Fundamentals of Capillary Electrophoresis

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Buenos Aires, Argentina
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Presentation Outline

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

BREAK

- Troubleshooting strategies and solutions
- Review of some specific problems
- Questions

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

NIST and NIJ Disclaimer

Funding: Interagency Agreement between the National Institute of Justice and NIST Office of Law Enforcement Standards

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

Steps in DNA Analysis

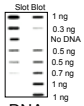
Usually 1-2 day process (a minimum of ~5 hours)

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



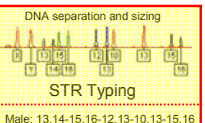
Blood Stain Buccal swab
Sample Collection & Storage


DNA Extraction



DNA Quantitation


Multiplex PCR Amplification

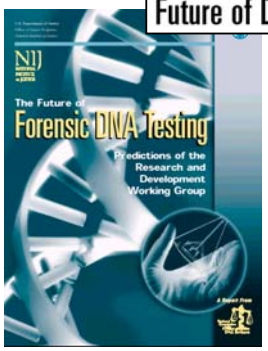


Biology / Technology

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

National Commission on the Future of DNA Evidence



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

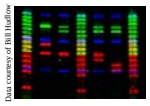
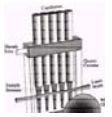
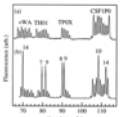
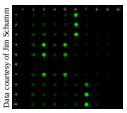
- Report published in Nov 2000
- Asked to estimate where DNA testing would be 2, 5, and 10 years into the future

Conclusions

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

STR 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

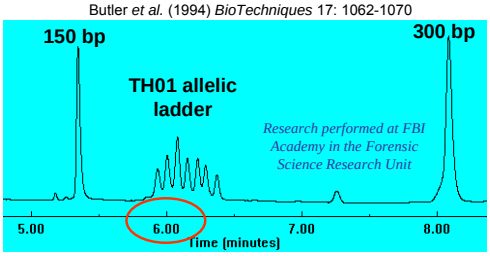
Early Work with CE and STRs

- 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

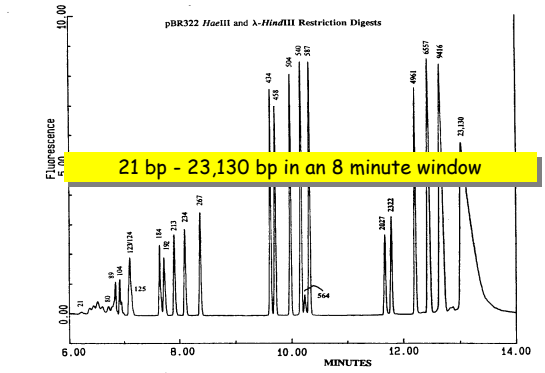
Butler *et al.* (1994) *BioTechniques* 17: 1062-1070



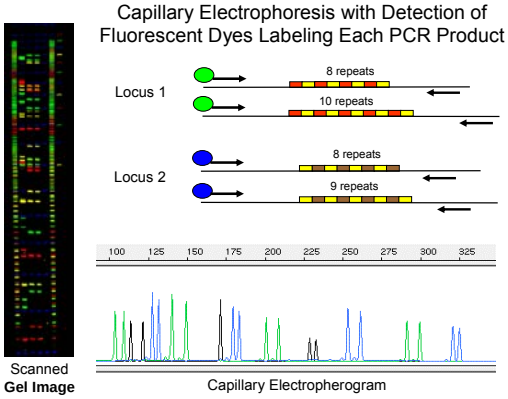
Performed in December 1993

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



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

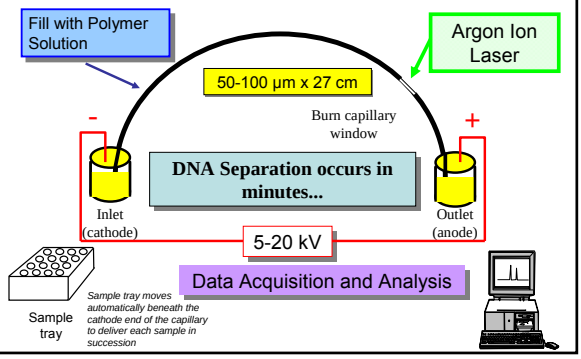


Why Use CE for DNA Analysis?

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)



Typical Instruments Used for STR Typing

GeneAmp 9700

Thermal Cycler for PCR Amplification

Capillary electrophoresis instruments for separating and sizing PCR products

single capillary

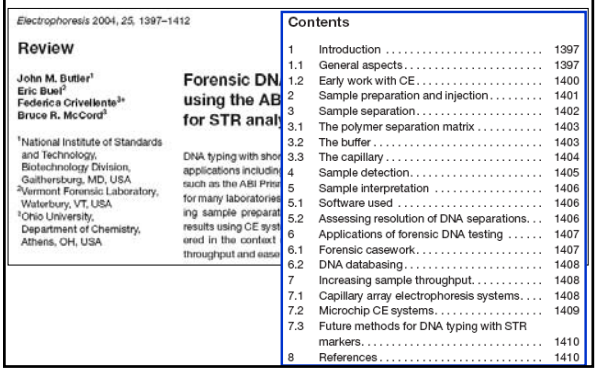
ABI 310

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

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Forensic DN
using the AB
for STR anal

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

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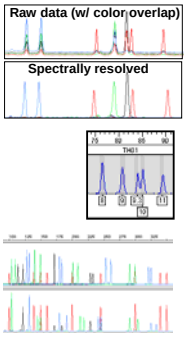
Analytical Requirements for STR Typing

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…

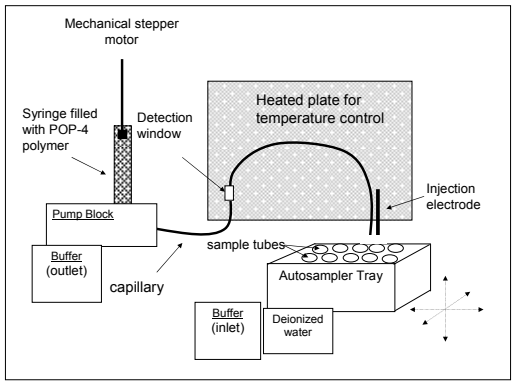
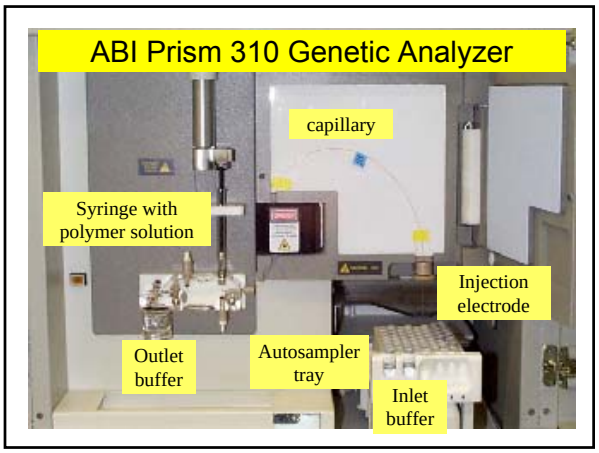
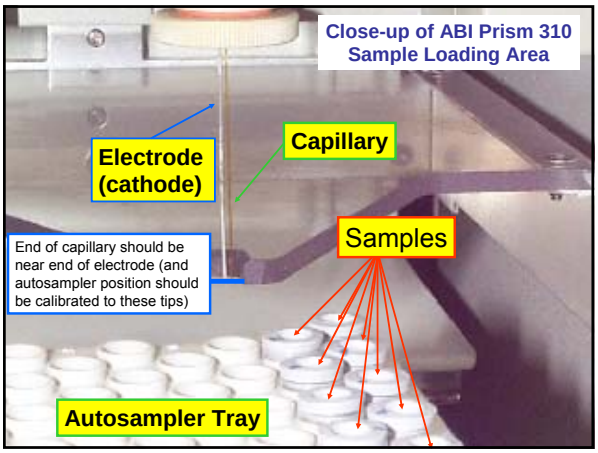
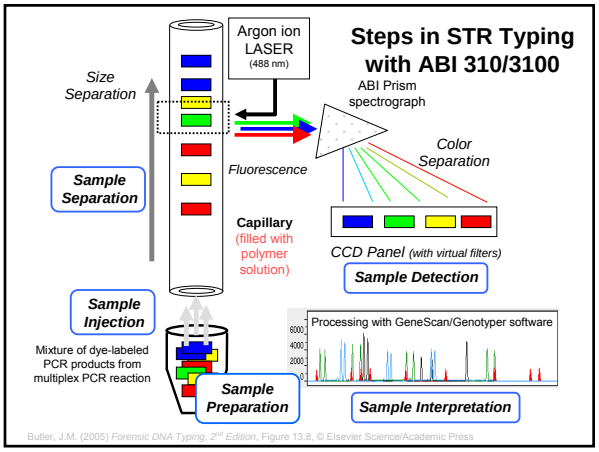
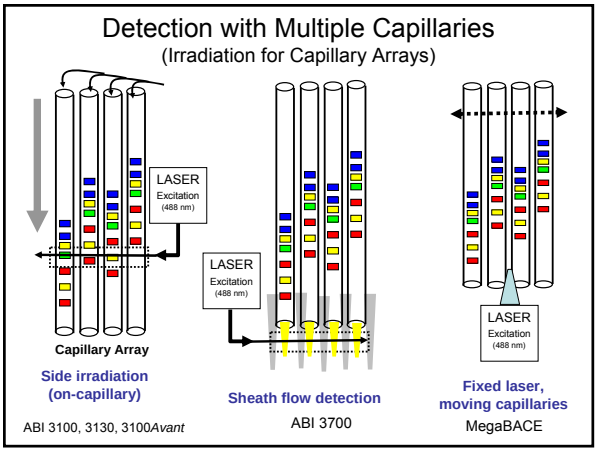


Figure 14.1, J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition © 2005 Elsevier Academic Press









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

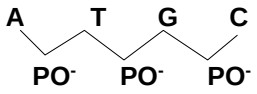
Ohm's Law

- 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!

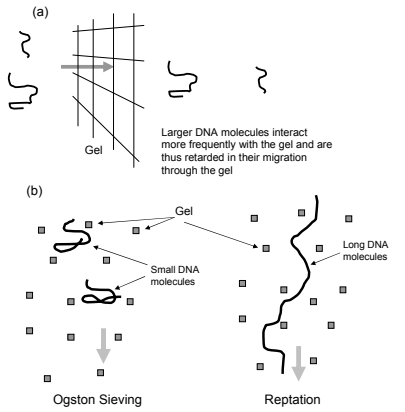
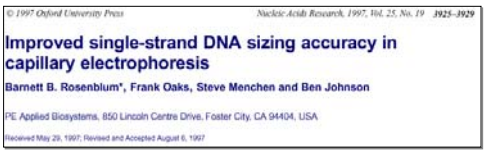


Figure 12.4, J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition © 2005 Elsevier Academic Press

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 ºC helps reduce secondary structure on DNA and improves precision. (Temperature control affects DNA sizing)

What is in POP-4 and Genetic Analyzer Buffer?

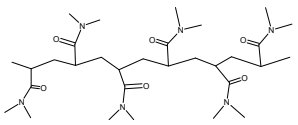


See also Wenz *et al.* (1998) *Genome Research* 8: 69-80

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

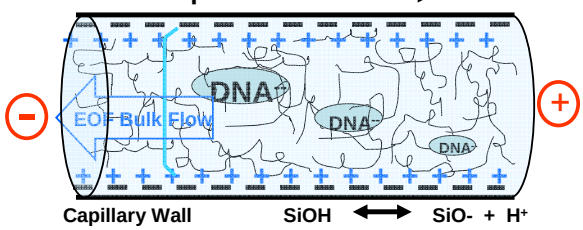
US Patent 5,552,028 covers POP-4 synthesis

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*



Capillary Wall Coatings Impact DNA Separations

Electrophoretic flow →



← Electroosmotic flow (EOF)

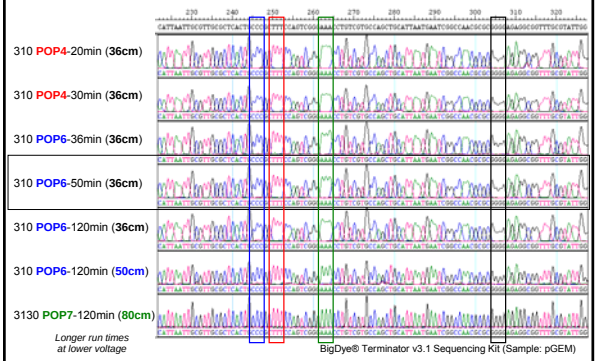
Solvated ions drag solution towards cathode in a flat flow profile

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

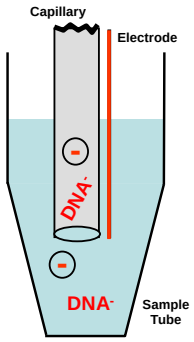
Impact of Capillary Length and Polymer Concentration on DNA Sequencing Resolution



Data collected at NIST by Tomohiro Takamaya (Japanese guest researcher, fall 2007)

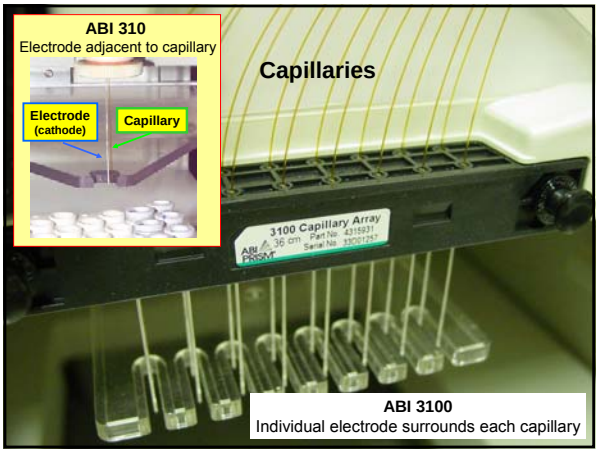
Injection

Electrokinetic Injection Process



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

Salty samples result in poor injections



Sample Conductivity Impacts Amount Injected

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

$[DNA_{inj}]$ 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

Steps Performed in Standard Module

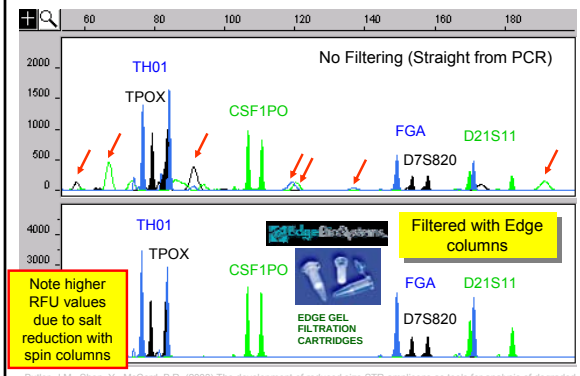
See J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition; Chapter 14

- **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

Comments on Sample Preparation

- Use high quality formamide (<100 μS/cm)
- Denaturation with heating and snap cooling is not needed (although most labs still do it…)
- **Post-PCR purification reduces salt levels** and leads to more DNA injected onto the capillary

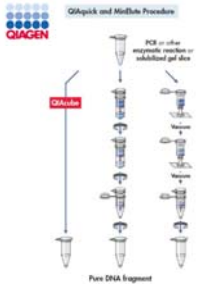
Removal of Dye Artifacts Following PCR Amplification



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.

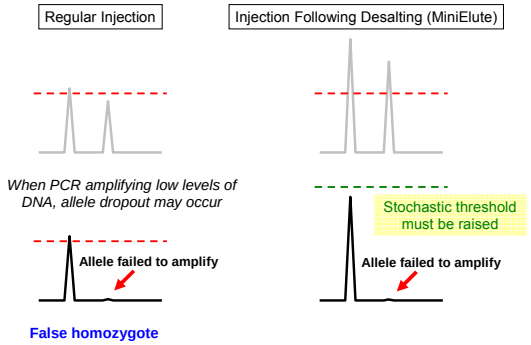
Why MiniElute increases peak heights

- QIAGEN MiniElute **reduces salt levels in samples causing more DNA to be injected**
- **Requires setting a higher stochastic threshold** to account for the increased sensitivity



Smith, P.J. and Ballantyne, J. (2007) Simplified low-copy-number DNA analysis by post-PCR purification. *J. Forensic Sci.* 52: 820-829

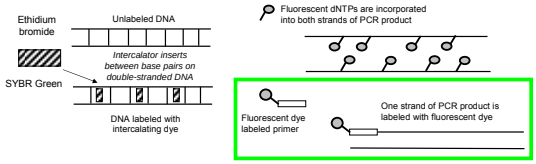
Stochastic Effects and Thresholds



Detection

Methods for Fluorescently Labeling DNA

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

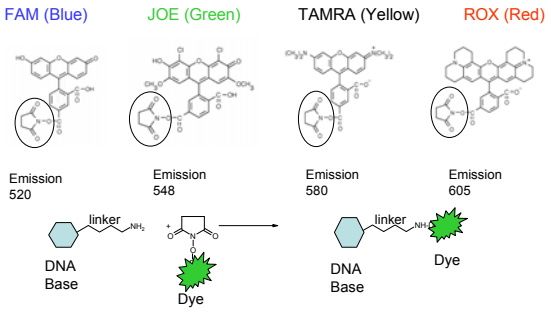


Butler, J.M. (2001) *Forensic DNA Typing*, Figure 10.2, ©Academic Press

Fluorescent Labeling of PCR Products

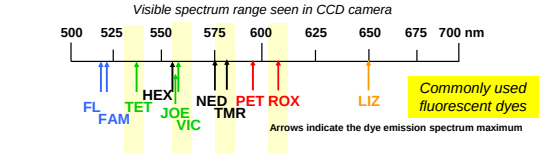
- Dyes are attached to one primer in a pair used to amplify a STR marker
- Dyes are coupled to oligonucleotides (primers) through NHS-esters and amine linkages on the 5'end of the primer: Dye-$(CH_2)_6$-primer
- Dye-labeled oligonucleotides are incorporated during multiplex PCR amplification giving a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

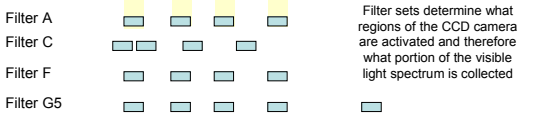
Amine Reactive Dyes used in Labeling DNA



The succinimidyl ester reacts rapidly with amine linkers on DNA bases

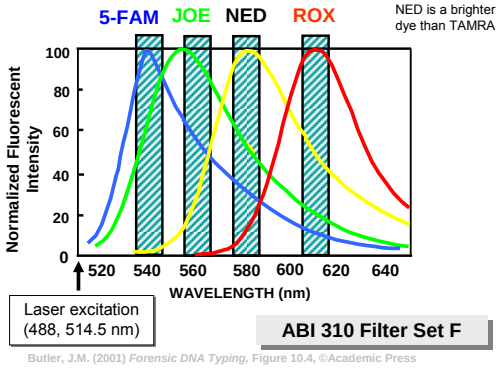
Virtual Filters Used in ABI 310





	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

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 ?

If there are 5 dyes

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

For Example

$I_{540} = bx_b + gy_b + yz_b + rw_b$ 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
$I_{610} = bx_r + gy_r + yz_r + rw_r$ 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)

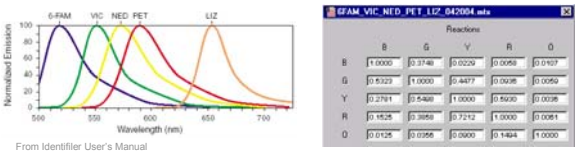
POP4STRMODF

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

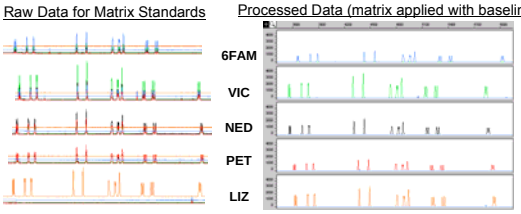
5 x 5 matrix for 5-dye analysis on ABI 310



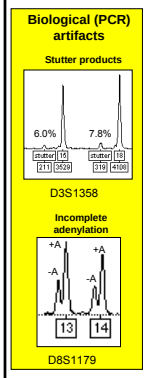
From Identifiler User's Manual

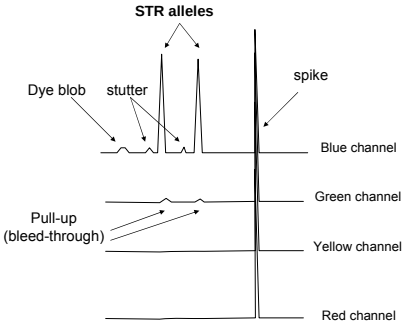
Raw Data for Matrix Standards

Processed Data (matrix applied with baselining)



Deciphering Artifacts from the True Alleles



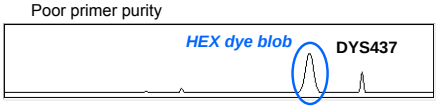


Butler, J.M. (2005) *Forensic DNA Typing, 2nd Edition*, Figure 15.4, © Elsevier Science/Academic Press

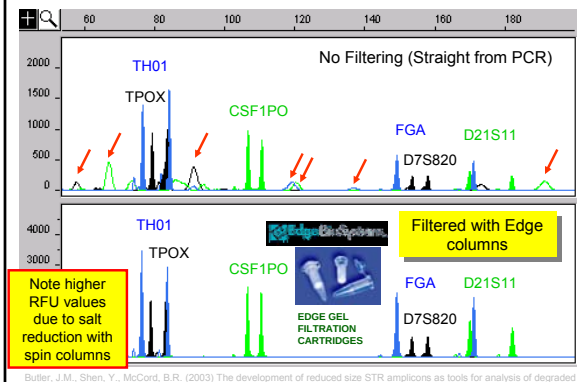
Dye Blobs ("Artifacts")

- 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)

Poor primer purity



Removal of Dye Artifacts Following PCR Amplification

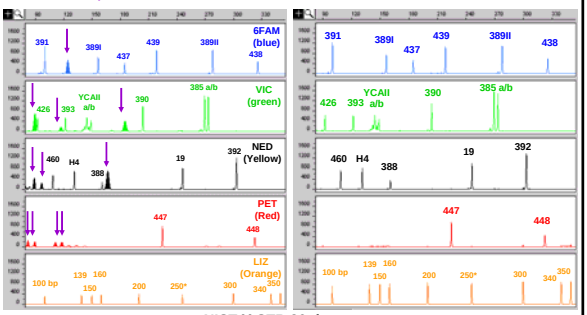


Butler, J.M., Shen, Y., McCord, B.R. (2003) The development of reduced size STR amplicons as tools for analysis of degraded DNA. *J. Forensic Sci* 48(5) 1054-1064.

Dye Artifacts Can Be Removed With Filtration

Residual dye artifacts

Dye blob removal with Edge columns



NIST Y-STR 20plex assay

From 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.*

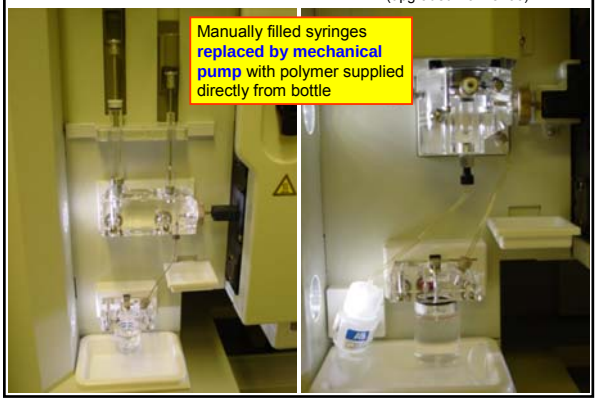
ABI Genetic Analyzer Usage at NIST

- ABI 310 x 2 (originally with Mac, then NT)
  - 1st was purchased in 1996
  - 2nd was purchased in June 2002

  Not really used any more
- ABI 3100 (Data collection v1.0.1)
  - Purchased in June 2002
  - Original data collection software retained

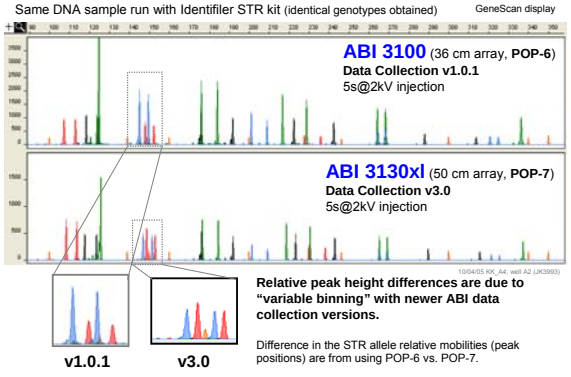
  Jan 2007 – upgraded to 3130xl with data collection v3.0
- ABI 3130xl upgrade (Data collection v3.0)
  - Purchased in April 2001 as ABI 3100
  - Upgraded to ABI 3130xl in September 2005
  - Located in a different room

ABI 3100 | ABI 3130xl (upgraded from 3100)



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.

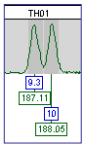
NIST Use of the ABI 3100/3130xl

- Data collection software:
  - version 1.0.1 (Apr 2001- Jan 2007)
  - version 3.0 (Jan 2007-present)
- **POP-6** with 36 cm capillary array
  - **STR kits** and in-house assays for autosomal STRs, Y-STRs, and miniSTRs
  - **SNaPshot assays**: mtSNPs, Y-SNPs, and autosomal SNPs
  - **DNA sequencing** for mtDNA and STR repeat sequencing

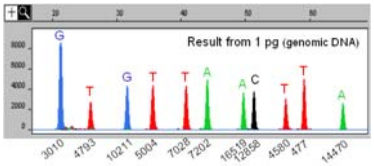
We used to routinely get more than 400 runs per capillary array by not changing the polymer between applications

NIST ABI 3100 Analysis Using POP-6 Polymer

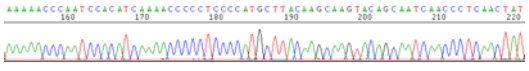
High Resolution STR Typing



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



mtDNA Sequencing (HV1)



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

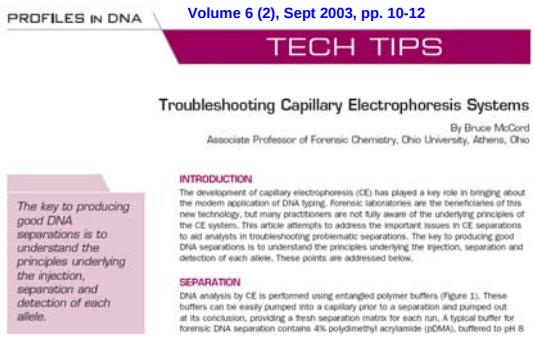
YOU MUST BE CLEAN AROUND A CE!

Protocols Used for STR Typing

- Most forensic DNA laboratories follow PCR amplification and CE instrument protocols provided by the manufacturer
- Comments
  - Lower volume reactions may work fine and reduce costs
  - No heat denaturation/snap cooling is required prior to loading samples into ABI 310 or ABI 3100
  - Capillaries do not have to be thrown away after 100 runs
  - POP-4 polymer lasts much longer than 5 days on an ABI 310
  - **Validation does not have to be an overwhelming task**

Troubleshooting:
Strategies and Solutions

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


Applied Biosystems

Forensic News

October 2007 FAS Corner

http://marketing.appliedbiosystems.com/images/forensic/volume11/docs/52808_FN_FAS_r3.pdf

Troubleshooting Amplification and Electrophoresis of the AmpF/STR® Kits

One of the key responsibilities of our Human Identification Field Application Specialists is to troubleshoot results obtained using any of the AmpF/STR® kits on any Applied Biosystems validated instrument platform.

Troubleshooting Electrophoresis

Below are some common observations that may be seen during electrophoresis of AmpF/STR® kit PCR products:

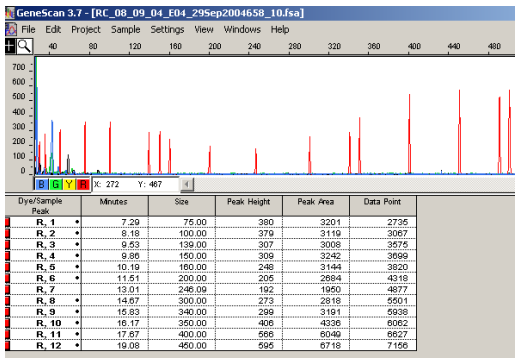
- Spikes/Extraneous peaks
- No signal or low signal
- Loss of resolution
- Arcing
- Low reproducibility
- Contamination
- Baseline issues
- Poor peak morphology

Spikes or Extraneous Peaks

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


GeneScan 3.7 - [RC_08_09_04_E04_29Sep2004658_10.fsa]

File Edit Project Sample Settings View Windows Help

Dye/Sample Peak		Minutes	Size	Peak Height	Peak Area	Data Point
R, 1	•	7.29	76.00	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	•	17.67	400.00	566	6049	6627
R, 12	•	19.08	460.00	595	6718	7156

In sample- shadow peaks appearing to left of allelic peaks

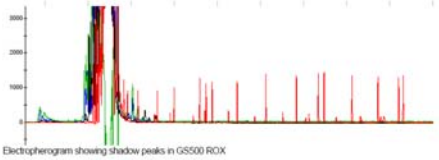
December 21, 2007

What does ABI Say?

Dear Valued Customer,

We are writing this letter in response to inquiries from customers regarding artifact peaks that appear as "shadow peaks" to true DNA peaks observed in the electropherogram. In most cases, these artifacts appear to be the most prevalent in the dye channel corresponding to the size standard and do not affect accurate sizing of the size standard peaks.

An example electropherogram is shown below:

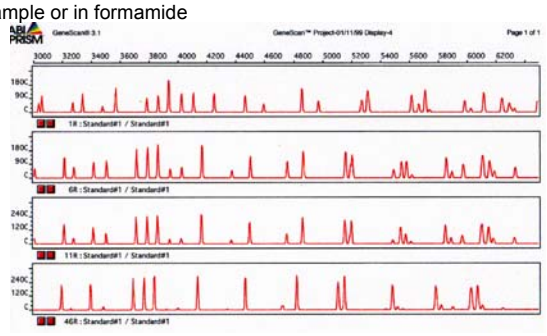


Electropherogram showing shadow peaks in GS500 ROX

The occurrence of these "shadow peaks" has been replicated at Applied Biosystems. We also observed during the testing process that higher shadow peak heights result from longer injection times. We are in the process of investigating the occurrence of these "shadow peaks" to determine the root cause and address the issue.

Applied Biosystems is committed to providing the highest quality products available for use in DNA typing. Thank you for your valued feedback. Your input is extremely valuable to us in our efforts to improve the quality of our products. Please feel free to contact HID Technical Support at 1.888.821.4HID (4443), #1 for further information.

What is it really?
Incomplete denaturation of standard due to excessive salt in sample or in formamide



ds DNA migrates faster and over time with this set of runs ds DNA replaced the ssDNA

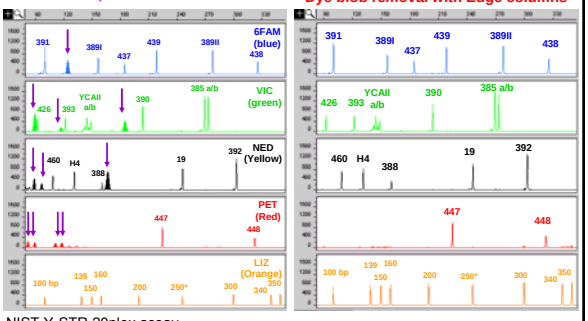
Post PCR manipulation

- 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

Residual dye artifacts

Dye blob removal with Edge columns



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.*

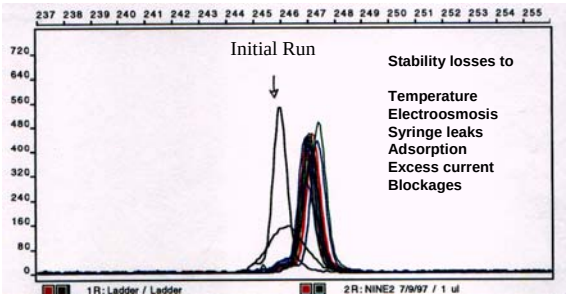
3. External Factors

- 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.

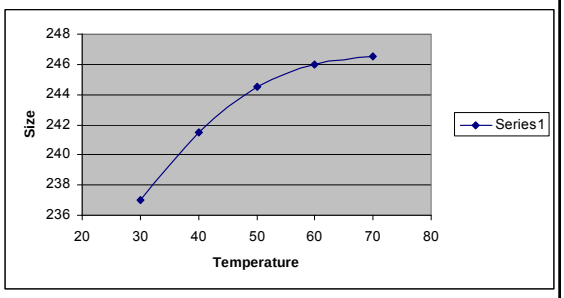
Temperature effects

- 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

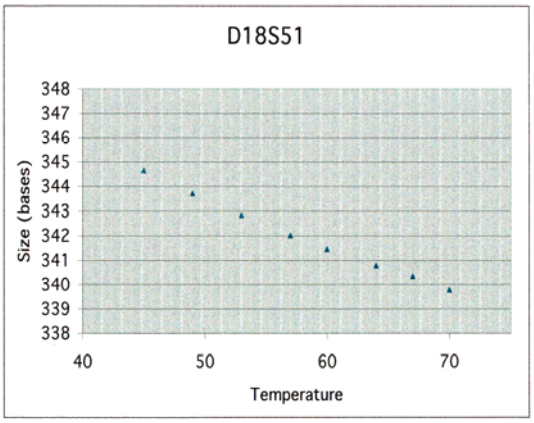
Due to its structure and its non-calibration, the "250"peak can be used to indicate stability

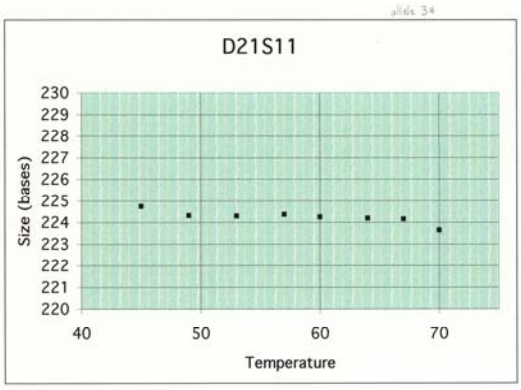


Change in size of GS 250 peak with Temperature (Tamra Std)

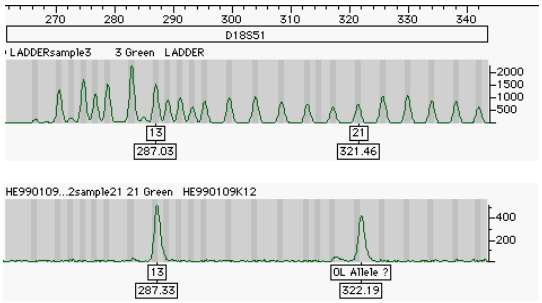


4% pDMA with 8M urea and 5% 2-pyrrolidinone
***Rosenblum et al., Nucleic Acids Res.(1997) 25,19, 2925**

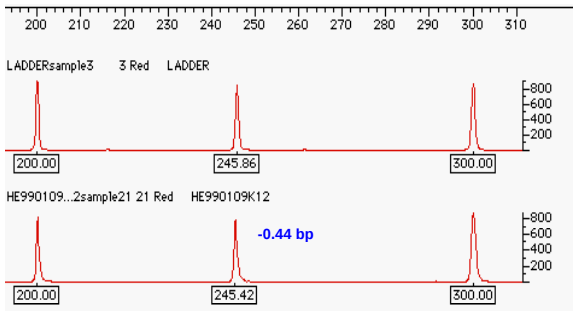




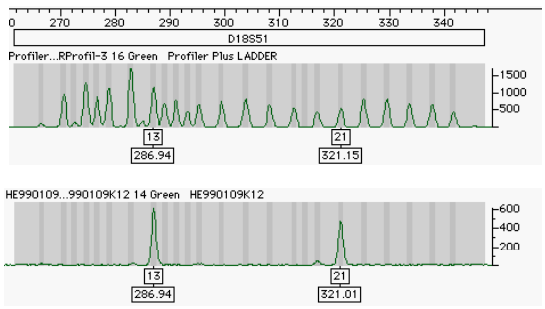
"OL Alleles"



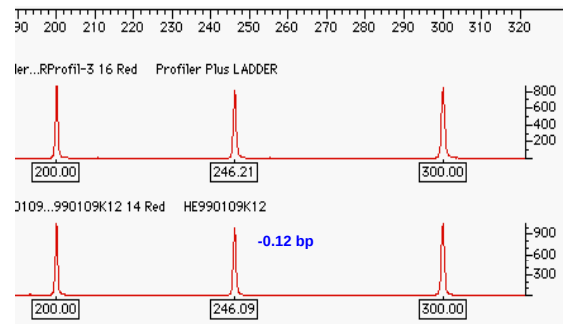
"OL alleles " - look at the 250 peak



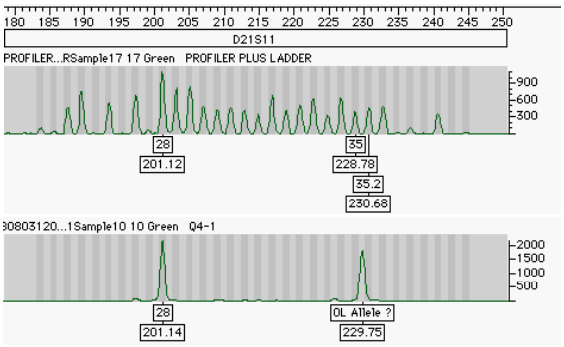
"OL allele re-injected"



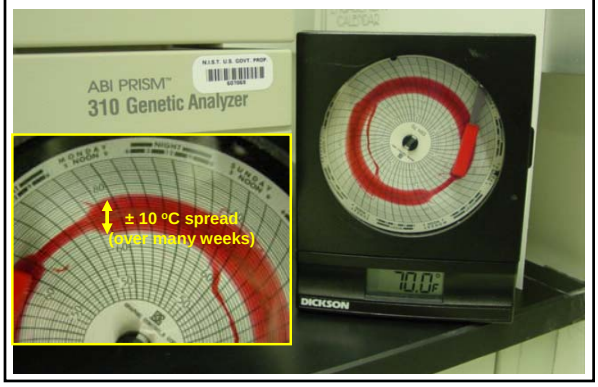
And the 250 peak...



True off-ladder alleles



Monitoring Room Temperature Over Time



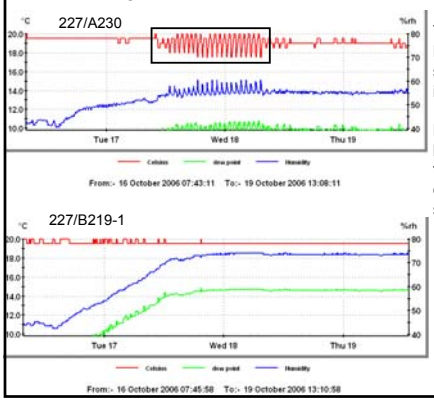
Temperature Probes

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

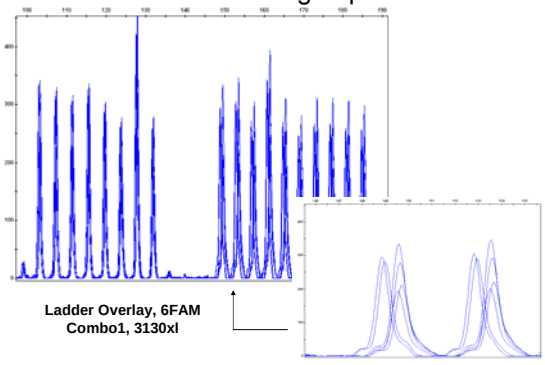
Monitoring Instrument Room Temperature Fluctuations



Temperature Monitoring of two separate instrument rooms.

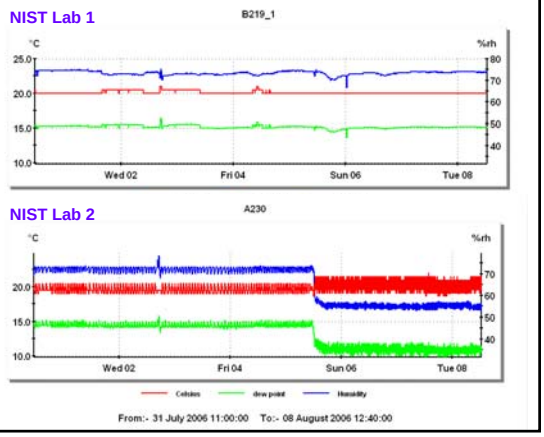
Box area is a 24 hour period where temperature control is not stable.

Poor Temperature Control Causes DNA Sizing Imprecision

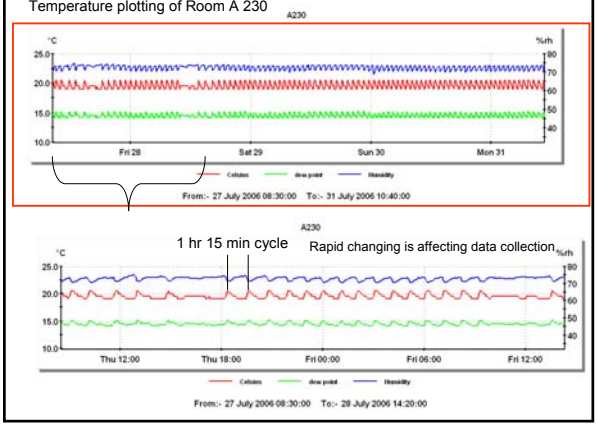


Ladder Overlay, 6FAM Combo1, 3130xl

NIST Lab 1 / NIST Lab 2



Temperature plotting of Room A 230

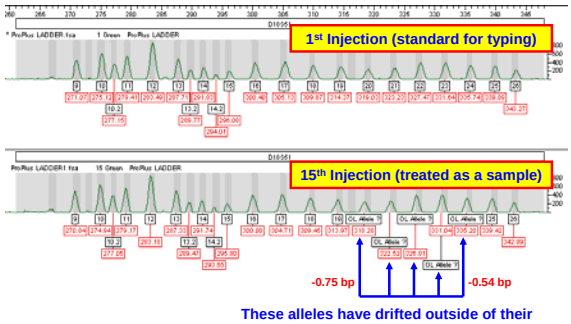


What to do if calibration is lost?

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

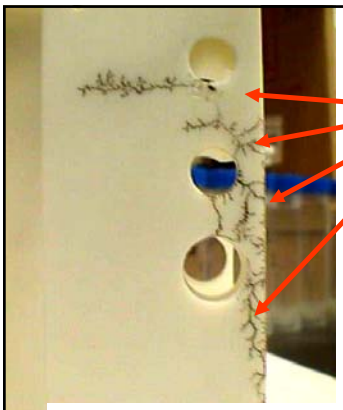
Use of Second Allelic Ladder to Monitor Potential Match Criteria Problems



These alleles have drifted outside of their genotyping bins due to temperature shifting over the course of the sample batch

Cleanliness

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

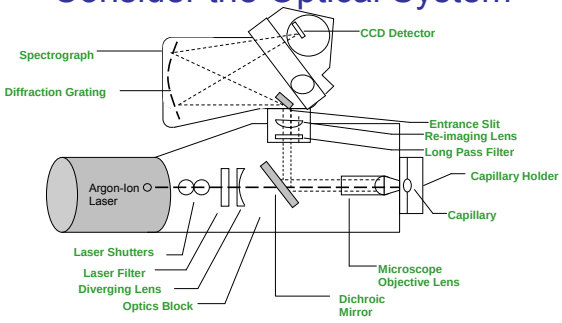


Keep Your System Clean and Dry!

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

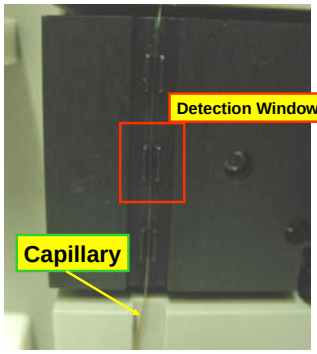


Watts, D. Genotyping STR Loci using an automated DNA Sequencer. In *Forensic DNA Profiling Protocols;* Lincoln, P.J.; Thomson, J. Eds.; Humana Press Inc.: Totowa, NJ, 1998; Vol. 48, pp 193-208.

Issues with the Optical System

- 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, optics: 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.**

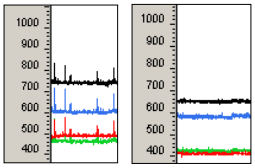
The Detection Window



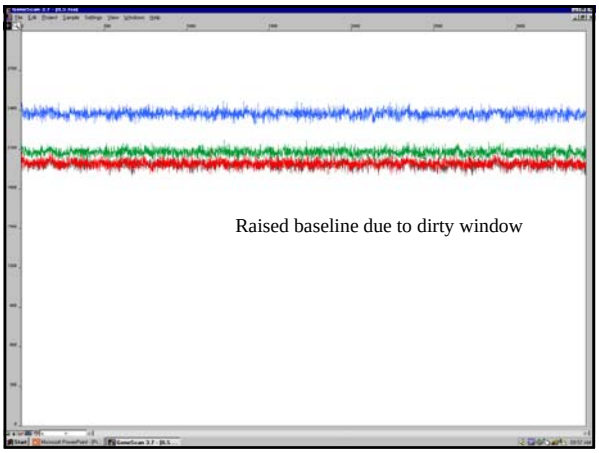
Make sure that the capillary window is lined up (if it is not, then no peaks will be seen)

Window may need to be cleaned with ethanol or methanol

Review Start of Raw Data Collection



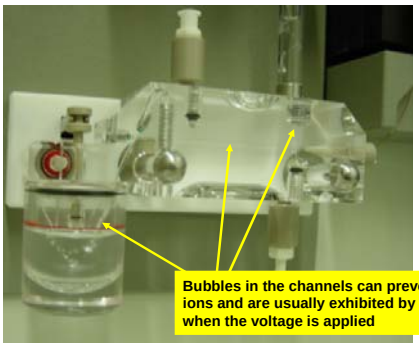
Little spikes indicate need to change buffer… check current

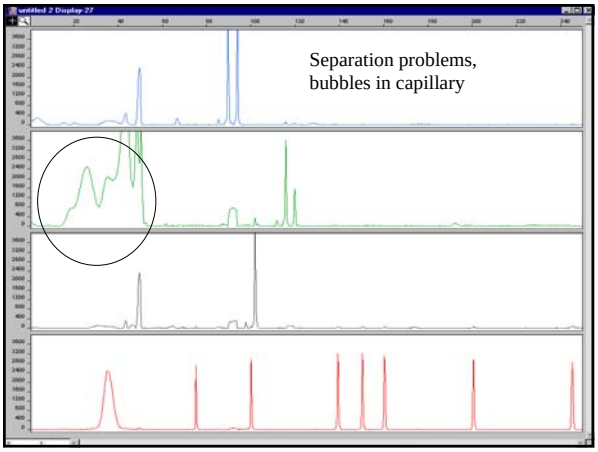


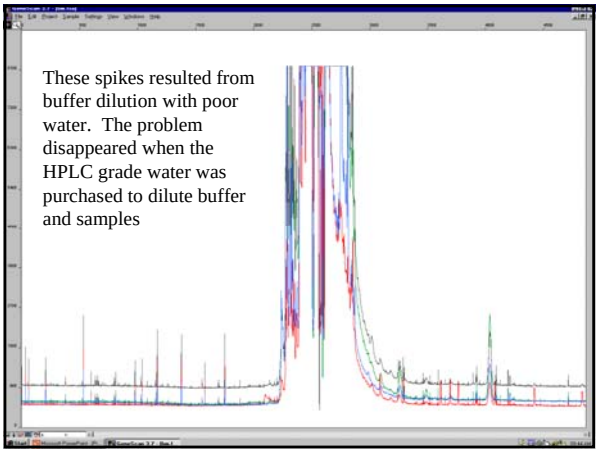
Buffer Issues

- 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

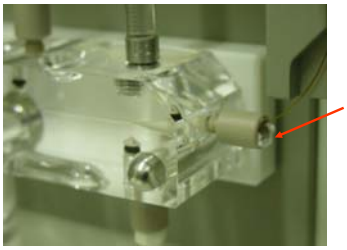
Remove all bubbles from the channels







Beware of Urea Crystals



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

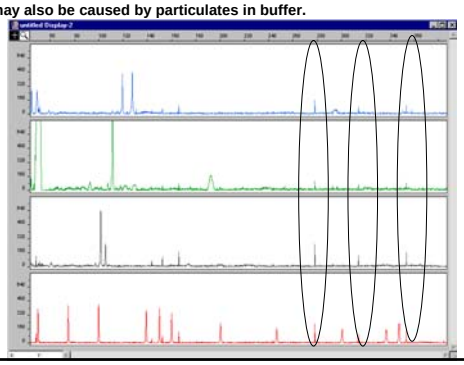
Urea sublimates and can evaporate to appear elsewhere

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

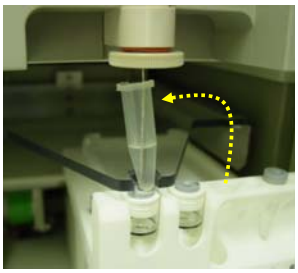
Pump block should be well cleaned to avoid problems with urea crystal formation

Current Spikes

Generally appear in all lanes and are sharper than regular peaks
These are a natural consequence of the application of high voltage in CE and may also be caused by particulates in buffer.



Storage when ABI 310 is not in use

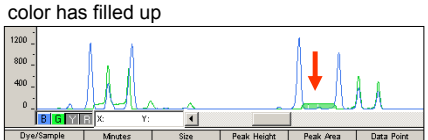


- Keep inlet of capillary in water…if it dries out then urea crystals from the polymer will clog the opening
- The waste vial (normally in position 3) can be moved into position
- A special device can be purchased from Suppelco to rinse the capillary off-line
- Store in distilled water
- Note that the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

Remember that the water in the open tube will evaporate over time…
Also this will destroy the electode if turned on without removing the tube

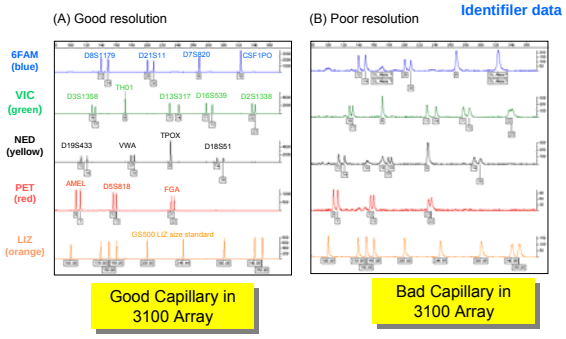
Matrix Problems

- 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	16.86	138.58	160	1645	4325
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	1863	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.

ABI Letter to Customers – July 2008

Applied Biosystems

July 29, 2008

Dear Valued Customer,

A limited number of customers have reported incidents of peak broadening and a decline in the number of runs per array when using Applied Biosystems' 3100 and 3130-series Genetic Analyzer instruments, in some cases achieving less than 100 runs/array. This letter is intended to address customer concerns and provide further information regarding the steps taken at Applied Biosystems to address this issue.

Each report has been investigated by field personnel to determine the root cause of the loss of peak resolution and decrease in the number of runs per array, including verifying that the appropriate maintenance procedures are in place and that recommended protocols have been adhered to. In addition, all relevant consumables lot numbers have been collected in an effort to link any related complaints to a specific consumable, or set of consumables to determine if there is a root cause common to all reported incidents.

ABI Solution to Polymer Problem

The preliminary results of our investigation suggest that recent lots of 31xx POP4 polymer may be contributing to some of the reported incidents and, as such, additional efforts have focused on polymer as a potential root cause. While our root cause investigation is still on-going, a cross-functional team has been established to review all polymer testing data. The team meets regularly and is actively looking into aspects of polymer manufacturing and the relationship of polymer to other consumables that may impact data quality.

As a proactive effort, the rate of polymer production has been increased in order to help meet customer demand and all recently released POP4 polymer has been subjected to additional functional testing to ensure polymer performance. We have confirmed that all released lots of POP4 polymer have passed internal quality control testing.

We are also pleased to inform you that most reported incidents have been successfully resolved through the efforts of the local support teams utilizing the following procedure:

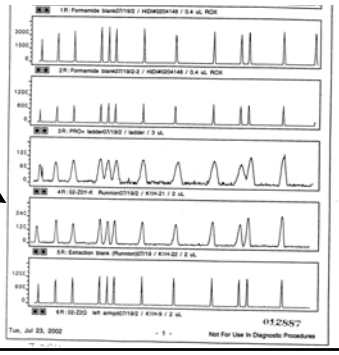
- Flush the system 10 to 15 times with warm (40°C) deionized system water wash prior to array/polymer replacement. Using a high purity bottled water source may help to eliminate water as a potential contributing factor.
- The warm water wash should be followed immediately by replacement of the capillary array and consumables lots (e.g. polymer, buffer and water) as advised by your Applied Biosystems Field Applications Specialist.
- In extreme cases, replacement of the lower block or front end may also be required to recover performance.

The most recent reports are specific to low quality data on the 3100 instrument platform. Internal testing indicates that packaging is contributing to this low quality data. We are focusing our efforts on variation in packaging between our different bottle configurations. To help minimize packaging variation we are evaluating an alternative bottle plastic for 3100 POP4, which will be more similar to the 3130 POP4 bottle configuration. We are also closely monitoring shipping conditions to determine any potential impacts from shipping.

Effect of contaminant in reference sample

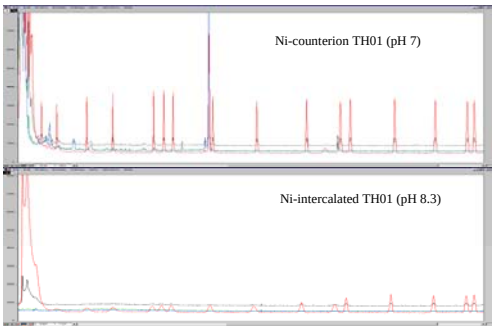
Contamination results in problems in subsequent analyses

Effect is transitory



Metal Ions in the Sample

DNA clumps and injects poorly. Effect is pH and EDTA dependent



1 µl TH01 added to 10 µl of 3.0 mM $NiCl_2$ 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 can be the result of

- Bad formamide
- Bubbles in the sample vial
- 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 in sample

A permanent loss of resolution may mean

- Adsorptive sites on a capillary
- Initiation of electroosmotic flow
- Conductivity changes in buffer/polymer
- Wrong buffer formulation
- Bad formamide or internal lane standard
- Contaminated syringe

http://marketing.appliedbiosystems.com/images/forensic/volume11/docs/52808_FN_FAS_r3.pdf

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)

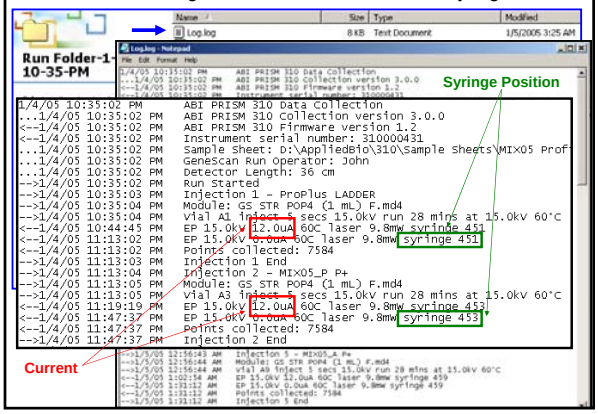
Measurement of Current

- 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

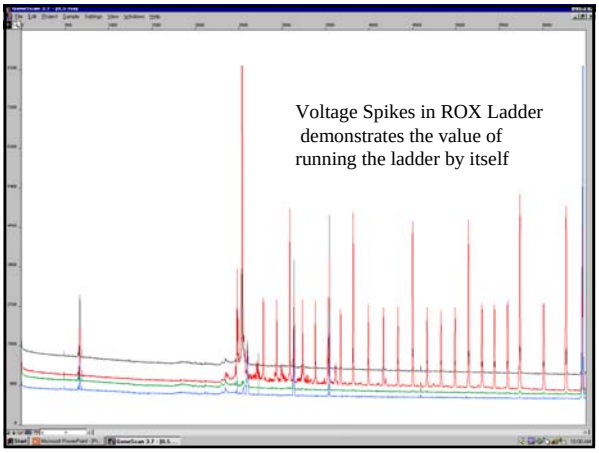
- The ABI 310 instrument also keeps track of the position of the syringe (in the log file)
- Depending on the resistance to flow, the syringe will travel different lengths
- Syringe leaks may be reflected in a longer distance traveled prior to each injection
- These leaks occur around the barrel of the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

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

Example Problems Seen and Provided by Others

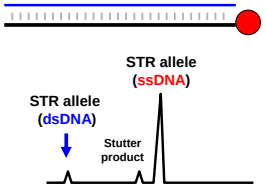
For Promega 2008
Troubleshooting Workshop

Why dsDNA migrates through CE capillary faster than ssDNA…

- DNA molecule separation depends on interactions with the polymer
  - Higher polymer concentration (or longer polymer molecules) permits more polymer interactions and provides better resolution (i.e., POP-6 vs POP-4)
- **Single-stranded DNA (ssDNA) is more flexible than double-stranded DNA (dsDNA)** and therefore moves more slowly through the capillary because it is interacting with polymer strands more

dsDNA vs ssDNA CE Migration

- If a small amount of the complementary strand re-hybridizes to the labeled STR allele strand, then a little peak will be seen in-front of each internal lane standard peak and



•Height of dsDNA peak will depend on amount of re-hybridization between the two strands (**some loci will re-hybridize more readily giving rise to larger dsDNA peaks**)

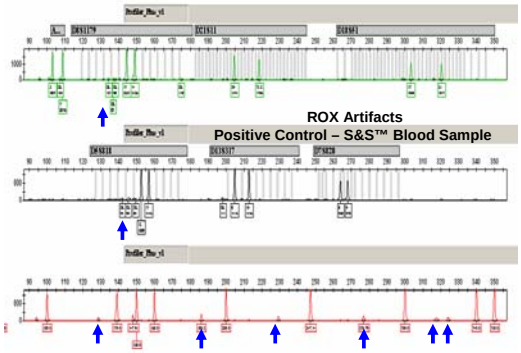
•Local temperature environment of capillary impacts amount of re-hybridization (may change over time)

Sample Renaturation (minor dsDNA peaks running in front of primary ssDNA STR alleles)

ROX Artifacts
Comparison Casework Blood Sample

Data from Peggy Philion (RCMP)

More Sample Renaturation



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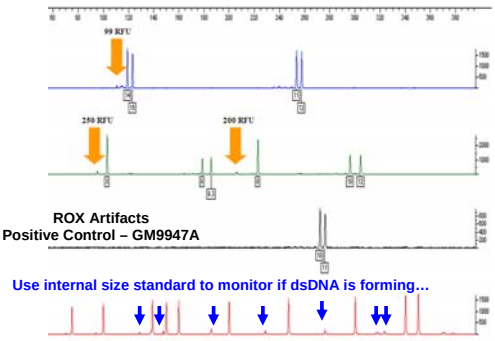
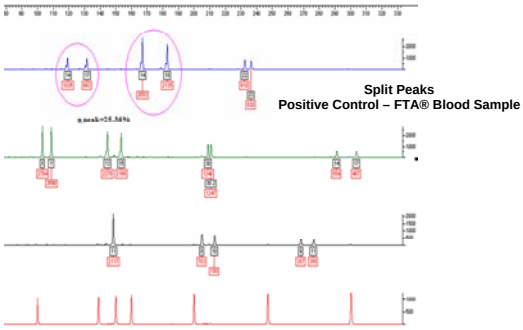


Data from Peggy Philion (RCMP)

Split Peaks (amplification reagents starting to go bad – dNTPs, polymerase, etc.)



Data from Peggy Philion (RCMP)

Acknowledgments

NIST Human Identity Project Team

Leading the Way in Forensic DNA…

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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
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