 ng
0.3 ng
No DNA
0.5 ng
0.5 ng
0.7 ng
1 ng
1 ng
DNA Quantitation

Biology

Multiplex PCR Amplification

Genetics

If a match occurs, comparison of DNA profile to population allele frequencies to generate a case report with probability of a random match to an unrelated individual

DNA Database Search

Technology

DNA separation and sizing
STR Typing

Male: 13,14-15,16-12,13-10,13-15,16
Interpretation of Results

Presentation Outline

- History and background on CE
- Separation
- Injection and sample preparation
- Detection
- Troubleshooting the ABI 310/3100/3130xl

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

Background of Participants…

Name
Experience (years) with DNA typing
Something memorable about yourself
What you hope to learn from this workshop

STR Typing Technologies

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

Gels	**Capillary Electrophoresis**	Capillary Arrays
J. Forensic Sci. (1998) 43: 1168-1180	*Electrophoresis.* (1998) 19: 86-93	*Nucleic Acids Res.* (1999) 27: e36
Microchip CE	**Mass Spectrometry**	Hybridization Arrays
PNAS (1997) 94: 10273-10278	*Int. J. Legal Med.* (1998) 112: 45-49	*Nucleic Acids Res.* (2000) 28: e17

Pioneers of Capillary Electrophoresis

Stellan Hjertén
Uppsala University

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

James Jorgenson
University of North Carolina

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

Barry Karger
Northeastern University

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

Stellan Hjertén

Uppsala University (Sweden)

With first fully automated capillary free zone electrophoresis apparatus in 1967

In 2003 at age 75

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

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 allelic 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

My Experience with CE, STRs, etc. (cont.)

- 1996-1997 Developed STRBase while a postdoc at NIST
- Nov 1998 – GeneTrace Systems purchased a 310; typed several hundred samples with Profiler Plus and Cofiler kits and compared results to mass spec STR analysis
- **1999-present – Run thousands of samples with all STR kits available (except PP 1.2) and developed a number of new STR multiplex systems**
- Jan 2001 – Published "*Forensic DNA Typing: Biology and Technology behind STR Markers*" (2nd Edition in Feb 2005)
- April 2001-present – Use of ABI 3100 16-capillary array system

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
  - Themal degradation
- Alternative was to not use a gel at all
  - Refillable sieving polymers
  - However, resolution was poor early on

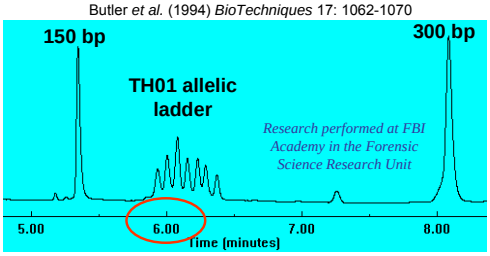
Early Work with CE and STRs

- 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
- Beckman P/ACE 2050 is introduced in 1992 as the first commercially available CE coupled to a laser to enable fluorescence detection
- John Butler and Bruce McCord (1993-1995)
  - **First STR typing with single color CE using intercalating dyes and dual bracketing internal size standards**
- Rich Mathies' group (1995)
  - 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**

First Rapid STR Typing with Capillary Electrophoresis

Single color detection with dual internal size standards

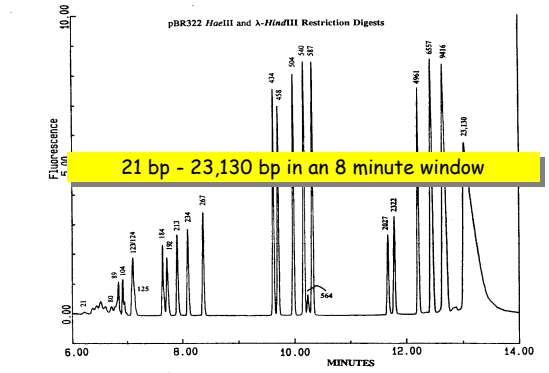
Butler *et al.* (1994) *BioTechniques* 17: 1062-1070



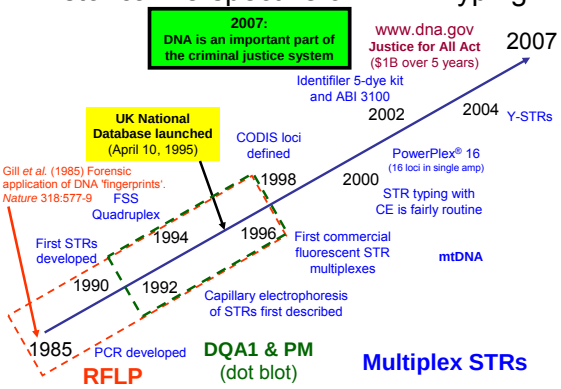
Performed in December 1993

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

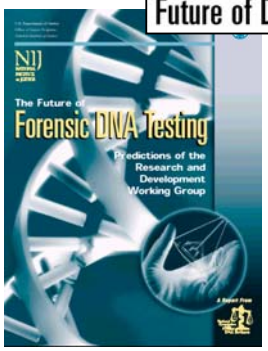
Results from 1995 Butler Ph.D. Dissertation



Historical Perspective on DNA Typing



National Commission on the Future of DNA Evidence



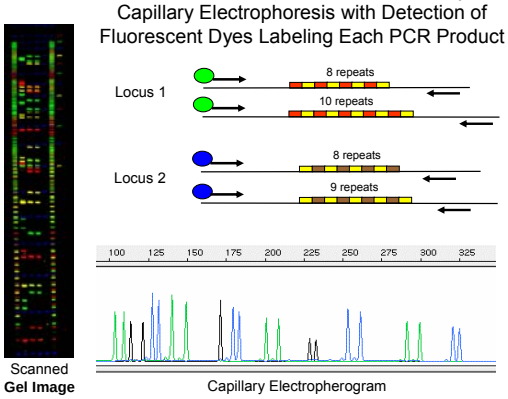
http://www.ojp.usdoj.gov/nij/pubs-sum/183697.htm

- Report published in Nov 2000
- Asked to estimate where DNA testing would be 2, 5, and 10 years into the future

Conclusions

STR typing is here to stay for a few years because of DNA databases that have grown to contain millions of profiles

STR Allele Separation Can Be Performed by Gel or Capillary Electrophoresis with Detection of Fluorescent Dyes Labeling Each PCR Product

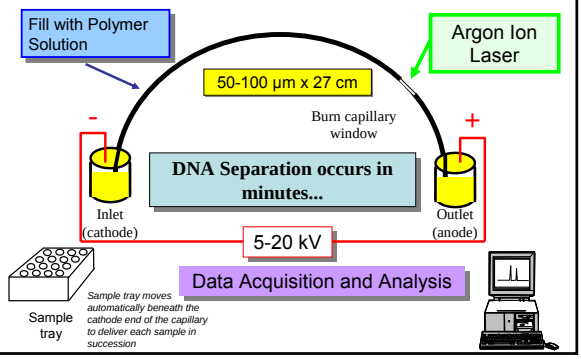


Why Use CE for DNA Analysis?

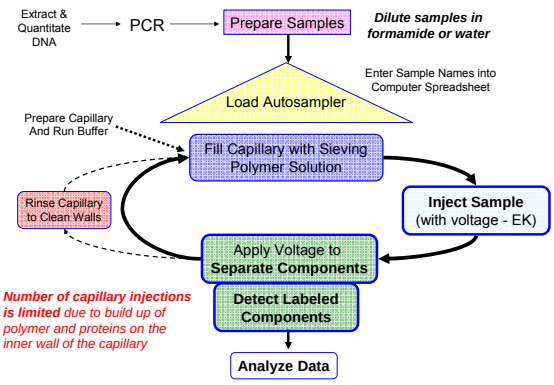
1. **Injection, separation, and detection are automated.**
2. **Rapid separations are possible**
3. **Excellent sensitivity and resolution**
4. **The time at which any band elutes is precisely determined**
5. **Peak information is automatically stored for easy retrieval**

Symbol first used in Oct 1994 at the Promega meeting when I had a poster introducing the use of CE for STR typing

Capillary Electrophoresis (CE)



Flowchart of CE Sample Analysis



Typical Instruments Used for STR Typing

GeneAmp 9700

Thermal Cycler for PCR Amplification

Capillary electrophoresis instruments for separating and sizing PCR products

single capillary

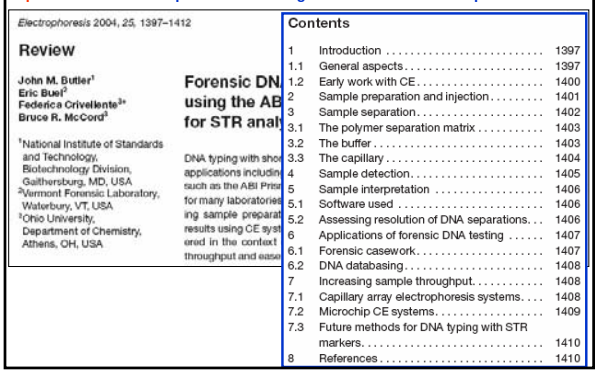
ABI 310

16-capillary array

ABI 3100

Review Article on STRs and CE

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



Electrophoresis 2004, *25*, 1397–1412

Review

Forensic DN using the AB for STR anal

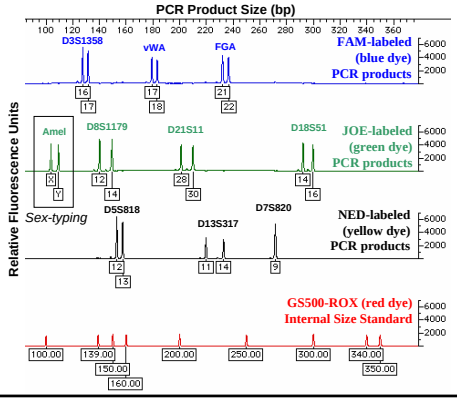
John M. Butler[1]
Eric Buel[2]
Federica Crivellente[3]*
Bruce R. McCord[3]

[1]National Institute of Standards and Technology, Biotechnology Division, Gaithersburg, MD, USA
[2]Vermont Forensic Laboratory, Waterbury, VT, USA
[3]Ohio University, Department of Chemistry, Athens, OH, USA

DNA typing with shor applications includin such as the ABI Prism for many laboratories ing sample preparat results using CE syst ored in the context throughput and ease

Contents

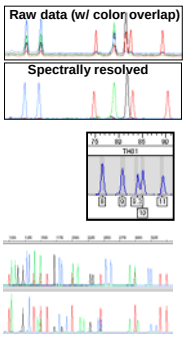
Genotype Results with Profiler Plus™ kit



Analytical Requirements for STR Typing

Butler *et al.* (2004) Electrophoresis 25: 1397-1412

- 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



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…

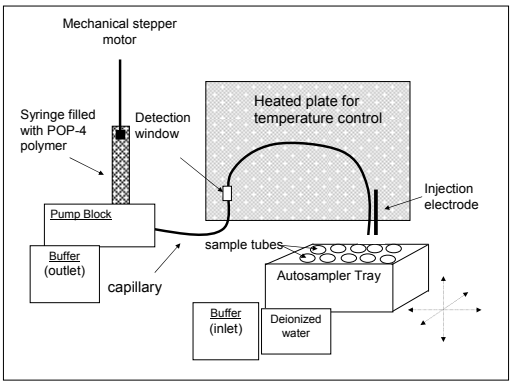
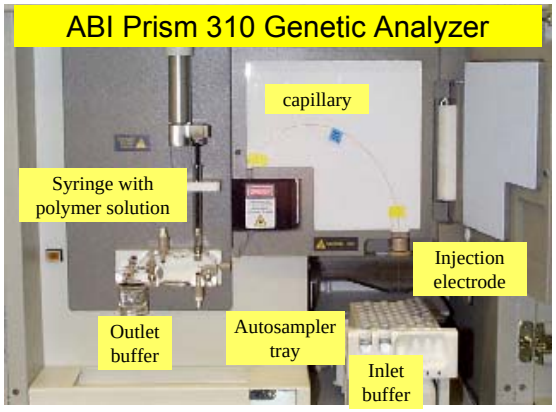
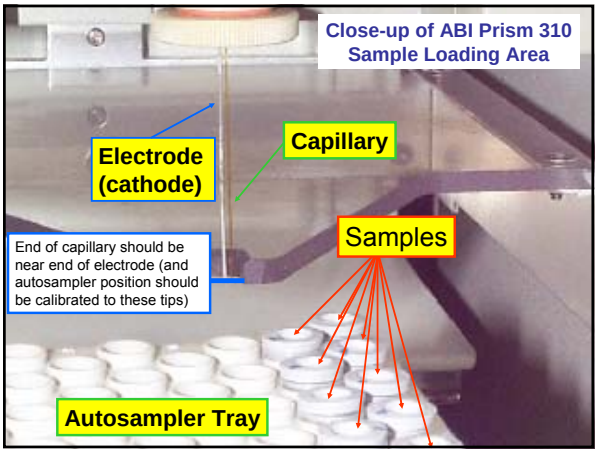
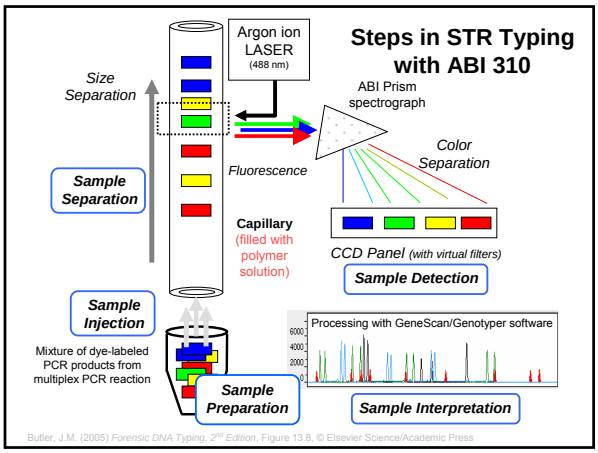
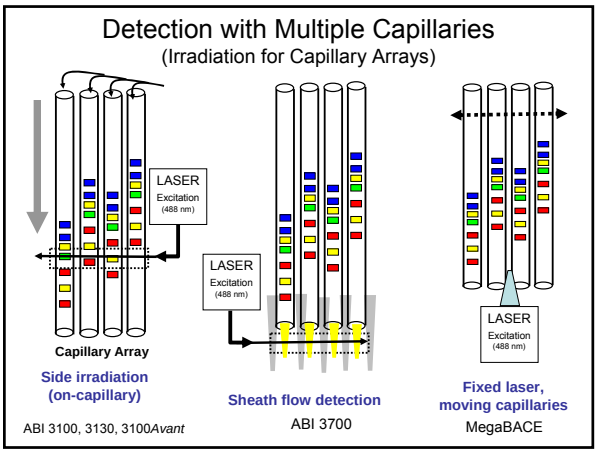


Figure 14.1, J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition © 2005 Elsevier Academic Press









Process Involved in 310/3100 Analysis

- Separation
  - Capillary – 50um fused silica, 43 cm length (36 cm to detector)
  - POP-4 polymer – Polydimethyl acrylamide
  - Buffer - TAPS pH 8.0
  - Denaturants – urea, pyrolidinone
- Injection
  - electrokinetic 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

Separation

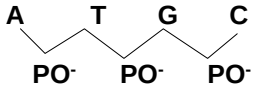
Ohm's Law

- 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

$\mu_{ep} = q/6\pi\eta r$ **small ions with high charge move fastest**



As size increases so does charge!

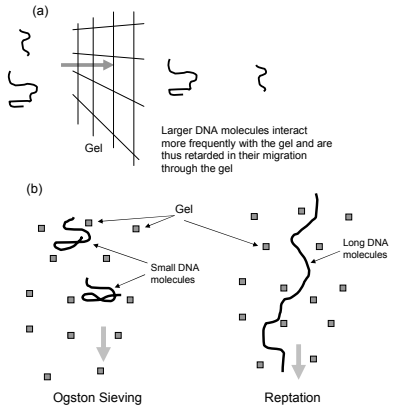


Figure 12.4, J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition © 2005 Elsevier Academic Press

Separation Issues

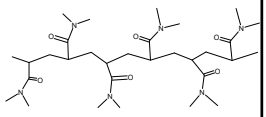
- Electrophoresis buffer –
  - Urea for denaturing and viscosity
  - Buffer for consistent pH
  - Pyrolidinone for denaturing DNA
  - EDTA for stability and chelating metals
- Polymer solution -- POP-4 (but others work also)
- Capillary wall coating -- dynamic coating with polymer
  - Wall charges are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

DNA Separations in Entangled Polymer Sieving Solutions

- Size based separation due to interaction of DNA molecules with entangled polymer strands
- Polymers are not cross-linked (as in slab gels)
- "Gel" is not attached to the capillary wall
- Pumpable -- can be replaced after each run
- Polymer length and concentration determine the separation characteristics

Entangled Polymer Solutions

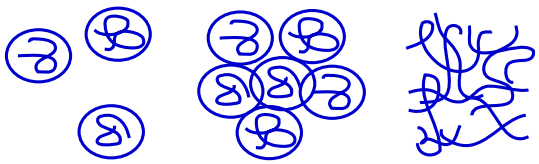
- Polymers are not cross-linked (above entanglement threshold)
- "Gel" is not attached to the capillary wall
- Pumpable -- can be replaced after each run
- Polymer length and concentration determine the separation characteristics
- Examples:
  - 1% HEC (hydroxyethyl cellulose)
  - 4% polyvinyl pyrolidinone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



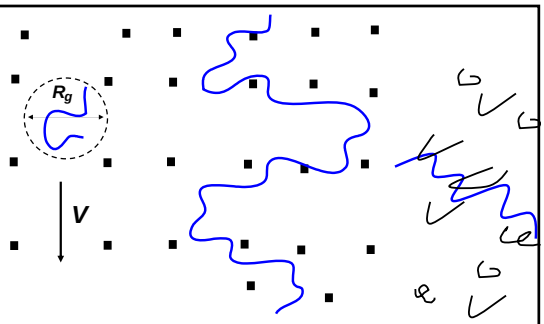
POP4 Polymer

Polydimethyl acrylamide

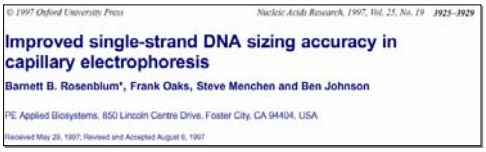
Transient Pores Are Formed Above the Entanglement Threshold.



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



What is in POP-4 and Genetic Analyzer Buffer?



© 1997 Oxford University Press — Nucleic Acids Research, 1997, Vol. 25, No. 19 3925–3929

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

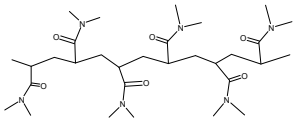
Received May 29, 1997; Revised and Accepted August 6, 1997

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

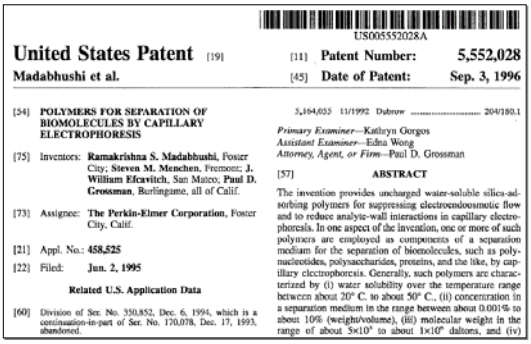
POP-4 (4% poly-dimethylacrylamide, 8 M urea, 5% 2-pyrrolidinone)

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



US Patent Covering POP-4



US005552028A

United States Patent [19]

Madabhushi et al.

[11] **Patent Number: 5,552,028**

[45] **Date of Patent: Sep. 3, 1996**

[54] **POLYMERS FOR SEPARATION OF BIOMOLECULES BY CAPILLARY ELECTROPHORESIS**

[75] Inventors: **Ramakrishna S. Madabhushi**, Foster City; **Steven M. Menchen**, Fremont; **J. William Efcavitch**, San Mateo; **Paul D. Grossman**, Burlingame, all of Calif.

[73] Assignee: **The Perkin-Elmer Corporation**, Foster City, Calif.

[21] Appl. No.: **458,525**

[22] Filed: **Jun. 2, 1995**

Related U.S. Application Data

[60] Division of Ser. No. 350,852, Dec. 6, 1994, which is a continuation-in-part of Ser. No. 170,078, Dec. 17, 1993, abandoned.

5,164,055 11/1992 Dubrow 204/180.1

Primary Examiner—Kathryn Gorgos
Assistant Examiner—Edna Wong
Attorney, Agent, or Firm—Paul D. Grossman

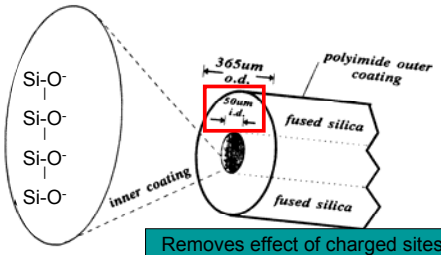
[57] **ABSTRACT**

The invention provides uncharged water-soluble silica-adsorbing polymers for suppressing electroendoosmotic flow and to reduce analyte-wall interactions in capillary electrophoresis. In one aspect of the invention, one or more of such polymers are employed as components of a separation medium for the separation of biomolecules, such as polynucleotides, polysaccharides, proteins, and the like, by capillary electrophoresis. Generally, such polymers are characterized by (i) water solubility over the temperature range between about 20° C. to about 50° C., (ii) concentration in a separation medium in the range between about 0.001% to about 10% (weight/volume), (iii) molecular weight in the range of about 5×10^3 to about 1×10^6 daltons, and (iv)

Why TAPS instead of Tris-borate (TBE) buffer?

- TBE is temperature/pH sensitive
  - as temperature increases, the pH decreases (0.02 pH units with every 1 °C); this is the principle by which TaqGold activation works
- At lower pH, fluorescence emission of dyes decreases
  - see Singer and Johnson (1997) *Proceedings of the Eighth International Symposium on Human Identification*, pp. 70-77
- Thus when running at 60 °C on the ABI 310, if Tris-borate was used, fluorescent intensity of PCR products would be lower

Capillary Coating

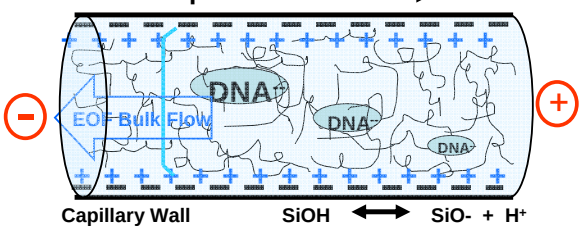


Removes effect of charged sites-eliminates EOF, sample adsorption

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

Capillary Wall Coatings Impact DNA Separations

Electrophoretic flow →



Capillary Wall **SiOH ⟷ SiO- + H⁺**

← Electroosmotic flow (EOF)

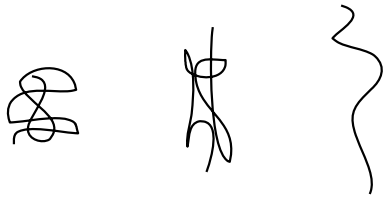
Solvated ions drag solution towards cathode in a flat flow profile

How to Improve Resolution?

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

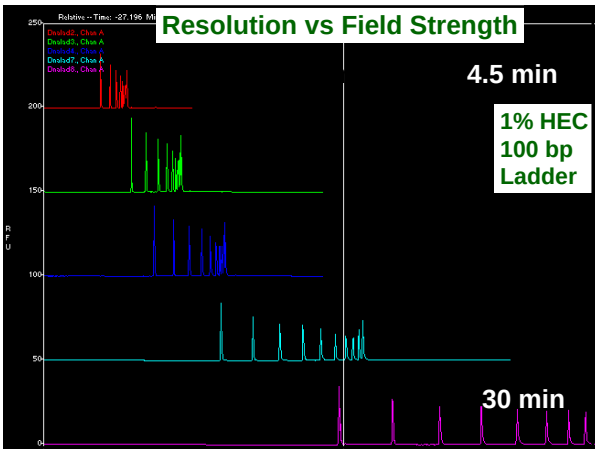
All of these come at a cost of longer separation run times

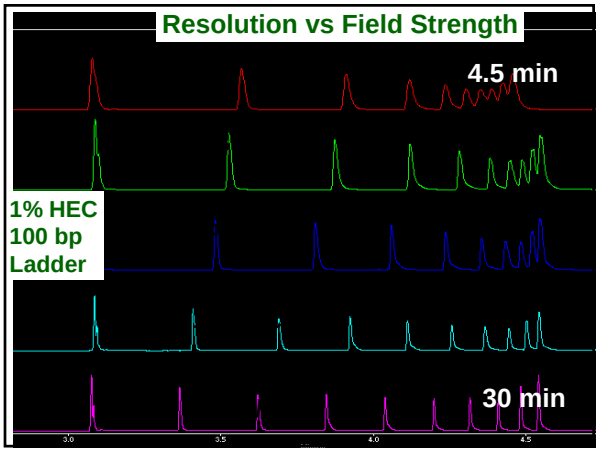
The electric field strength can influence the shape of the DNA molecule.



low *moderate* *high*

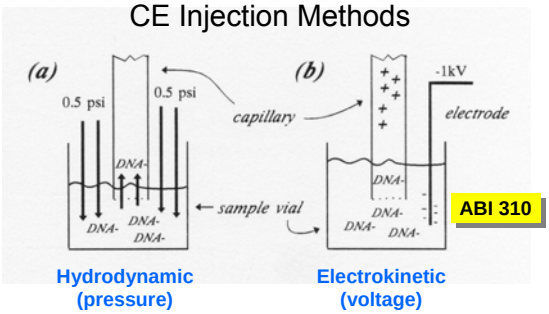
Optimal separations usually 180-200 V/cm





Injection

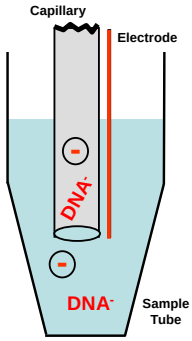
CE Injection Methods



Ulfelder K. J.; McCord, B. R. (1996) Capillary Electrophoresis of DNA, In *Handbook of Capillary Electrophoresis* (Landers, J., ed.), CRC Press: NY, pp. 347-378.

Butler, J.M. (1997) Effects of sample matrix and injection on DNA separations. *Analysis of Nucleic Acids by Capillary Electrophoresis* (Heller, C., ed.), Vieweg: Germany, Chapter 5, pp. 125-134

Electrokinetic Injection Process



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

Salty samples result in poor injections

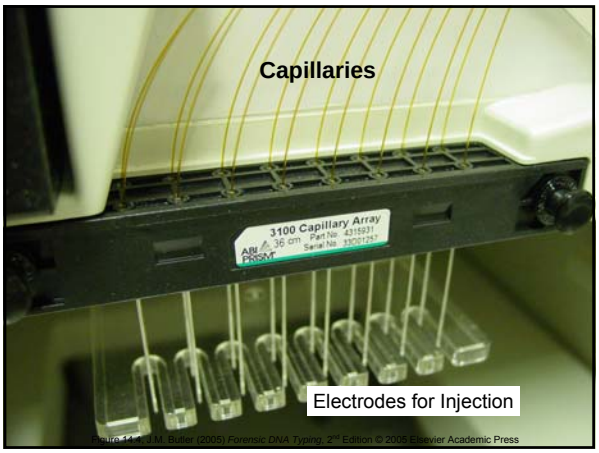


Figure 14.6, J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition © 2005 Elsevier Academic Press

Sample Conductivity Impacts Amount Injected

$$[DNA_{inj}] = \frac{Et(\pi r^2)\,(\mu_{ep} + \mu_{eof})[DNA_{sample}]\,(\lambda_{buffer})}{\lambda_{sample}}$$

$[DNA_{inj}]$ is the amount of sample injected

E is the electric field applied

t is the injection time

r is the radius of the capillary

μ_{ep} is the mobility of the sample molecules

μ_{eof} is the electroosmotic mobility

$[DNA_{sample}]$ is the concentration of DNA in the sample

λ_{buffer} is the buffer conductivity

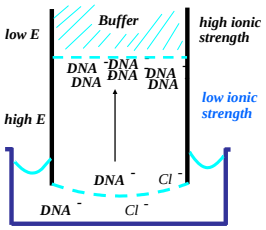
λ_{sample} is the sample conductivity

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

Butler *et al.* (2004) Electrophoresis 25: 1397-1412

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 = μ_{ep} = velocity/ electric field



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; 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 no spectral deconvolution of the different dye colors; the matrix is applied during Genescan analysis

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

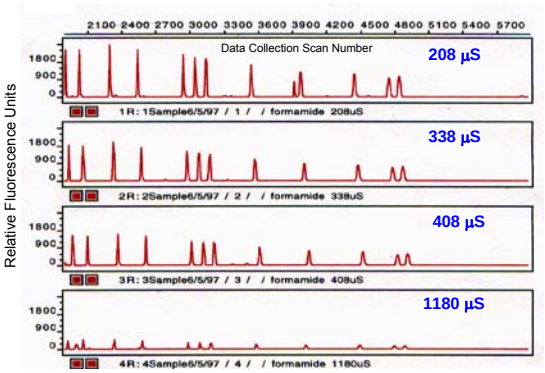


Figure 14.3, J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition © 2005 Elsevier Academic Press

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
  - Biega and Duceman (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...

January 6, 2005 Letter from Applied Biosystems to ABI 310 Customers

- "Testing has shown that Hi-Di Formamide denatures DNA **without the need to heat samples**…"
- In other words, no heat denaturation and snap cooling needed!

Applied Biosystems Okays Use of Deionized Water for DNA Sequencing

Technical Bulletin #1 **Issued August 2006**

Applied Biosystems 3730/3730*xl* DNA Analyzer

Subject: Influence of Sequencing Injection Solution on 3730/3730xl DNA Analyzer Performance

In this Bulletin:

- Three Loading Solutions Tested on Page 1
- Loading Solution Test Data on Page 2
- Recommendations on Page 6
- Guidelines for Use on Page 6

Three Loading Solutions Tested

Loading Solution Background

Applied Biosystems presently recommends the use of Hi-Di™ Formamide as the sample-loading solution for all Applied Biosystems DNA sequencers to ensure sample preservation and resistance to evaporation. However, many users of the 3730 choose either deionized water or dilute EDTA solutions. These choices are driven largely by cost and safety/hazardous material considerations.

Detection

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)

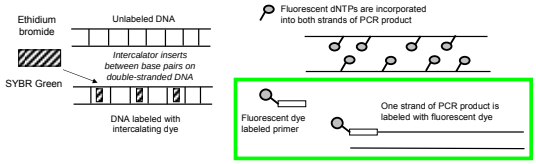
Filters determine which wavelengths of light are collected onto the CCD camera

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

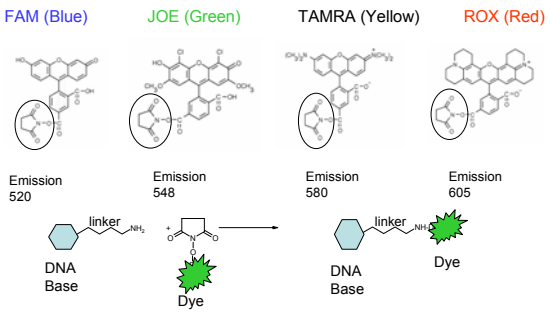


Butler, J.M. (2001) *Forensic DNA Typing*, Figure 10.2, ©Academic Press

Fluorescent Labeling of PCR Products

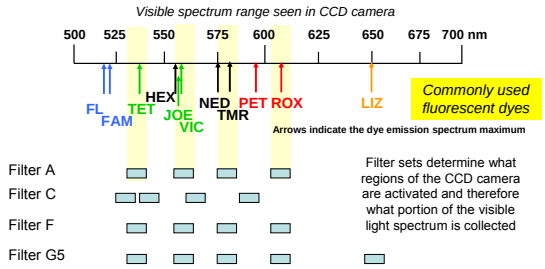
- Dyes are attached to one primer in a pair used to amplify a STR marker
- Dyes are coupled to oligonucleotides (primers) through NHS-esters and amine linkages on the 5'end of the primer: Dye-$(CH_2)_6$-primer
- Dye-labeled oligonucleotides are incorporated during multiplex PCR amplification giving a specific color "tag" to each PCR product
- PCR products are distinguished using CCD imaging on the 310

Amine Reactive Dyes used in Labeling DNA



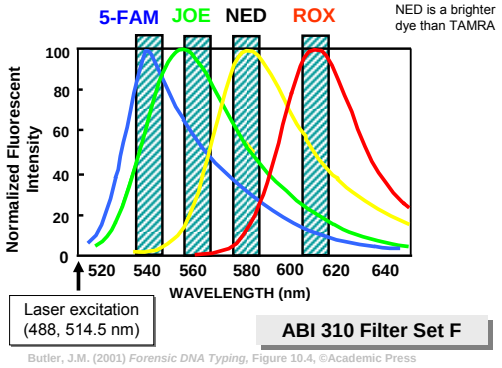
The succinimidyl ester reacts rapidly with amine linkers on DNA bases

Virtual Filters Used in ABI 310



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

Fluorescent Emission Spectra for ABI Dyes



Butler, J.M. (2001) *Forensic DNA Typing*, Figure 10.4, ©Academic Press

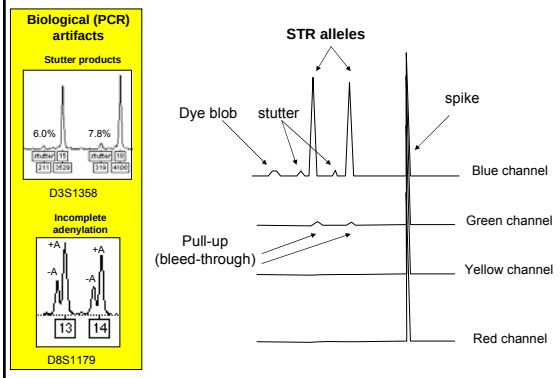
Please Note!

- 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

Comments on Matrices/Spectral Calibration (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

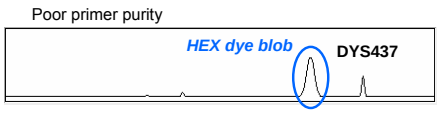
Deciphering Artifacts from the True Alleles



Butler, J.M. (2005) *Forensic DNA Typing, 2nd Edition*, Figure 15.4, © Elsevier Science/Academic Press

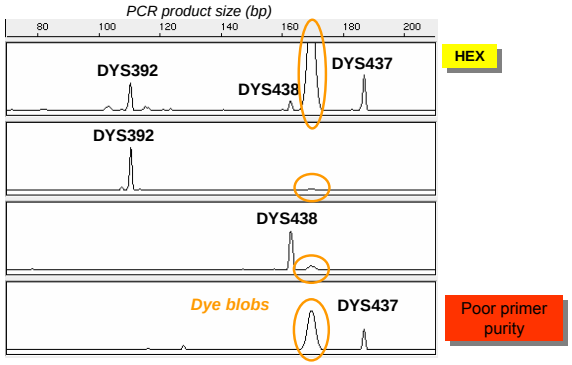
Dye Blobs ("Artifacts")

- Free dye (not coupled to primer) can be injected into the CE capillary and interfere with detection of true STR alleles
- **Dye blobs are wider and usually of less intensity** than true STR alleles (amount depends on the purity of the primers used)
- Dye blobs usually appear at an apparent size that is unique for each dye (e.g., FAM ~120 bp, PET ~100 bp)

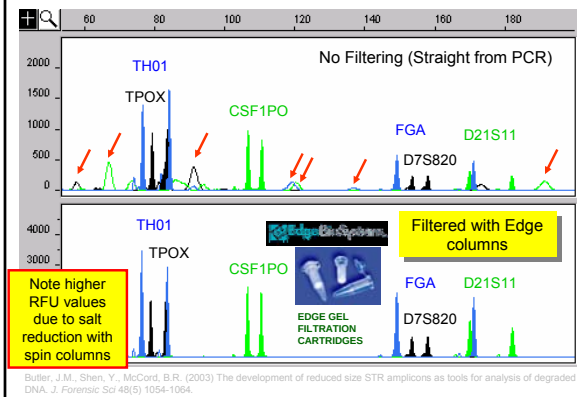


Dye Blob Problems with Some PCR Primers

Individual Y-STR Locus Amplifications



Removal of Dye Artifacts Following PCR Amplification



Butler, J.M., Shen, Y., McCord, B.R. (2003) The development of reduced size STR amplicons as tools for analysis of degraded DNA. *J. Forensic Sci* 48(5) 1054-1064.

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

Practical Aspects of ABI 310/3100 Use

ABI Genetic Analyzer Usage at NIST

- ABI 310 x 2 (originally with Mac, then NT)
  - 1st was purchased in 1996
  - 2nd was purchased in June 2002
- ABI 3100 (Data collection v1.0.1)
  - Purchased in June 2002
  - Original data collection software retained

Jan 2007 – upgraded to 3130xl with data collection v3.0

- ABI 3130xl upgrade (Data collection v3.0)
  - Purchased in April 2001 as ABI 3100
  - Upgraded to ABI 3130xl in September 2005
  - Located in a different room

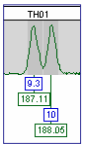
Our Use of the ABI 3100

- Data collection software, version 1.0.1
- **POP-6** with 36 cm capillary array
- STR kits and in-house assays for autosomal STRs, Y-STRs, and miniSTRs
- SNaPshot assays for mtDNA SNPs, Y-SNPs, and autosomal SNPs
- DNA sequencing for mtDNA and STR repeat sequencing

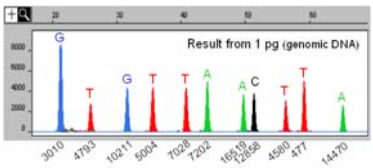
We can routinely get more than 400 runs per capillary array by not changing the polymer between applications

NIST ABI 3100 Analysis Using POP-6 Polymer

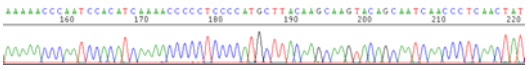
High Resolution STR Typing



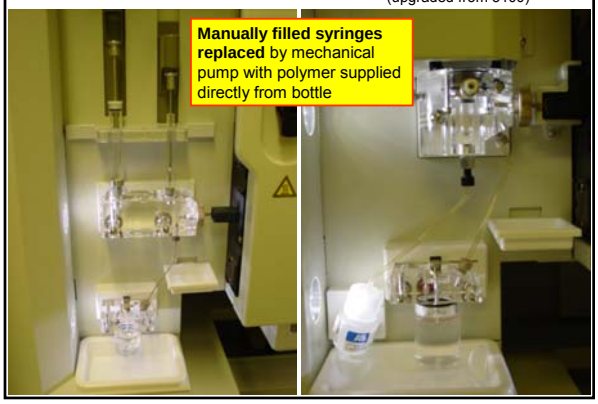
SNaPshot SNP Typing
(Coding Region mtSNP 11plex minisequencing assay)



mtDNA Sequencing (HV1)



ABI 3100	ABI 3130xl (upgraded from 3100)

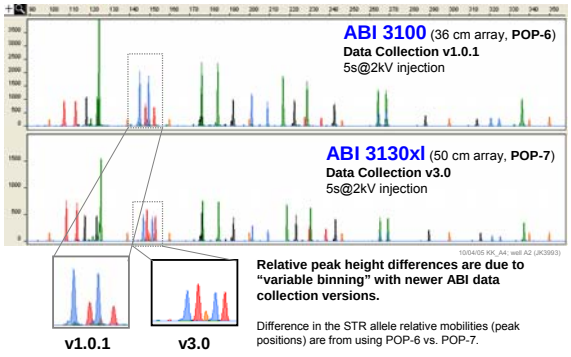


Manually filled syringes replaced by mechanical pump with polymer supplied directly from bottle

Comparison of ABI 3100 Data Collection Versions

Same DNA sample run with Identifiler STR kit (identical genotypes obtained)

GeneScan display



Relative peak height differences are due to "variable binning" with newer ABI data collection versions.

Difference in the STR allele relative mobilities (peak positions) are from using POP-6 vs. POP-7.

Consumables for ABI 310/3100

What we use at NIST

- A.C.E.™ Sequencing Buffer 10X (Amresco)
  - $155/L = $0.0155/mL 1X buffer **(costs 20 times less!)**
  - http://www.amresco-inc.com
- 3700 **POP-6 Polymer** (Applied Biosystems)
  - $530 / 200 mL = $2.65/mL **(costs 20 times less!)**

What ABI protocols suggest

- 10X Genetic Analyzer Buffer with EDTA
  - $78/25 mL = $0.312/mL 1X buffer (ABI)
- 3100 POP-4 Polymer
  - $365 / 7 mL = $52/mL

2004 prices

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

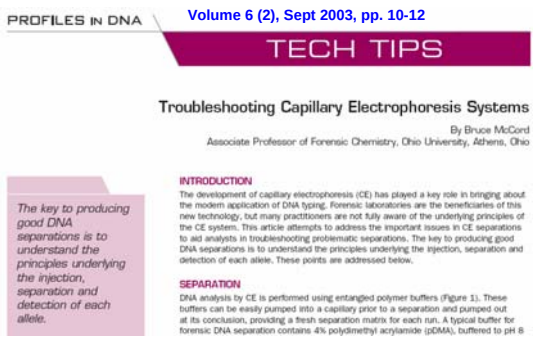
YOU MUST BE CLEAN AROUND A CE!

Troubleshooting

Questions?

- What are your biggest challenges with keeping your ABI 310/3100/3130 running?
- What kind of signal intensity variation are you seeing between your different instruments?
- Have anyone seen uneven injection across a sample plate? (We believe this to be an autosampler calibration issue…e.g., position G10 or H12 does not inject properly)

Bruce McCord's *Profiles in DNA* Article


PROFILES IN DNA

Volume 6 (2), Sept 2003, pp. 10-12

TECH TIPS

Troubleshooting Capillary Electrophoresis Systems

By Bruce McCord
Associate Professor of Forensic Chemistry, Ohio University, Athens, Ohio

The key to producing good DNA separations is to understand the principles underlying the injection, separation and detection of each allele.

INTRODUCTION

The development of capillary electrophoresis (CE) has played a key role in bringing about the modern application of DNA typing. Forensic laboratories are the beneficiaries of this new technology, but many practitioners are not fully aware of the underlying principles of the CE system. This article attempts to address the important issues in CE separations to aid analysts in troubleshooting problematic separations. The key to producing good DNA separations is to understand the principles underlying the injection, separation and detection of each allele. These points are addressed below.

SEPARATION

DNA analysis by CE is performed using entangled polymer buffers (Figure 1). These buffers can be easily pumped into a capillary prior to a separation and pumped out at its conclusion, providing a fresh separation matrix for each run. A typical buffer for forensic DNA separation contains 4% polydimethyl acrylamide (pDMA), buffered to pH 8

Outline for This Section

CE Troubleshooting — Bruce McCord, AAFS 2006 Workshop (Seattle, WA) February 20, 2006

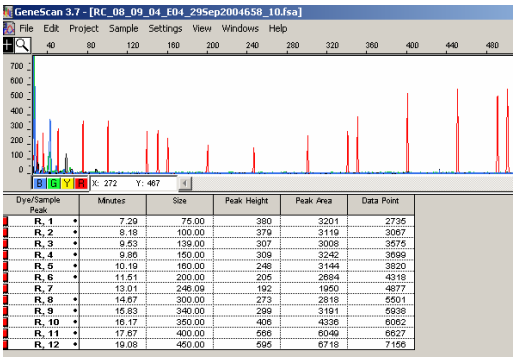
1. ~~Chemistry/molecular biology problems – stutter, -A, degradation, inhibition, low copy #~~
2. Sample and buffer problems – formamide, urea, water, salt concentration, free dye ("dye blobs")
3. External factors – power supply, room temperature, cleanliness, voltage leaks
4. Instrument problems – optical system, capillary clogging, air bubbles, syringe leaks
5. Troubleshooting benchmarks/QC monitoring

2. Sample Issues

- Formamide Conductivity
- Excessive salt in sample due to evaporation
- Metal ion contamination
- Sensitivity issues with Microcon cleanup (salt removal)
- Dye "blobs" – artifacts from primer synthesis

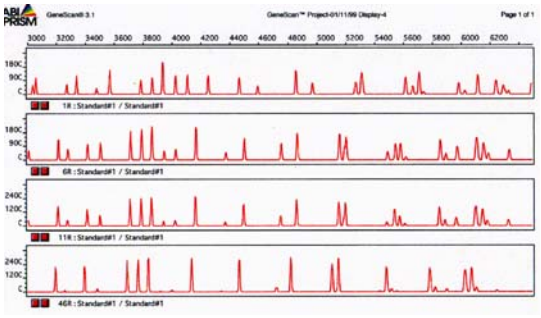
Golden Gate Effect

Attributed to poor formamide



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
R, 1	7.29	75.00	380	3201	2735
R, 2	8.18	100.00	379	3119	3067
R, 3	9.53	139.00	307	3008	3575
R, 4	9.86	150.00	309	3242	3699
R, 5	10.19	160.00	248	3144	3820
R, 6	11.51	200.00	205	2684	4318
R, 7	13.01	246.09	192	1950	4877
R, 8	14.67	300.00	273	2818	5501
R, 9	15.83	340.00	299	3191	5938
R, 10	16.17	350.00	406	4336	6062
R, 11	17.87	400.00	566	6049	6627
R, 12	19.08	450.00	595	6718	7156

Answer: Incomplete denaturation of standard due to poor quality formamide

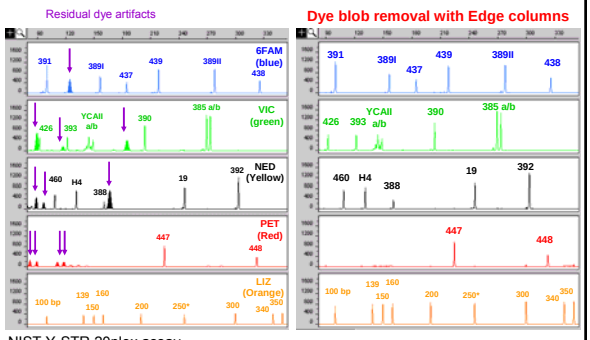


320 V/cm 47 cm uncoated capillary
POP4 Polymer

Post PCR manipulation

- Reprocessing post PCR to concentrate samples can improve signal but be careful
  - PCR sample is concentrated but:
    - Spin filtration may result in removal of background salts,
    - This can greatly enhance sensitivity due to the stacking process
    - Best idea- remake sample up in buffer, not water to avoid reading stochastic effects.

Dye Blobs and their Removal



NIST Y-STR 20plex assay

Butler, J.M. (2005) Constructing STR multiplex assays. *Methods in Molecular Biology: Forensic DNA Typing Protocols* (Carracedo, A., ed.), Humana Press: Totowa, New Jersey, *297: 53-66.*

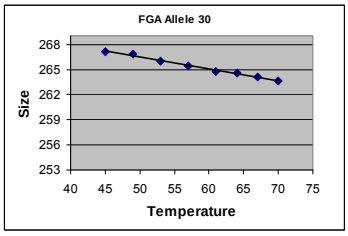
3. External Factors

- Room temperature
  - Variations in room temperature can cause mobility shifts with band shifts and loss of calibration
  - Temperature is also important due to effects of high humidity on electrical conductance
- Cleanliness
  - Urea left in sample block can crystallize and catalyze further crystal formation causing spikes, clogs and other problems.
  - Best bet is to keep polymer in system and not remove or change block until polymer is used up.

Temperature effects

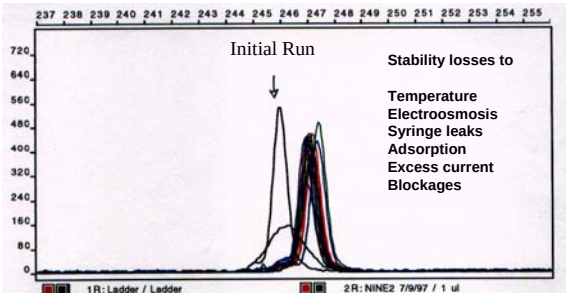
- Viscosity – mobility shift
  - $\mu_{ep} = q/6\pi\eta r$
- Diffusion – band broadening
  - ← DNA →
- Conformation – DNA size based sieving
  - vs $\mu_{ep} = q/6\pi\eta r$
- Current – Power
  - $P = VI = I^2R$
  - Increased current → internal temperature rise → diffusion → band broadening

Effect of Temperature on allele size

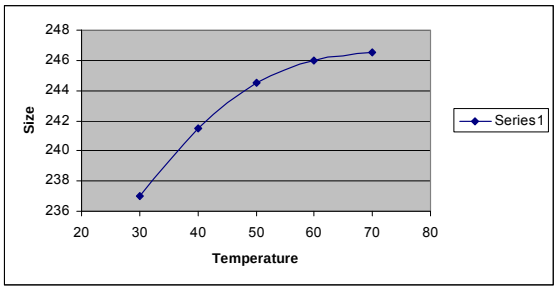


Slope is 0.14 bases/degree centigrade
Therefore a small change in temperature has a big effect
(A 1-2 degree shift in temperature of the heat plate can produce an OL allele)

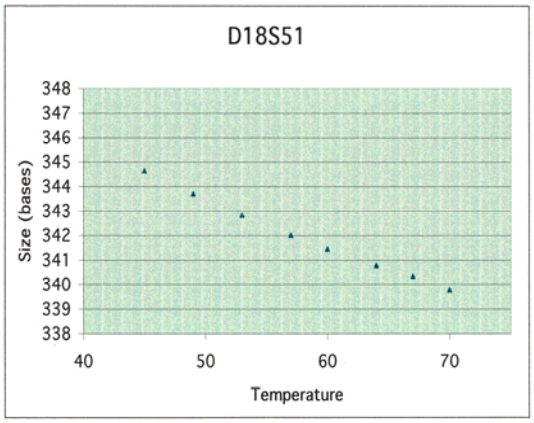
Due to its structure and its non-calibration, the "250"peak can be used to indicate stability

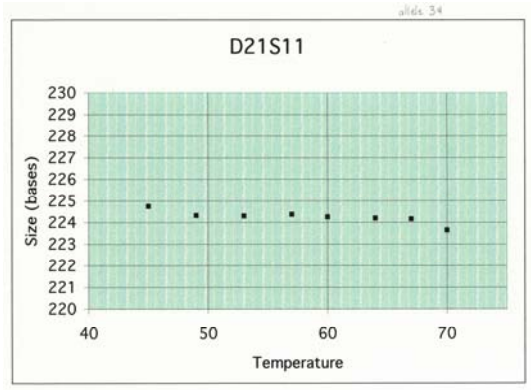


Change in size of GS 250 peak with Temperature (Tamra Std)

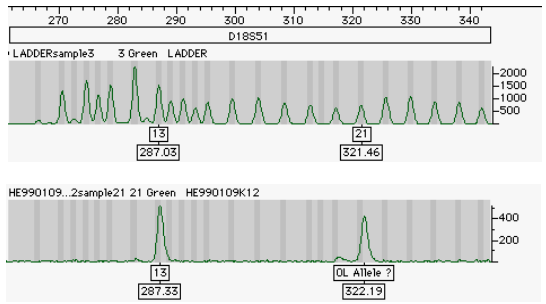


4% pDMA with 8M urea and 5% 2-pyrrolidinone
***Rosenblum et al., Nucleic Acids Res.(1997) 25,19, 2925**

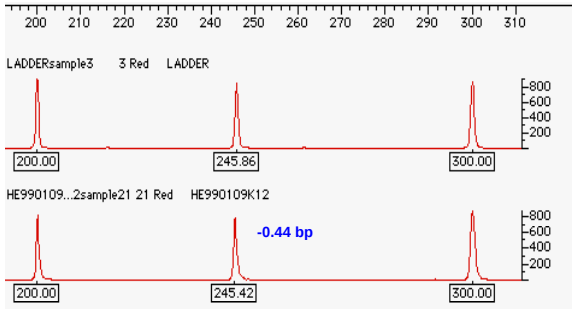




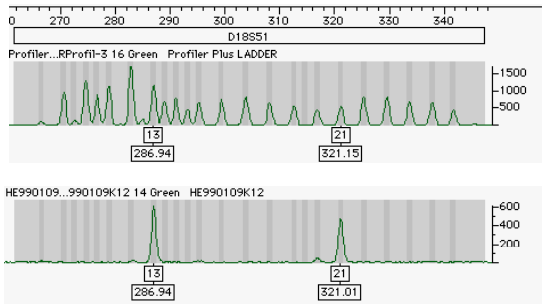
"OL Alleles"



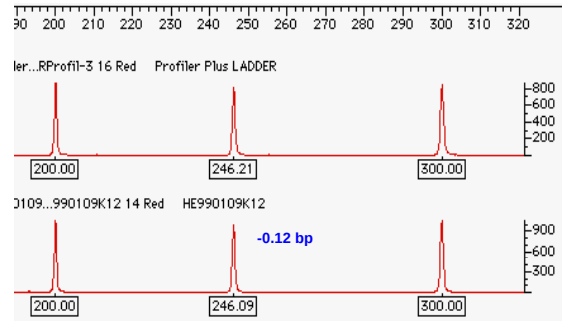
"OL alleles " - look at the 250 peak



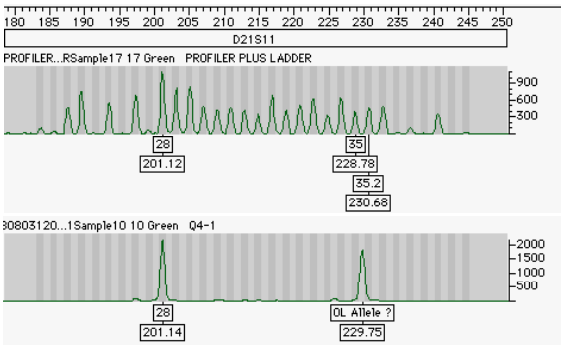
"OL allele re-injected"



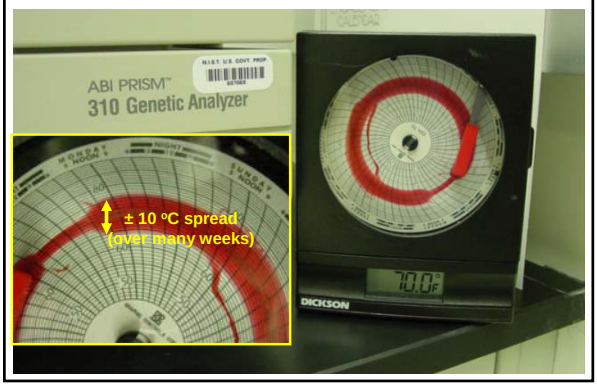
And the 250 peak...



True off-ladder alleles



Monitoring Room Temperature Over Time



Temperature Probes

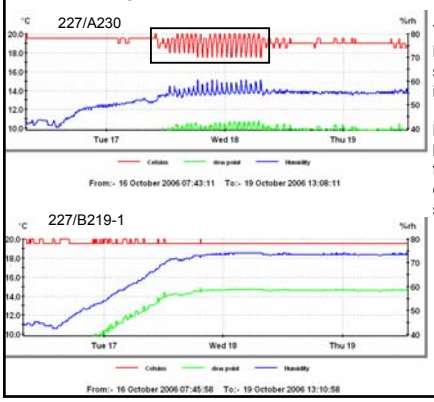
Refrigerator and freezer monitoring

Frig/Freeze Monitors $240
#DT-23-33-80 – USB Temperature Datalogger PLUS Software $79.00 (#DT-23-33-60)

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

Room temperature monitoring

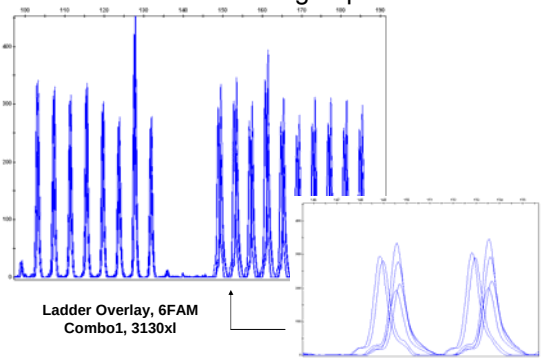
Monitoring Instrument Room Temperature Fluctuations



Temperature Monitoring of two separate instrument rooms.

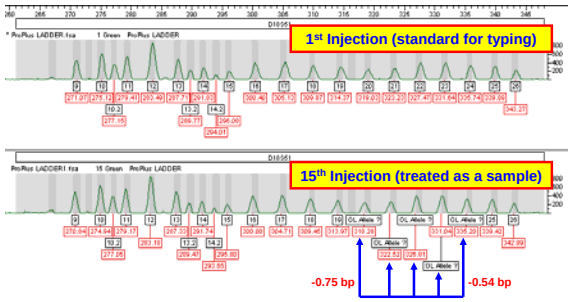
Box area is a 24 hour period where temperature control is not stable.

Poor Temperature Control Causes DNA Sizing Imprecision



Ladder Overlay, 6FAM Combo1, 3130xl

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

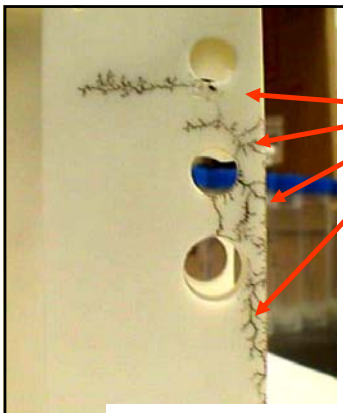
What to do if calibration is lost?

The 310 only calibrates to the first run ladder
this ladder sample may have been run at a different temperature!

- If protocol permits
  - Go to the next ladder
  - Rerun sample
  - Check current
  - Check allelic ladder
- Always check the ROX size standard
  - Look for extra bands
  - Check peak height
  - Check parameters and alignment

Cleanliness

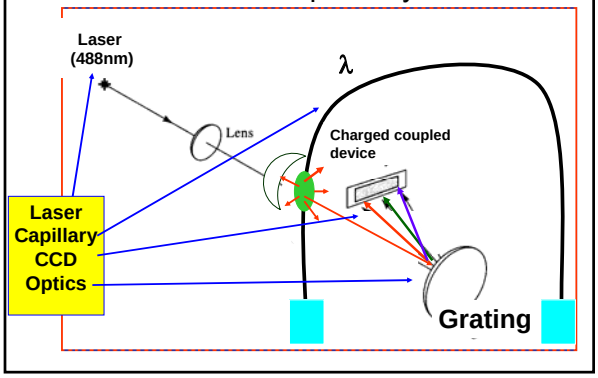
- Urea sublimates and breaks down to ionic components - these find a path to ground
- Similarly wet buffer under a vial creates paths to ground
- Capillary windows must be clear or matrix effects will occur
- Laser will often assist in this process
- Vial caps will transfer low levels of DNA to capillary



4. Instrumental Factors

- Optical System
  - Sensitivity changes with age, capillary diameter, capillary cleanliness, instrument calibration
- Fluidic System
  - Effects of bubbles, dust, urea crystals, leaks in syringe and capillary ferrule
- Matrix Calculations
  - Changes in buffer, optics, sample dye can alter the software calibrations
- Capillary Problems
  - Chemisorbed materials on capillary surface can produce osmotic flow, DNA band broadening and inconsistent resolution (meltdowns)

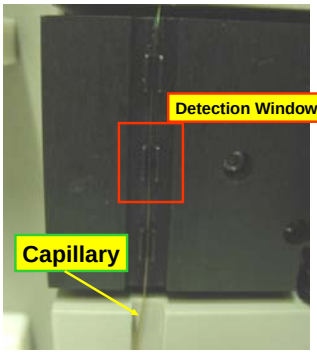
Consider the optical system



Issues with the Optical System

- **Pay attention to signal to noise, not absolute peak intensity**
- Argon Ion lasers outgas and eventually loose intensity; **take note of laser current and monitor it over time**
- Fluorescence expression:
  $I_f = I_0 k \varepsilon b C \varphi$ - changes in input intensity, I_0
  - changes in capillary diameter, b
  - cleanliness of capillary, k
  - All these things directly affect peak RFUs, however, baseline noise is more affected by detector.
- **Thus by monitoring signal to noise, you can get a better picture of your optical system.**

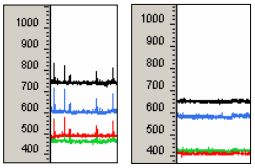
The Detection Window



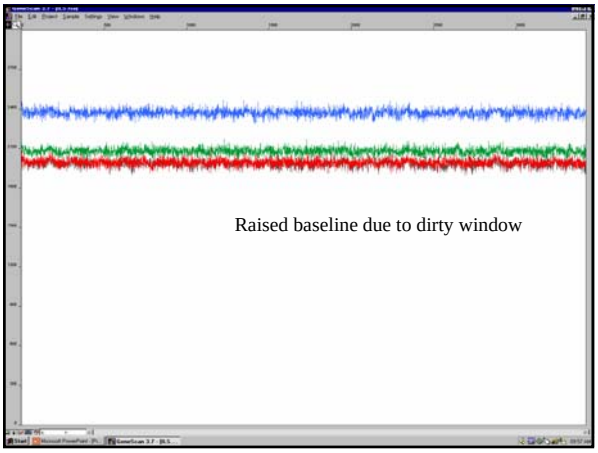
Make sure that the capillary window is lined up (if it is not, then no peaks will be seen)

Window may need to be cleaned with ethanol or methanol

Review Start of Raw Data Collection



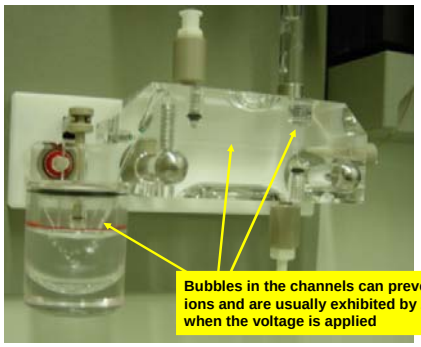
Little spikes indicate need to change buffer… check current

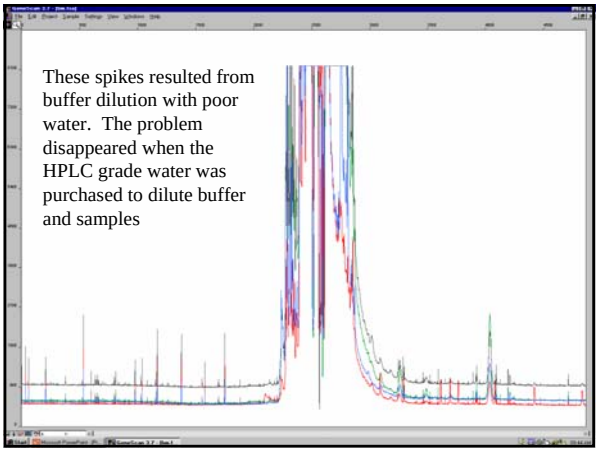


Buffer Issues

- 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

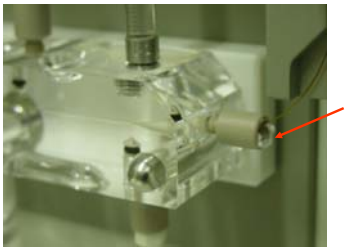
Remove all bubbles from the channels





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

Beware of Urea Crystals



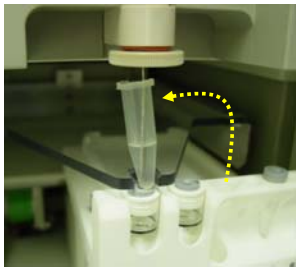
Urea crystals have formed due to a small leak where the capillary comes into the pump block

Urea sublimates and can evaporate to appear elsewhere

Use a small balloon to better grip the ferrule and keep it tight

Pump block should be well cleaned to avoid problems with urea crystal formation

Storage when ABI 310 is not in use

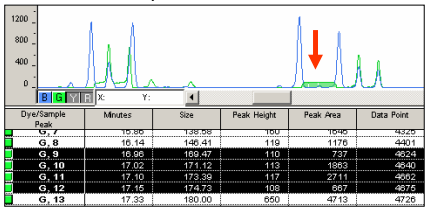


Remember that the water in the open tube will evaporate over time…

- Keep inlet of capillary in water…if it dries out then urea crystals from the polymer will clog the opening
- The waste vial (normally in position 3) can be moved into position
- A special device can be purchased from Suppelco to rinse the capillary off-line
- Store in distilled water
- Note that the laser is on when the instrument is on

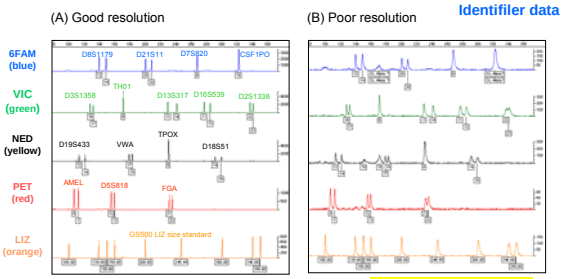
Matrix Problems

- A poor matrix can lead to raised baseline and therefore calling of too many peaks
- Larger sized alleles will not be identified as peaks because the GeneScan table for a particular dye color has filled up



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
G, 7	15.85	138.58	160	1545	4326
G, 8	16.14	146.41	119	1176	4401
G, 9	16.96	169.47	110	737	4624
G, 10	17.02	171.12	113	1063	4640
G, 11	17.10	173.39	117	2711	4662
G, 12	17.15	174.73	108	667	4675
G, 13	17.33	180.00	650	4713	4726

Capillary Meltdowns



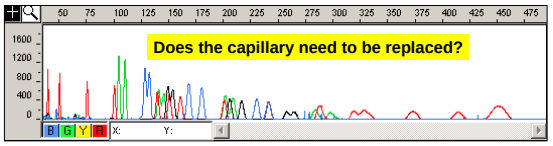
Good Capillary in 3100 Array

Bad Capillary in 3100 Array

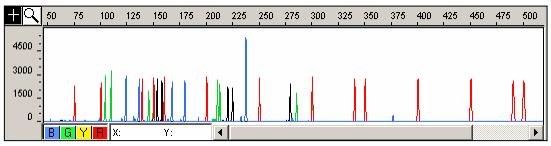
Butler, J.M., Buel, E., Crivellente, F., McCord, B.R. (2004) Forensic DNA typing by capillary electrophoresis: using the ABI Prism 310 and 3100 Genetic Analyzers for STR analysis. *Electrophoresis*, 25: 1397-1412.

Meltdowns can be permanent or transitory

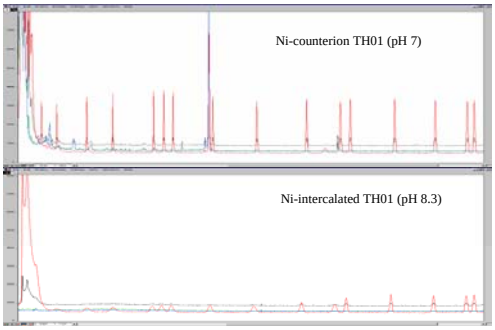
as we have seen these may result from sample contamination effects



No! The next injection looks fine…



CE: Effect of pH 7 vs. 8.3



1 µl TH01 added to 10 µl of 3.0 mM $NiCl_2$ in 10 mM Tris, pH 7 or pH 8.3. Sample allowed to interact for 1 hr and then 1 µl added to ROX/formamide.

Meltdowns may be the result of

- Bad formamide
- Excess salt in sample/renaturation
- Water in the polymer buffer
- Syringe leak or bottom out
- Poisoned capillary
- Conductive polymer buffer due to urea degradation
- Crack/shift in capillary window
- Detergents and metal ions

A permanent loss of resolution may mean

- Adsorptive sites on a capillary
- Initiation of electroosmotic flow
- Conductivity changes in buffer
- Wrong molecular weight or concentration of sieving polymer (viscosity)

5. Troubleshooting benchmarks

- **Monitor run current**
- Observe syringe position and movement during a batch
- Examine ILS (ROX) peak height with no sample
- Observe "250 bp" peak in GS500 size standard
- Monitor resolution of TH01 9.3/10 in allelic ladder and size standard peak shapes
- **Keep an eye on the baseline signal/noise**
- Measure formamide conductivity
- Reagent blank – are any dye blobs present?
- See if positive control DNA is producing typical peak heights (along with the correct genotype)

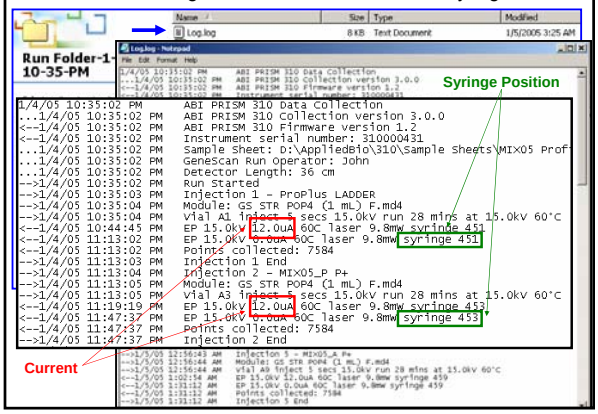
Measurement of Current

- V/I = R where R is a function of capillary diameter, [buffer], and buffer viscosity
- In a CE system the voltage is fixed, thus changes in resistance in the capillary will be reflected in the current observed
- Air bubbles, syringe leaks, alternate paths to ground, changes in temperature, changes in zeta potential, and contamination, will be reflected in the current
- A typical current for a CE system with POP4 buffer is **8-12 µA** (microamps)

Syringe Travel

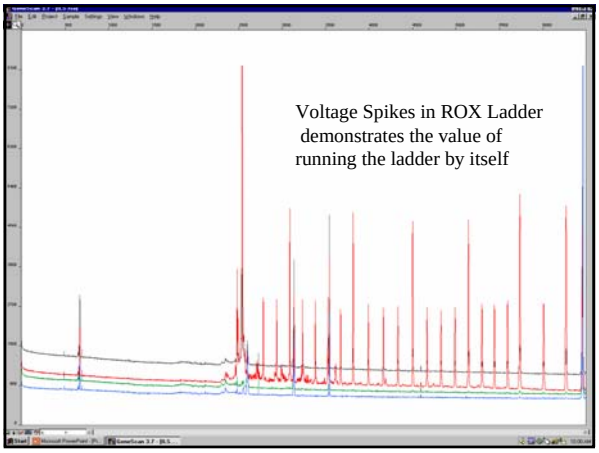
- The ABI 310 instrument also keeps track of the position of the syringe (in the log file)
- Depending on the resistance to flow, the syringe will travel different lengths
- Syringe leaks may be reflected in a longer distance traveled prior to each injection
- These leaks occur around the barrel of the syringe and at the connection to the capillary block

Use of ABI 310 Log File to Monitor Current and Syringe Travel



ROX Ladder QC procedures

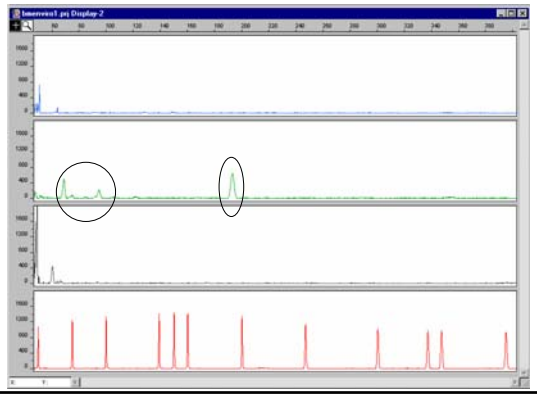
- A recommended sequence for initial operation of the 310
  - Rox ladder – initial injection - throwaway
  - Rox ladder- QC to test peak intensity and look for problems in blank
  - Allelic ladder- to determine resolution and to provide standard
  - 10-15 samples
  - Allelic ladder
  - 10-15 samples
  - Allelic ladder



Measurement of Signal and Noise Ratio

- You can also use the ROX size standard to keep track of sensitivity
  - For a given set of runs determine the average peak height of the ROX standard
  - Monitoring this signal level will help determine if any major loss of sensitivity has occurred
  - You can also measure the P-P noise level in the same way and compare the two values.

Dye Blobs in the Negative Control Sample



Question: What is a real blank?

- Because of the stacking effect, injections of pure water or formamide can produce extreme sensitivity
- This will allow you to detect small amounts of DNA clinging to the capillary, leading to a false impression that carry-over is a problem
- Instead, inject ROX plus formamide as your blank. In this case the added salt and fluorescent DNA drowns out these spurious peaks

Measuring Formamide Conductivity

(not this way)

The key is to measure the bottle when it comes in or buy the good stuff and immediately pipette it out into small tubes with or without ROX already added. Then freeze the tubes.

Do not ever open a cold bottle of formamide. Water will condense inside and aid in the formation of conductive formic acid.

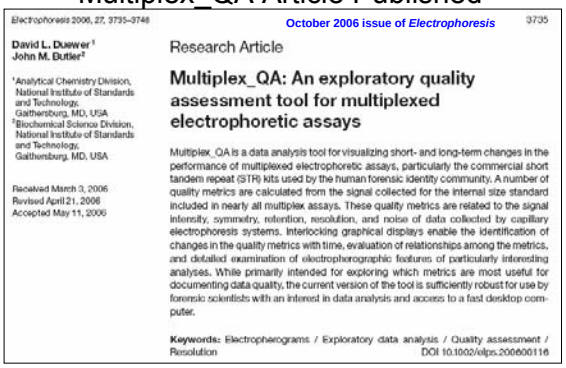
Conclusion:

Troubleshooting is more than following the protocols

It means keeping watch on all aspects of the operation

1. Monitoring conductivity of sample and formamide
2. Keeping track of current and syringe position in log.
3. Watching the laser current
4. Watching and listening for voltage spikes
5. Monitoring room temperature and humidity

Multiplex_QA Article Published



Electrophoresis 2006, 27, 3735–3748 — October 2006 issue of *Electrophoresis* — 3735

David L. Duewer[1] John M. Butler[2]

[1]Analytical Chemistry Division, National Institute of Standards and Technology, Gaithersburg, MD, USA
[2]Biochemical Science Division, National Institute of Standards and Technology, Gaithersburg, MD, USA

Received March 3, 2006
Revised April 21, 2006
Accepted May 11, 2006

Research Article

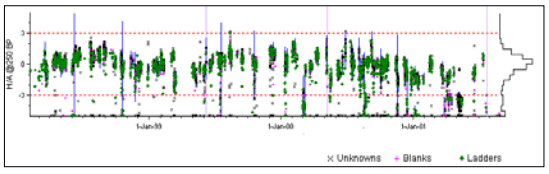
Multiplex_QA: An exploratory quality assessment tool for multiplexed electrophoretic assays

Multiplex_QA is a data analysis tool for visualizing short- and long-term changes in the performance of multiplexed electrophoretic assays, particularly the commercial short tandem repeat (STR) kits used by the human forensic identity community. A number of quality metrics are calculated from the signal collected for the internal size standard included in nearly all multiplex assays. These quality metrics are related to the signal intensity, symmetry, retention, resolution, and noise of data collected by capillary electrophoresis systems. Interlocking graphical displays enable the identification of changes in the quality metrics with time, evaluation of relationships among the metrics, and detailed examination of electropherographic features of particularly interesting analyses. While primarily intended for exploring which metrics are most useful for documenting data quality, the current version of the tool is sufficiently robust for use by forensic scientists with an interest in data analysis and access to a fast desktop computer.

Keywords: Electropherograms / Exploratory data analysis / Quality assessment / Resolution
DOI 10.1002/elps.200600116

User manual (127 pages) available for download from STRBase

Multiplex_QA Overview



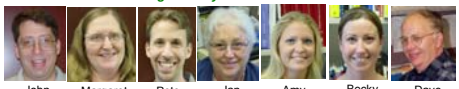
- **Research tool** that **provides quality metrics to review instrument performance over time** (e.g., examines resolution and sensitivity using internal size standard peaks)
- Runs with Microsoft Excel macros. Requires STR data to be converted with NCBI's BatchExtract program into numerical form.

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

Acknowledgments

NIST Human Identity Project Team

Leading the Way in Forensic DNA…



John Butler (Leader) | Margaret Kline | Pete Vallone | Jan Redman | Amy Decker | Becky Hill | Dave Duewer

Funding from interagency agreement 2003-IJ-R-029 between the National Institute of Justice and the NIST Office of Law Enforcement Standards

Many wonderful collaborators from industry, university, and government laboratories.

Bruce McCord (Florida International University) for many of the slides

Thank you for your attention…

Our team publications and presentations are available at:
http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm

Questions?

See also http://www.dna.gov/research/nist

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

john.butler@nist.gov