Troubleshooting Common Laboratory Problems

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Purpose of This Workshop

- DNA labs often encounter challenges when working with the many variable aspects of STR analysis and capillary electrophoresis separation/detection.
- This workshop will explore common challenges experienced by forensic laboratories and suggest solutions for fixing various problems.
- Participants are invited to suggest problems that they would like to have reviewed in advance of the workshop.
- Tried to have limited enrollment to encourage group discussion.

Presentation Plan

- 15 min • Introductions
- 30 min (John) • Fundamentals of CE
  - sample prep, injection, separation, detection
- 45 min (Bruce/John) • Setting instrument parameters and thresholds
  - applying validation data
  - mixture interpretation
- 15 min BREAK
- 30 min (Bruce) • Troubleshooting strategies and solutions
- 30 min (John/Bruce) • Review of some specific problems
- 15 min (John/Bruce) • Questions

Our Backgrounds

John Butler

- NIST Fellow - **National Institute of Standards and Technology**
- PhD in Analytical Chemistry from University of Virginia (1995)
- Family: wife Terilynne and six children
- Hobbies: reading, writing, and making PowerPoint slides

Bruce McCord

- Professor of Analytical/Forensic Chemistry – **Florida International University**
- PhD in Analytical Chemistry from University of Wisconsin (1986)
- Family: wife Margie and three children
- Hobbies: dixieland jazz, windsurfing, sailing and editing John's slides

Background of Participants…

Your name
Your organization
Instrumentation in use (e.g., ABI 310, 3100, 3130xl)
What you hope to learn from this workshop

NIST and NJ Disclaimer

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

Funding for Bruce McCord: National Institute of Justice

Points of view are the presenters 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.

These workshop slides will be available at:
http://www.cstl.nist.gov/biotech/strbase/training.htm

Review of Fundamentals
for STR Analysis and Capillary Electrophoresis

John

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

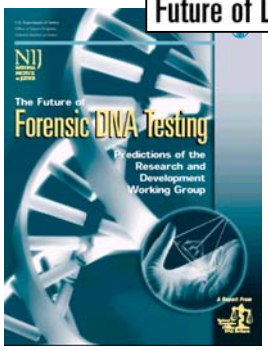
If a match occurs, comparison of DNA profile to population allele frequencies to generate a case report with probability of a random match to an unrelated individual

DNA Database Search

Technology

DNA separation and sizing
STR Typing
Male: 13,14-15,16-12,13-10,13-15,16
Interpretation of Results

National Commission on the Future of DNA Evidence



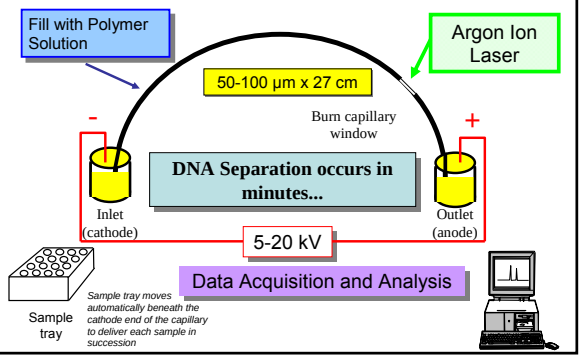
http://www.ojp.usdoj.gov/nij/pubs-sum/183697.htm

- 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

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

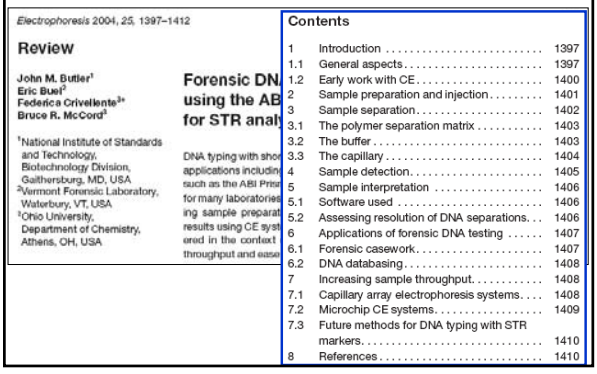
ABI 310

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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[3]Ohio University, Department of Chemistry, Athens, OH, USA

Forensic DN
using the AB
for STR anal

DNA typing with sho
applications including
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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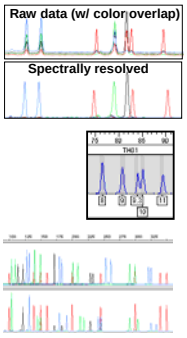
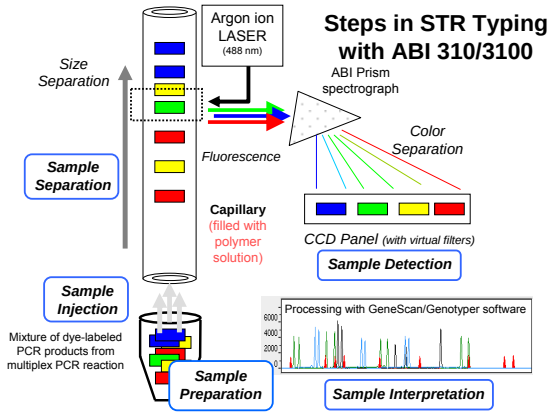
Analytical Requirements for STR Typing

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



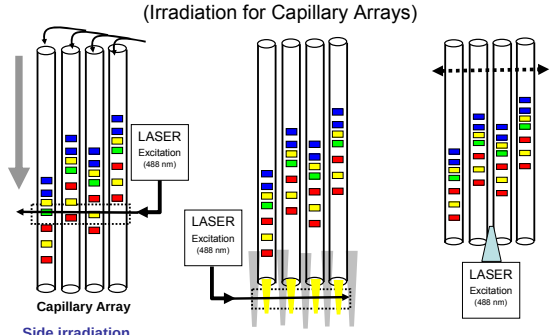
Steps in STR Typing with ABI 310/3100



Butler, J.M. (2005) *Forensic DNA Typing, 2nd Edition*, Figure 13.8, © Elsevier Science/Academic Press

Detection with Multiple Capillaries

(Irradiation for Capillary Arrays)



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

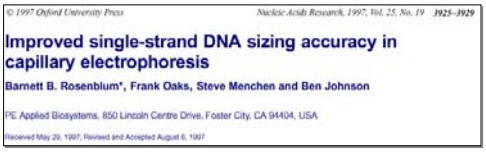
Ohm's Law

- 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)

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?

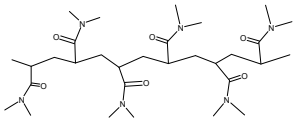


See also Wenz *et al.* (1998) *Genome Research* 8: 69-80

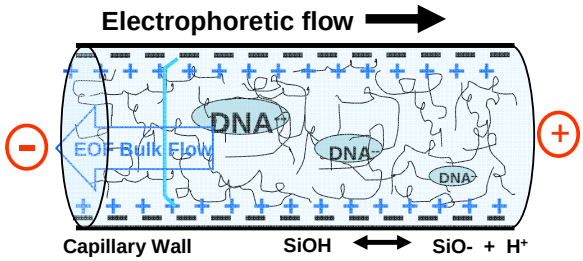
POP-4 (4% poly-dimethylacrylamide, 8 M urea, 5% 2-pyrrolidinone)

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



Electroosmotic flow (EOF)

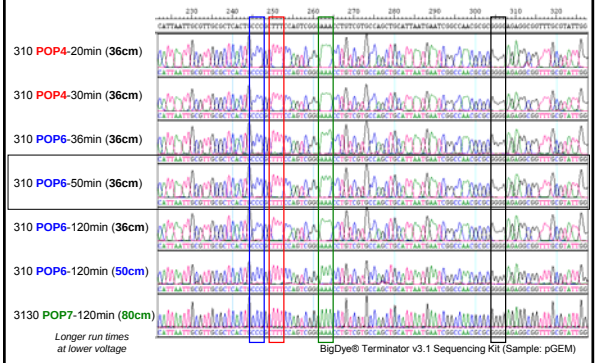
Solvated ions drag solution towards cathode in a flat flow profile

How to Improve Resolution?

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

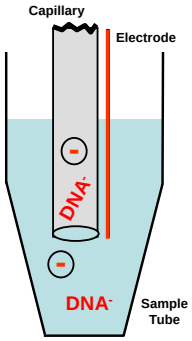
All of these come at a cost of longer separation run times

Impact of Capillary Length and Polymer Concentration on DNA Sequencing Resolution



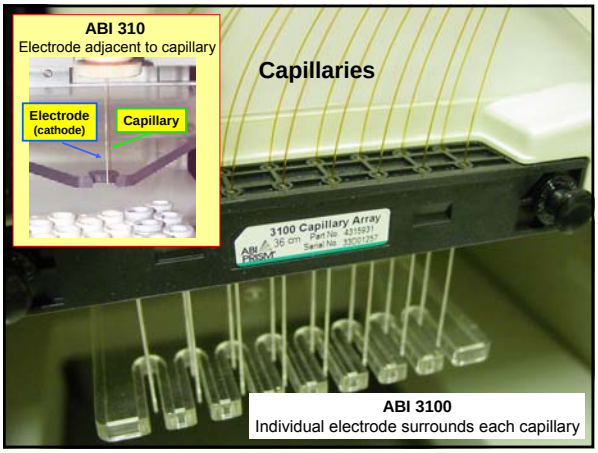
Data collected at NIST by Tomohiro Takamaya (Japanese guest researcher, fall 2007)

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

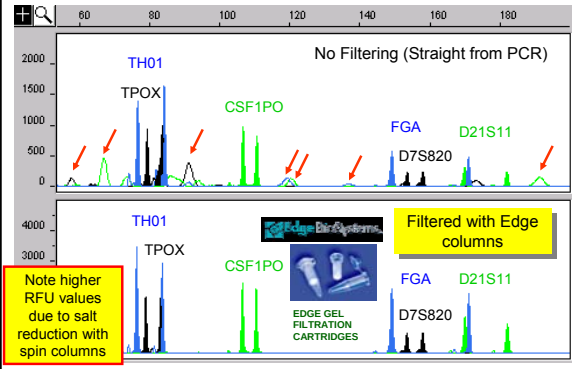
See J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition; Chapter 14

- **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

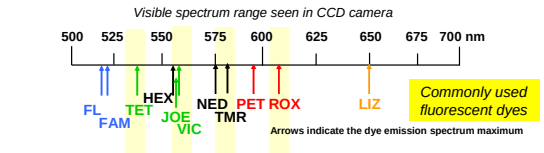
- Use high quality formamide (<100 μS/cm)
- Denaturation with heating and snap cooling is not needed (although most labs still do it…)
- Post-PCR purification reduces salt levels and leads to more DNA injected onto the capillary

Removal of Dye Artifacts Following PCR Amplification



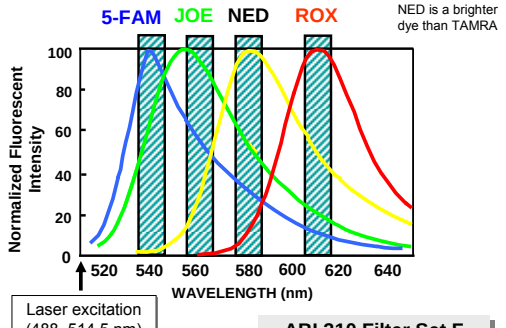
Butler, J.M., Shen, Y., McCord, B.R. (2003) The development of reduced size STR amplicons as tools for analysis of degraded DNA. *J. Forensic Sci* 48(5) 1054-1064.

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**
$I_{610} = bx_r + gy_r + yz_r + yw_r$ **intensity of red**

Where
b is the %blue labeled DNA
g is the %green labeled DNA, etc.

x,y,z,w are the numbers in the matrix (sensitivity to each color)

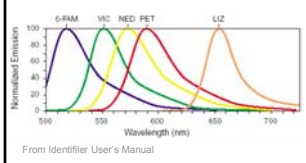
POP4STRMODF

Reactions

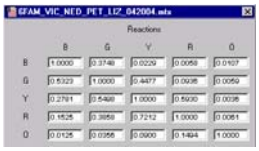
	B	G	Y	R
B	1.0000	0.8502	0.1380	0.0009
G	0.8300	1.0000	0.7622	0.0051
Y	0.6416	0.8324	1.0000	0.1102
R	0.4493	0.6484	0.7851	1.0000

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

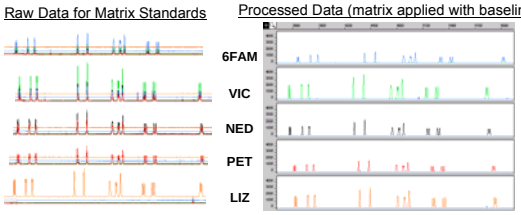


From Identifiler User's Manual



Raw Data for Matrix Standards

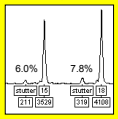
Processed Data (matrix applied with baselining)



Deciphering Artifacts from the True Alleles

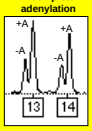
Biological (PCR) artifacts

Stutter products

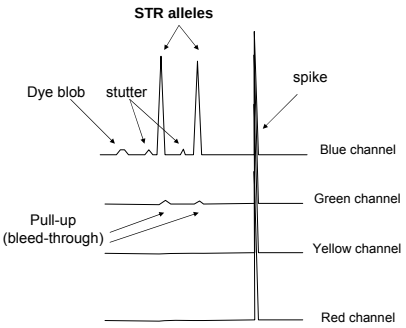


D3S1358

Incomplete adenylation



D8S1179



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

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

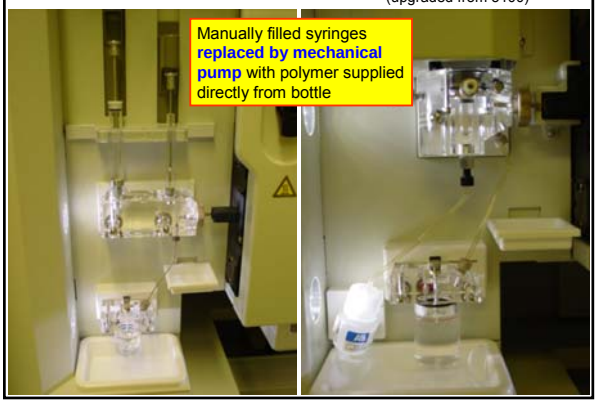
- ABI 3100 (Data collection v1.0.1)
  - Purchased in June 2002
  - Original data collection software retained

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)



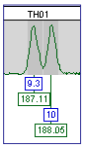
NIST Use of the ABI 3100/3130xl

- 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

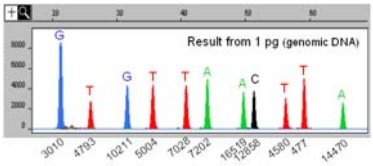
We can routinely get more than 400 runs per capillary array by not changing the polymer between applications

NIST ABI 3100 Analysis Using POP-6 Polymer

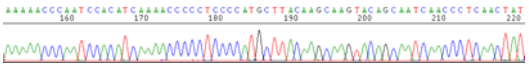
High Resolution STR Typing



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



mtDNA Sequencing (HV1)



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**

Questions?

- What are your biggest challenges with keeping your ABI 310/3100/3130 CE systems running?
- What kind of signal intensity variation are you seeing between your different instruments?
- Have you seen uneven injection across a sample plate?

Setting Instrument Parameters and Thresholds

Bruce & John

Spinal Tap Video

- The problem of instrument sensitivity
- Exists everywhere and is fundamental to the concept of signal to noise

Setting thresholds for the ABI 310/3100

- Where do current ideas on instrument thresholds for the ABI 310/3100 come from?
- How do I set these values in my laboratory?
- Why might they vary from one instrument to the next?
- How do these thresholds affect data interpretation?

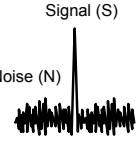
What is a true peak (allele)?

GeneScan function

Peak detection threshold

Signal (S)

Noise (N)



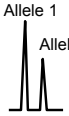
S/N >3

Genotyper function

Peak height ratio (PHR)

Allele 1

Allele 2



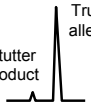
Heterozygote peak balance

PHR >60%

Stutter percentage

True allele

Stutter product



Stutter <15%

Provides range in which mixtures may reliably be detected

Fundamental Ideas behind **Threshold Settings** for the ABI 310/3100

Detection Limit: 3x the standard deviation of the noise.
Estimated using **2x peak to peak noise**. (approximately **35 - 50 RFUs**)
Peaks below this level may be random noise

Limit of Quantitation: 10x the standard deviation of the noise
Estimated using **7x peak to peak noise** (**150-200 RFUs**)
Below this point estimates of peak area or height are unreliable and may not be reliable indicators of mixture ratios

Stochastic Threshold: Level of quantifiable DNA below which peaks can show severe imbalance (peak height ratios below 60%) Approximately **150 -200 RFUs**. **(always greater than the LOQ)** Variance in peak height ratio is the sum of variance due to the stochastic amplification and instrumental noise.

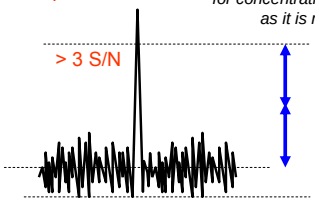
Limit of Detection (LOD)

- Typically 3 times the signal-to-noise (based on standard deviation of the noise)

Is this peak real?

Yes, it is a peak but you cannot rely on it for concentration determinations as it is not >10 S/N

> 3 S/N



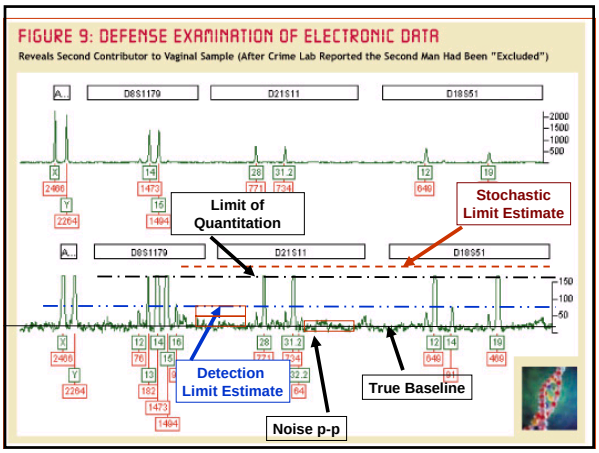
2X Np-p
Peak to peak noise

Approximates 3x sd of noise

The Scientific Reasoning behind the Concept of an Analytical Threshold (limit of detection/limit of quantitation)

- This is fundamentally an issue of reliability
- **For a peak intensity below the LOD there is a very real chance that such a signal is the result of a random fluctuation.**
- **For peaks below the LOQ, the variation in peak height from one run to the next is excessive. These results should not be used in mixture calculations.**

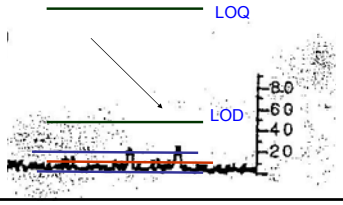
Abracadabra! It's an allele



So how to set thresholds?

- First determine the analytical threshold for your particular laboratory using the signal intensity from one or several CE systems

Analytical threshold for this instrument is approx. 50 RFUs, LOQ is approximately 180 RFUs.



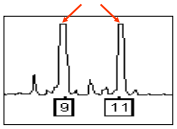
Alternate way to define LOD

- Run a blank with peak detection threshold set at 1
- Determine the standard deviation of the peak heights of all the noise peaks
- LOD is average intensity + 3x SD of the average intensity. LOQ is the baseline +10x average intensity
- This technique may produce lower estimate than the previous one

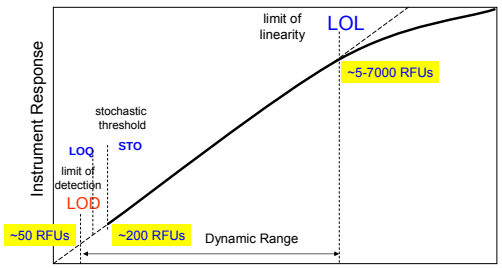
Limit of Linearity (LOL)

- Point of saturation for an instrument detector so that higher amounts of analyte do not produce a linear response in signal
- In ABI 310 or ABI 3100 detectors, the camera saturates leading to flat-topped peaks.

Off-scale peaks



Useful Range of an Analytical Method



LOD = 3x SD of blank
LOQ = 10x SD of blank
STO = peak balance threshold

Offscale Data

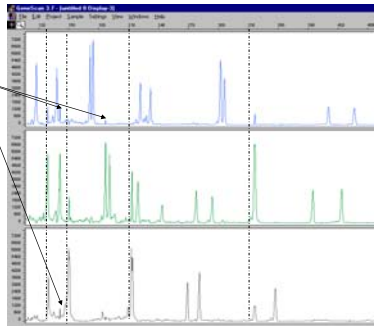
- Elevated baselines are seen with overloaded samples - Moretti et al (2001) JFS 46(3): 647-660
- Probably due to nonspecific amplification
- Stutter is artificially enhanced in such samples due to cutoff of peak top
- -A may also be apparent as a result of poor PCR conditions

Matrix Effects

overloaded samples stress the matrix calibration

Matrix problems also affect low level mixtures. Even with the best matrix you can expect 2-5% pullup!

The effect of pull-up on an overloaded sample



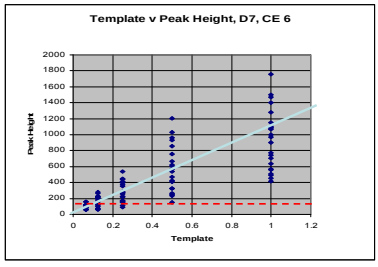
Sensitivity Study

(Debbie Hobson-FBI)

- 25 Individuals
  - 63 pg to 1 ng amplifications with Profiler Plus and Cofiler
  - amplicon run on five 310s
  - GeneScan Analysis threshold sufficient to capture all data
  - GenoTyper: category and peak height
- Import data into Excel
  - peak height ratios determined for heterozygous data at each locus

Sensitivity Study:

Profiler Plus (Debbie Hobson-FBI)



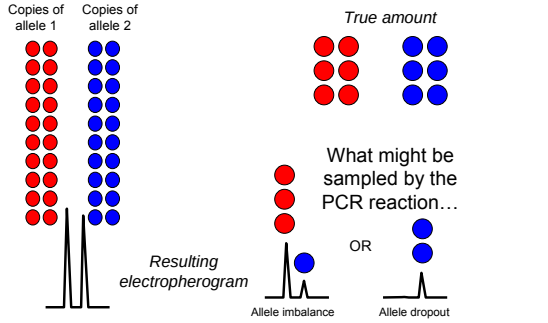
Observation: Peak height variation increases with concentration

Scientific Reasoning behind the **Stochastic Threshold**

- **When stochastic fluctuation is present, interpreting data becomes problematic due to the potential for:**
  - **Allele dropout**
  - **Poorly defined mixture ratios**
  - **Low copy # DNA**
- Bottom line: Input levels of DNA should be sufficiently high to avoid straddle data. Mixture interpretation must be done cautiously on low level data as peak intensities are highly variable.

How low can you go?

Stochastic Statistical Sampling



>20 copies per allele

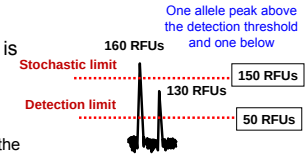
6 copies copies per allele (LCN)

Types of Results at Low Signal Intensity

(Stochastic amplification potential)

Straddle Data

- Only one allele in a pair is above the laboratory stochastic threshold



At low levels of input DNA, the potential for straddle data is high.

The issue is best avoided by re-amplifying the sample at higher input DNA

Otherwise **straddle data makes locus inconclusive**

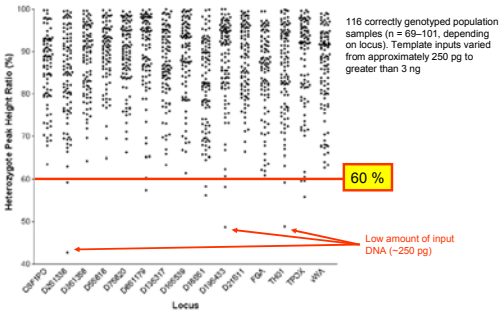
Straddle data may be caused by degradation, inhibition and low copy issues.

How to Determine the Stochastic Threshold

- Examine intensity and peak height ratio of 5 samples at three different low concentrations (e.g., 60, 75, and 125 pg)
- Observe variation in peak height ratio and peak intensity
- The stochastic threshold is the point at which this variation begins a rapid increase (change in slope of line relating std dev vs concentration)
- This can also be defined as the concentration at which a set percentage of peak height ratio values fall below 60%

Heterozygote Peak Height Ratios

Identifiler STR Kit Developmental Validation

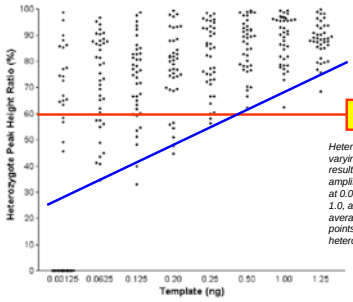


116 correctly genotyped population samples (n = 69–101, depending on locus). Template inputs varied from approximately 250 pg to greater than 3 ng

Collins PJ, Hennessy LK, Leibelt CS, Roby RK, Reeder DJ, Foxall PA. Developmental validation of a single-tube amplification of the 13 CODIS STR loci, D2S1338, D19S433, and amelogenin: the AmpFlSTR Identifiler PCR amplification kit. *J. Forensic Sci.* 2004; 49(6): 1265-1277.

Heterozygote Peak Height Ratios

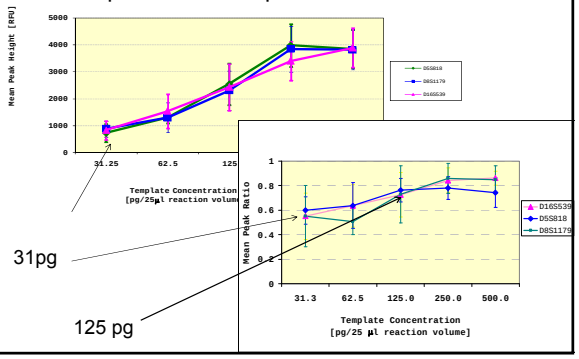
Identifiler STR Kit Developmental Validation



Heterozygote peak height ratios with varying inputs of template DNA. The results depicted are from three amplifications of a single genomic DNA at 0.03125, 0.0625, 0.125, 0.2, 0.25, 0.5, 1.0, and 1.25 ng. Multiple injections were averaged, resulting in a total of 39 data points per input amount (13 heterozygous markers × 3 repetitions).

Collins PJ, Hennessy LK, Leibelt CS, Roby RK, Reeder DJ, Foxall PA. Developmental validation of a single-tube amplification of the 13 CODIS STR loci, D2S1338, D19S433, and amelogenin: the AmpFlSTR Identifiler PCR amplification kit. *J. Forensic Sci.* 2004; 49(6): 1265-1277.

miniSTRs show the same effect
In spite of the improved sensitivity, peak balance is poor at low template concentration



Alternative Procedure (Mass State Police)

1. Since most estimates for LCN show up from 100-250 pg DNA, select a low level sample - say 150 pg as your stochastic limit.

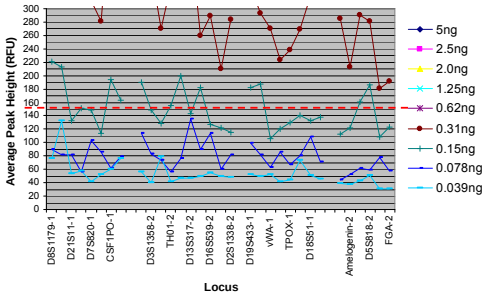
2. Amplify 2 or more samples at a range of concentrations (1.0-0.005) ng multiple times and score the intensity

3. The stochastic limit is the intensity (RFUs) at which half the alleles have intensity above this value and half are below

4. In this way you define straddle data as at the point 50% of your alleles will be above this mark

Reporting Threshold set at **red line** for partial profile @ 0.15ng & 3X noise (baseline + background)

CE011 Titration Sets: Average of All Sets and Replicates



Slide from Joanne Sgueglia (Mass State Police) – AAFS 2008 Mixture Workshop
http://www.cstl.nist.gov/biotech/strbase/training/AAFS2008_MixtureWorkshop.htm

What else can go wrong?

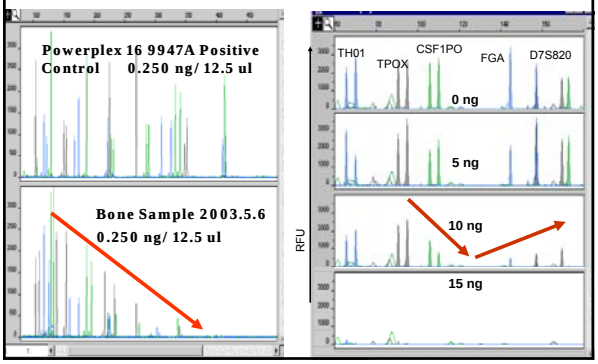
Yarr, Take care mates!

- Most validation studies are performed on pristine samples derived from clean sources.
- **DNA degradation** will result in dropped alleles from larger sized amplicons
- **DNA inhibition** will result in dropped alleles from any location and the effects are difficult to predict
- Inhibition and degradation can produce stochastic effects – peak balance issues and allele dropout.

Degradation vs Inhibition

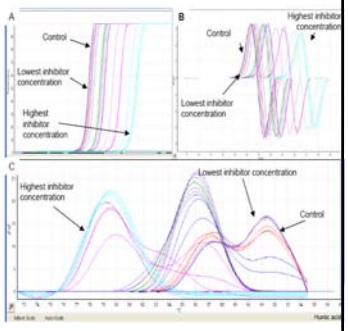
Degraded DNA Sample
Ski slope effect

Humic Acid Inhibited DNA Sample
Less predictable effects



qPCR Humic Acid Inhibition

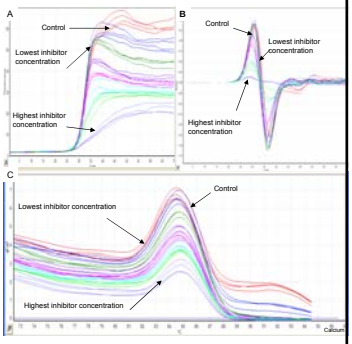
- Shift in take off cycle
- Change in melting curve
- No efficiency of amplification change
- Size effects on melt curve



Conclusion: Sequence specific Inhibitor

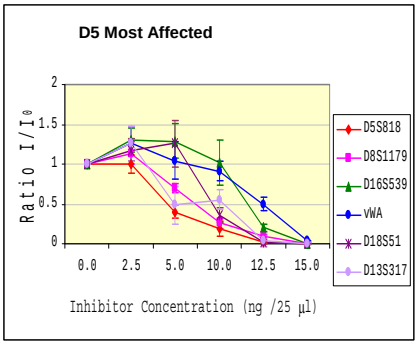
qPCR Calcium Inhibition

- No shift in take off cycle
- No change in melting curve
- Efficiency of amplification affected
- No difference for size or Tm

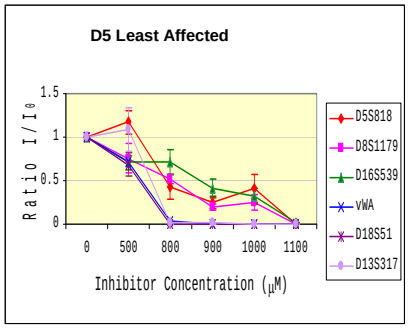


Conclusion: Taq Inhibitor

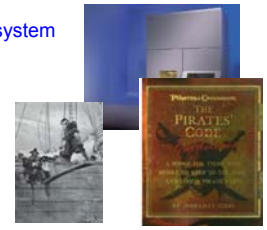
miniSTR Amplification w/ increasing Humic Acid



miniSTR Amplification w/ increasing Calcium



Fuzzy Logic in Data Interpretation

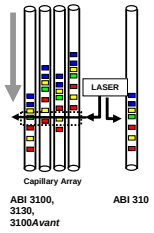


- The ABI 310/3100 is a dynamic system
- Sensitivity varies with
  - Allele size
  - Injection solvent
  - Input DNA
  - Instrument factors
  - Presence of PCR inhibitors
  - Gel matrix
- Thus interpretation must be conservative and data from these studies yields guidelines, not rules. These guidelines must be based on in-house validation. In addition the interpretation and its significance cannot be dissociated from the overall facts of the case.

Instrument factors

1. Because only signal is measured (RFUs) in forensic DNA analysis, many labs find that one instrument or another is more sensitive
2. There are also differences in sensitivity based on injection parameters, capillary illumination (single vs multiple) and laser intensity
3. Lastly the variation in qPCR sensitivity affects the output of any system
4. These differences should be corrected by proper setting of threshold parameters.

ABI 310 vs 3100



Sample
310 1.5 µL in 24 µL formamide
31xx 1 µL in 10 µL formamide

Injection

310	5s @15kV	= 75 kVs
3130 (4 cap)	5s @ 3kV	= 15 kVs
3100 (16cap)	10s @ 3kV	= 30 kVs

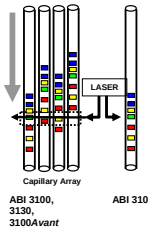
Irradiation

310	direct
3130 (4 cap)	side
3100 (16 cap)	both sides

Laser Power
10 mW (ABI 310)
25 mW (ABI 3130)
25 mW (ABI 3130xl)

Bottom line: you would expect to see
1. an approximate 3 fold difference in RFUs between a 310 and a 3130 (4 cap)
2. an approximate 2 fold difference between a 310 and a 3130xl (16 cap)

Additional Issues



Detection Threshold (ABI)

310	50 RFUs
31xx	30 RFUs

Stochastic Threshold

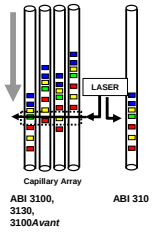
310	150 RFUs
31xx	90 RFU

Dynamic Range

310	4500 RFUs
31xx	3500 RFUs

Bottom line: 310 will appear more sensitive with a wider dynamic range unless proper validations are performed.

Bottom Line



Validate each class of instrument and expect differences in sensitivity/ signal to noise

Compensate for differences by choosing appropriate thresholds

Validate at 2 or more injection levels so that injection time can be increased- remembering that **longer injections risk drifting into LCN regime**

Calling thresholds involve sensitivity, dynamic range and the necessity to avoid low-copy number (LCN) data

Issues with Data Below the Stochastic Threshold

- PCR artifacts and stutter become prevalent
- Low levels of bleed through are possible
- Instrument spikes are more numerous
- -A peaks may appear
- Dye blobs become more significant in overall e-gram
- Low level 2nd contributors may show peaks

So why examine low level data at all?

•Detection of straddle data in which one allele is above threshold and the other is below

•Detection of the presence of low level mixtures

•Clues to the presence of inhibited samples or poor injections

•Aids in determination if a suspect is excluded as a contributor

Comparison of STR Kit Amplification SOP with LCN

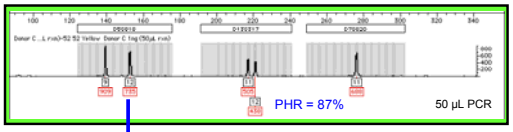
Using the Same DNA Donor

Input DNA

Data from Debbie Hobson (FBI) – LCN Workshop AAFS 2003

SOP

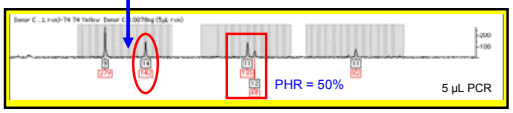
1ng



Allele Drop Out

LCN

8pg



Allele Drop In

Heterozygote Allele Imbalance

Stutter and Peak Height Ratios change at LCN

- Stutter increases with allele size:
- Stutter increases at low copy #

Leclair et. al (2004) Systematic analysis of stutter percentages and allele peak height and peak area ratios at heterozygous STR loci for forensic casework and database samples. J. Forensic Sci. 49(5): 968-980

Peak height (D5S818)

RESEARCH HIGHLIGHTS

Nature Reviews Genetics | AOP, published online 18 March 2008; doi:10.1038/nrg2362

Ethics watch

LCN DNA: PROOF BEYOND REASONABLE DOUBT?

Low copy number (LCN) DNA forensic profiling has lead to successful criminal prosecutions, including in the Peter Falconio case in Australia and the murder of the Swedish foreign minister, Anna Lindh. However, the technique has serious limitations, and few jurisdictions have followed the United Kingdom in accepting it as evidence in court. The discrediting of the LCN DNA evidence in the Omagh trial, which led the UK police to temporarily suspend their use of the method, has prompted further questioning of this technique and some scientists are claiming that criminal convictions based upon LCN DNA will soon start troubling the appeal courts.

Issues:

Was evidence collected with LCN in mind?

Is LCN evidence reliable?

Does the obtained profile result from the evidence?

Low copy number situations exist in many samples

- In a 1:1 mixture, each DNA source is at LCN when the total amount of DNA in the amplification reaction is ~ 0.125 ng.
- In a 1:9 mixture, the minor component could be at LCN **even when the total amount of DNA in the amplification is 1 ng**.

Robin Cotton, AAFS 2003 LCN Workshop
"Are we already doing low copy number (LCN) DNA analysis?"

Findley *et al.* (1997) *Nature* article

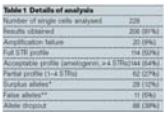


Table 1 Details of analysis

Number of single cells analysed	226
Results obtained	206 (91%)
Amplification failure	20 (9%)
Full STR profile	114 (50%)
Acceptable profile (amelogenin,>4 STRs)	144 (64%)
Partial profile (1-4 STRs)	62 (27%)
Surplus alleles*	28 (12%)
False alleles**	11 (5%)
Allele dropout	88 (39%)

*Additional allele present in conjunction with true alleles.

**Additional allele in place of true allele. Extra-allelic peaks could be caused by contamination, somatic mutation or PCR-generated non-allelic peaks. We never saw more than two additional peaks in a profile or in 18 negatives, minimizing the possibility of cellular contamination. When surplus alleles were observed we considered the locus, but not the profile, uninformative. We observed allele dropout in 39% of cells at a rate of ~10% in each allele. If two cells are analysed then the risk of allelic dropout and misinterpretation in cells is reduced to 1%, if three cells 0.1%, and so on. Wild-card designations and conservative statistical criteria are needed to ensure that evidential value can be properly assessed.

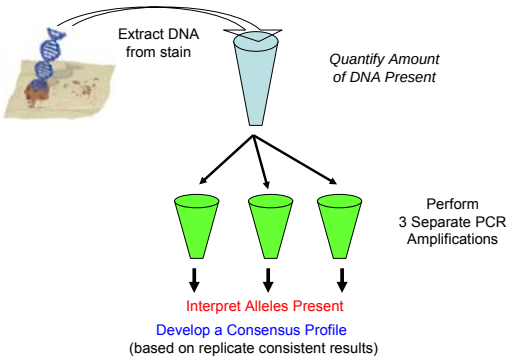
Some interpretational guidelines with LCN

- At least two* PCR amplifications from the same DNA extract
  *five is better; results are investigative
- An allele cannot be scored (considered real) unless it is present at least twice in replicate samples
- Extremely sterile environment is required for PCR setup to avoid contamination from laboratory personnel or other sources

Meatloaf Principle

- I want you
  I need you
  But -- there aint no way Im ever gonna love you
  Now dont be sad
  cause two out of three aint bad
  – Meatloaf
- You see an allele twice in 3 runs
- What if the the 4th measurement shows no allele?
- Is seeing an allele 50% of the time a measure of reliability. Is 66% ok?

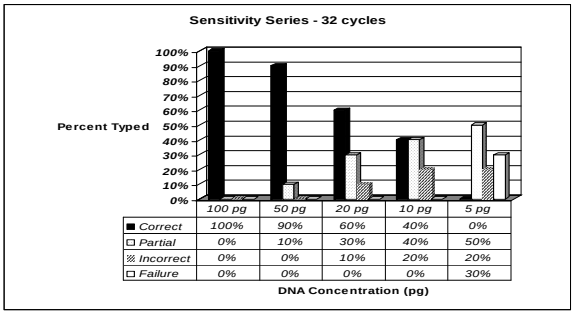
Typical LCN Procedure



Catch 22

- Note the Catch 22. Are two amplifications of 50pg better than 1 of 100pg?
- Are 3 amplifications of 17pg better than one of 50?
- Data shows that the lower the amount of the DNA amplified the more likely allele dropout and false alleles occur
- This somewhat calls in to question the idea that a sample should be split and run multiple times

Problems with Obtaining Correct Allele Calls at Low DNA Levels



	100 pg	50 pg	20 pg	10 pg	5 pg
Correct	100%	90%	60%	40%	0%
Partial	0%	10%	30%	40%	50%
Incorrect	0%	0%	10%	20%	20%
Failure	0%	0%	0%	0%	30%

Coble, M.D. and Butler, J.M. (2005) *J. Forensic Sci.* 50: 43-53

Replicate LCN Test Results from FSS

Gill, P. (2002) Role of short tandem repeat DNA in forensic casework in the UK--past, present, and future perspectives. *BioTechniques* 32(2): 366-385.

Table 2. Results of Six Replicate PCR Tests of a Sample Under Low Copy Number Analysis Conditions Compared to the Control Sample

	Amelo	D19	D3	D8	THO	VWA	D21	FGA	D16	D18	D2
CONTROL	X X	14,14	18,18	15,15	7 9.3	19,19	28 32.2	20,23	9,12	12,16	17,23
Sample											
1	--	14 F'	--	15 F'	--	--	28 32.2	20 F'	--	16 F'	--
2	X F'	--	18 F'	15 F'	--	19 F'	--	--	12 F'	--	--
3	X F'	--	--	15 F'	--	--	--	--	--	--	17 F'
4	X F'	14 F'	18 F'	--	--	--	--	--	9 12	--	--
5	X F'	--	18 F'	--	--	18 F'	--	--	--	--	--
6	X F'	14 F'	--	--	--	19 F'	28 32.2	20 F'	--	12 F'	--
Consensus	X F'	14 F'	18 F'	15 F'	--	19 F'	28 32.2	20 F'	12 F'	--	--

The consensus result is reported, provided that an allele is observed at least twice. If only one allele is observed, then an F' designation is given to denote the possibility of allele drop-out.

F' used to designate that allele drop-out of a second allele cannot be discounted when only a single allele is observed (OCME uses "Z")

Low Copy Number Limitations (cont):

From Bruce Budowle (2005) 1st International Human Identification E-Symposium

- Tissue source cannot be determined
- DNA may not be relevant – casual contact/transfer
- If victim and suspect have any common access…
- Old cases may not be viable – handling
- Not for post conviction analysis
- Rarely useful for database searching
- An intelligence tool

http://www.e-symposium.com/humid/archive/drbrucebudowle.php

The Report

- No nuclear profile due to insufficient or excessively degraded DNA
- Suspect is excluded based on results for 2 of 17 Y STR markers.
- Huh !?!
- My comments
  - 1. The result is clearly at low copy
  - 2. The pattern of alleles is not consistent with degradation as the cause of dropout.
  - 3. At low copy a scientist cannot express a strong opinion about how DNA arrived at the site where it was recovered. This DNA could just as easily come from thin air as it could come from the suspect.
- Bottom line: Why was this sample even run?

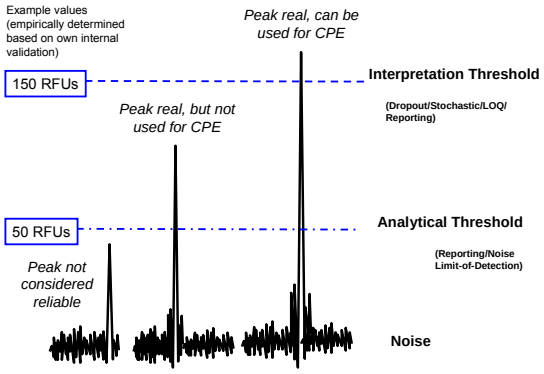
The Bottom Line:

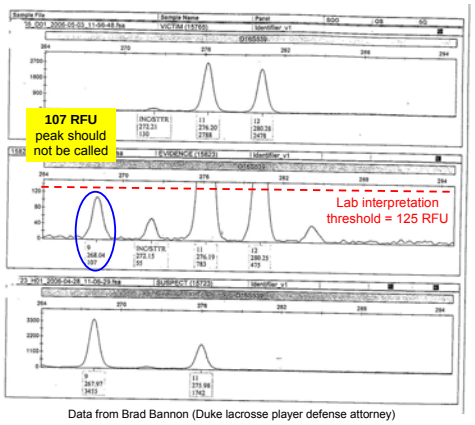
1. Low signal levels are bad because:
   a. They may indicate low copy # DNA = inconsistent or misleading results
   b. They often coincide with peak imbalance
   c. PCR and instrumental artifacts appear at these levels
2. Relying on signal level to determine DNA quantity can be misleading
   a. There is wide variation in signal strength of amplified DNA
   b. Inhibitors and mixtures complicate interpretation
      1. peak imbalance can occur even in single source samples due to inhibition and degradation
      2. instruments can vary in sensitivity

Conclusions

- Be conservative in interpretation
  - Set thresholds based on signal to noise and stochastic amplification (2 thresholds). Base these numbers on controlled in-house experiments
  - Understand that different instruments may vary in sensitivity – set thresholds high enough to encompass this variation
  - Understand that even with such guidelines issues such as degradation and inhibition can skew results.
- Leave room for the facts of the sample in your interpretation

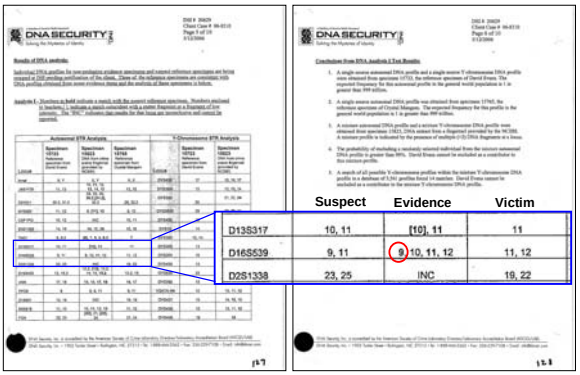
Threshold Illustration





Data from Brad Bannon (Duke lacrosse player defense attorney)

May 12, 2006: DNA Security Report

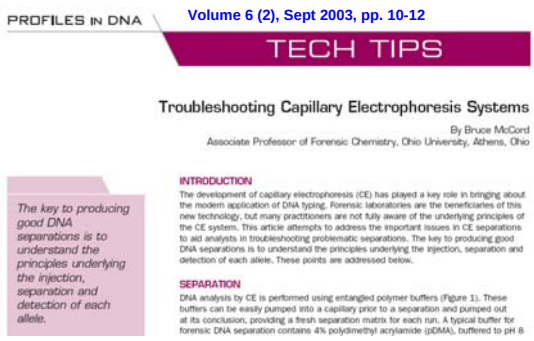


Data from Brad Bannon (Duke lacrosse player defense attorney)

Troubleshooting:
Strategies and Solutions

Bruce

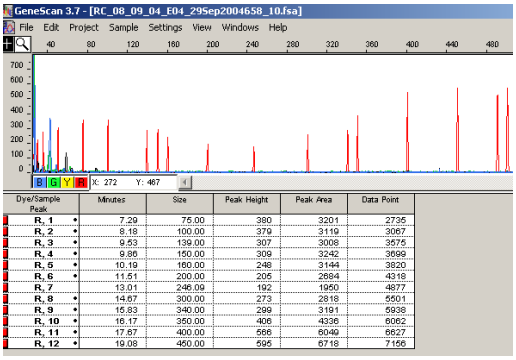
Bruce McCord's *Profiles in DNA* Article



2. Sample Issues

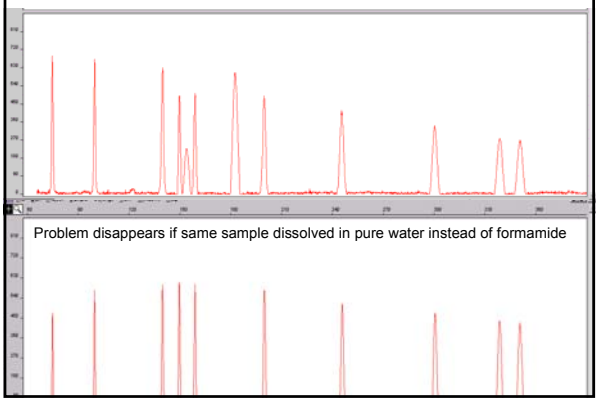
- Formamide Conductivity
- Excessive salt in sample due to evaporation
- Metal ion contamination
- Sensitivity issues with Microcon cleanup (salt removal)
- Dye "blobs" – artifacts from primer synthesis

Golden Gate Effect
Attributed to poor formamide



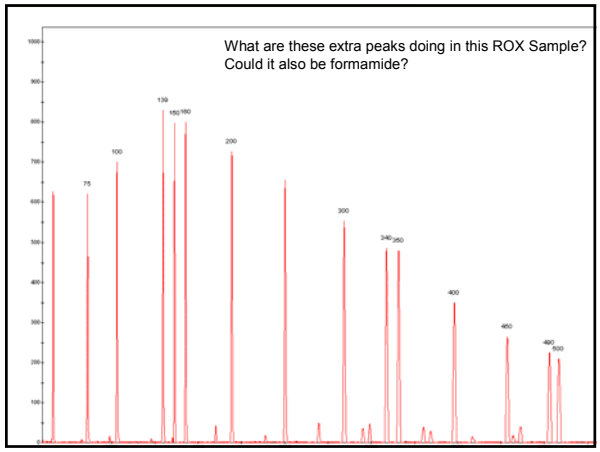
Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
R, 1	7.29	76.00	380	3201	2735
R, 2	8.18	100.00	379	3119	3067
R, 3	9.53	139.00	307	3008	3575
R, 4	9.86	150.00	309	3242	3699
R, 5	10.19	160.00	248	3144	3820
R, 6	11.51	200.00	205	2684	4318
R, 7	13.01	248.09	192	1950	4877
R, 8	14.67	300.00	273	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

Effect of bad HI-Dye Formamide - Note broad peaks and extra bands

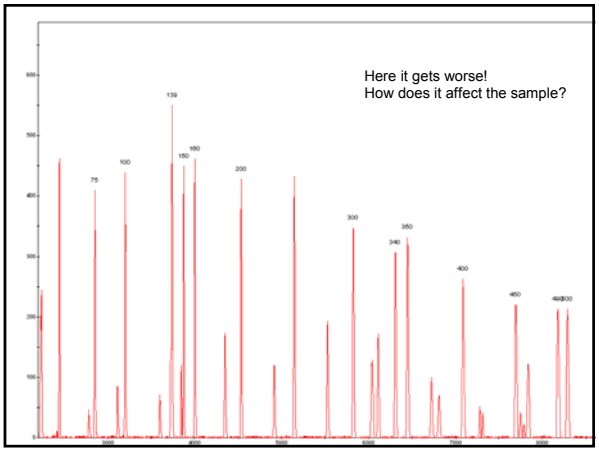


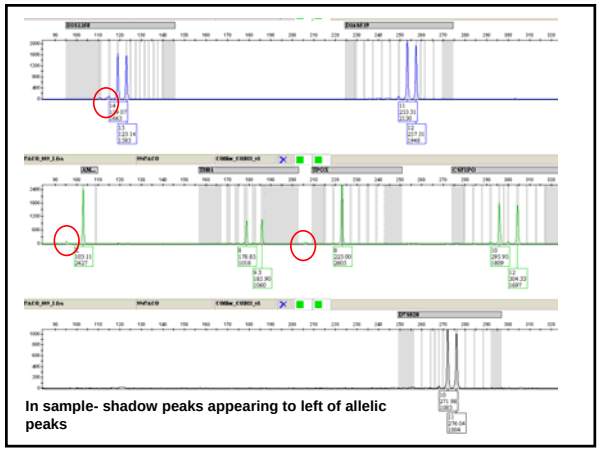
Problem disappears if same sample dissolved in pure water instead of formamide

What are these extra peaks doing in this ROX Sample?
Could it also be formamide?



Here it gets worse!
How does it affect the sample?





In sample- shadow peaks appearing to left of allelic peaks

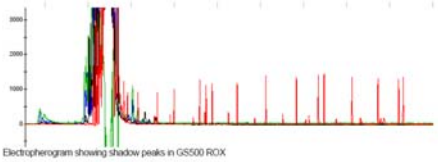
December 21, 2007

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:

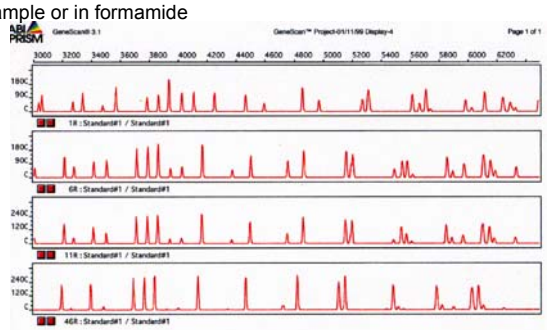


Electropherogram showing shadow peaks in GS500 ROX

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

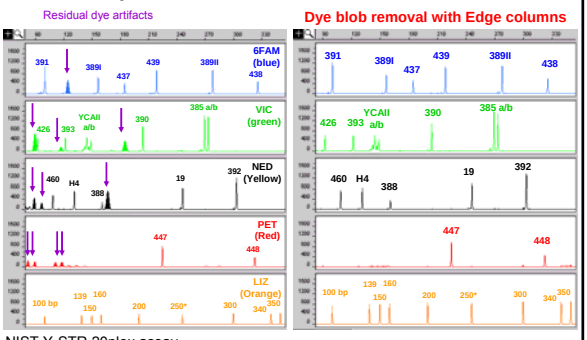


ds DNA migrates faster and over time with this set of runs ds DNA replaced the ssDNA

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



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*.

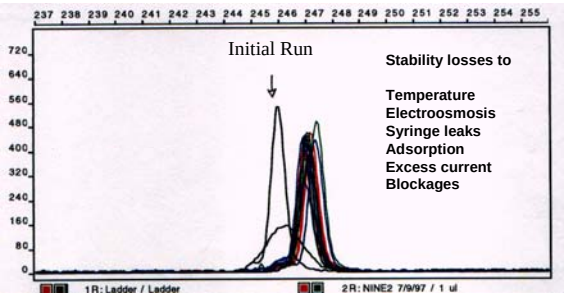
3. External Factors

- 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.

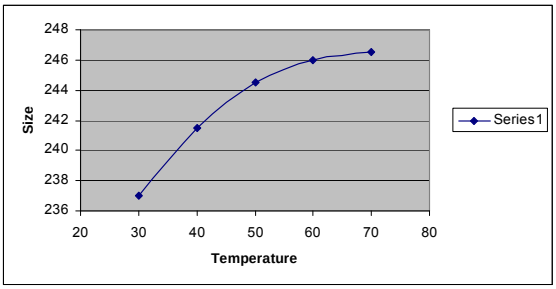
Temperature effects

- 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

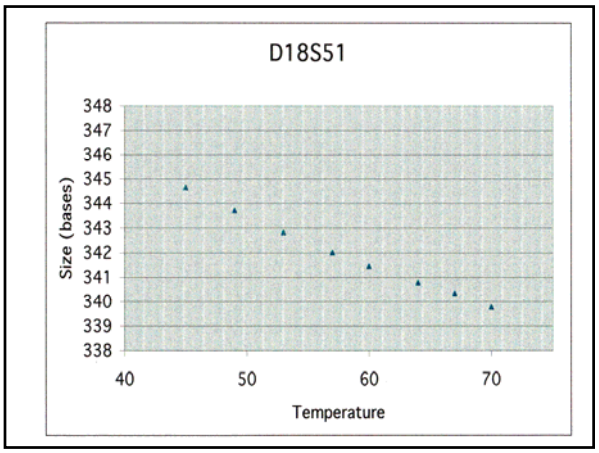
Due to its structure and its non-calibration, the "250"peak can be used to indicate stability

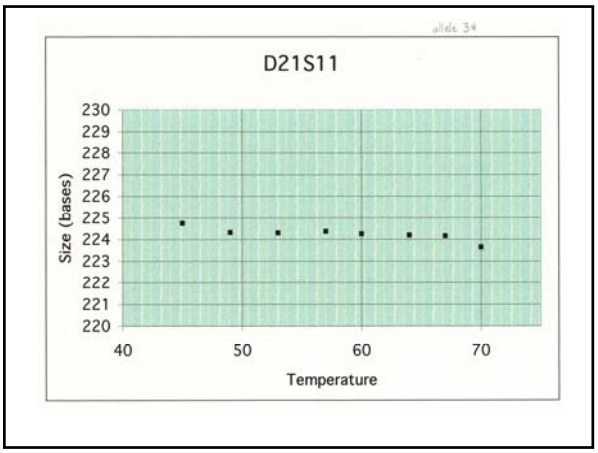


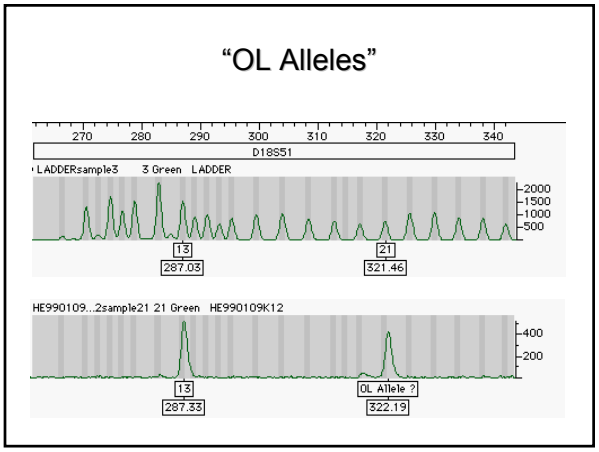
Change in size of GS 250 peak with Temperature (Tamra Std)

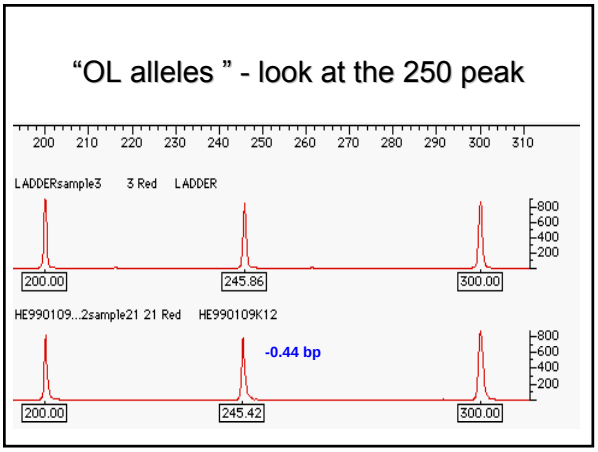


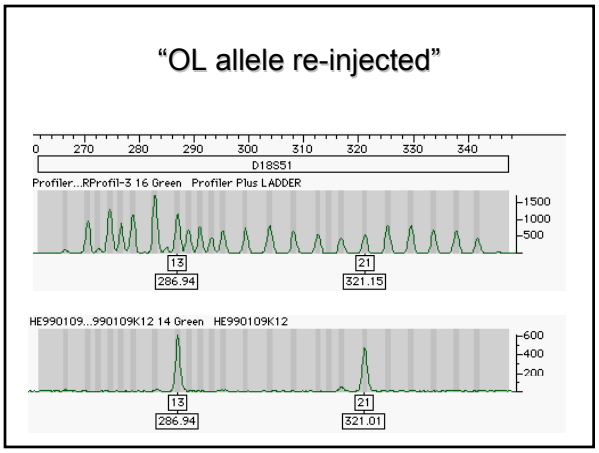
4% pDMA with 8M urea and 5% 2-pyrrolidinone
***Rosenblum et al., Nucleic Acids Res.(1997) 25,19, 2925**

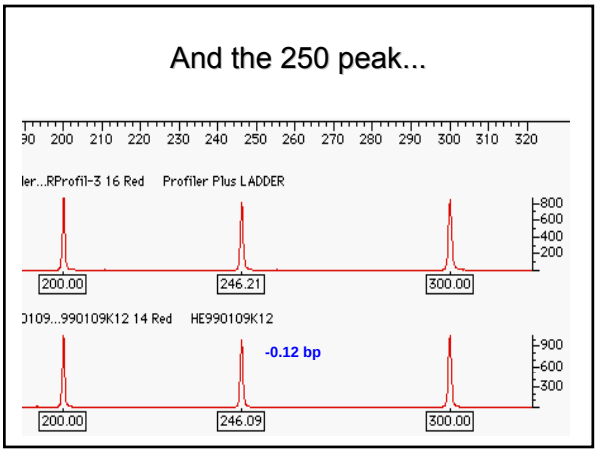

D18S51
Size (bases)
348
347
346
345
344
343
342
341
340
339
338
40
50
60
70
Temperature


D21S11
Size (bases)
230
229
228
227
226
225
224
223
222
221
220
40
50
60
70
Temperature

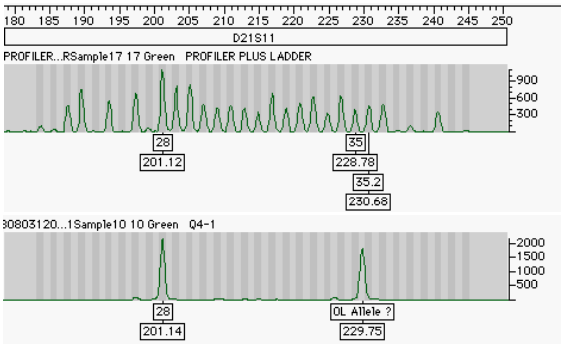

"OL Alleles"
D18S51
LADDERsample3 3 Green LADDER
13
287.03
21
321.46
HE990109...2sample21 21 Green HE990109K12
13
287.33
OL Allele ?
322.19


"OL alleles " - look at the 250 peak
LADDERsample3 3 Red LADDER
200.00
245.86
300.00
HE990109...2sample21 21 Red HE990109K12
-0.44 bp
200.00
245.42
300.00


"OL allele re-injected"
D18S51
Profiler...RProfil-3 16 Green Profiler Plus LADDER
13
286.94
21
321.15
HE990109...990109K12 14 Green HE990109K12
13
286.94
21
321.01


And the 250 peak...
ler...RProfil-3 16 Red Profiler Plus LADDER
200.00
246.21
300.00
0109...990109K12 14 Red HE990109K12
-0.12 bp
200.00
246.09
300.00

True off-ladder alleles



Monitoring Room Temperature Over Time

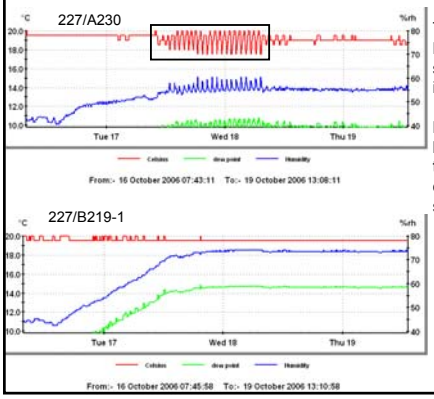
Temperature Probes

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

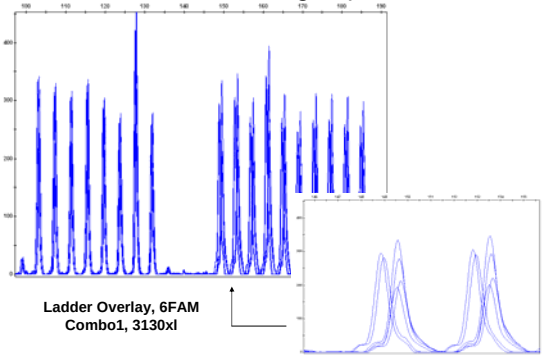
Monitoring Instrument Room Temperature Fluctuations



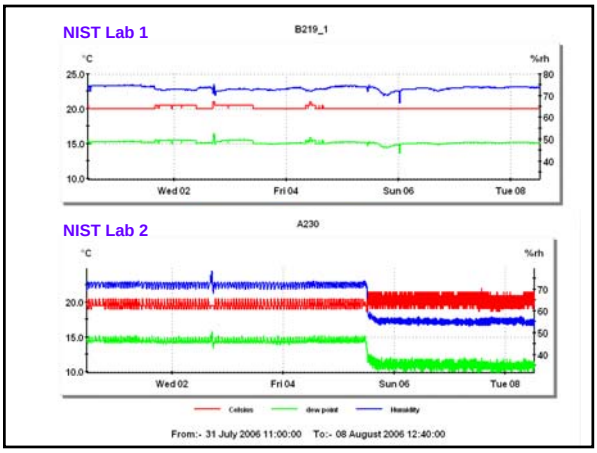
Temperature Monitoring of two separate instrument rooms.

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

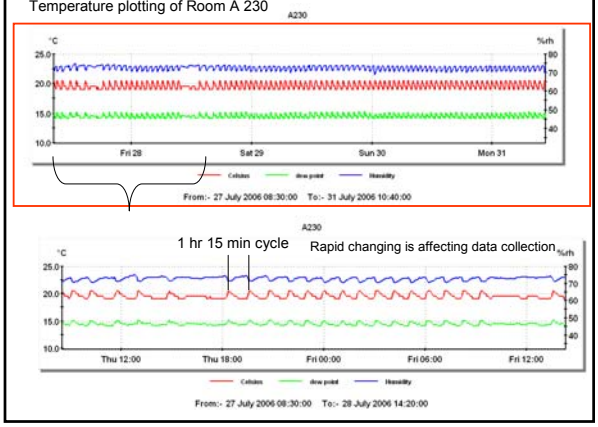
Poor Temperature Control Causes DNA Sizing Imprecision



Ladder Overlay, 6FAM Combo1, 3130xl



Temperature plotting of Room A 230

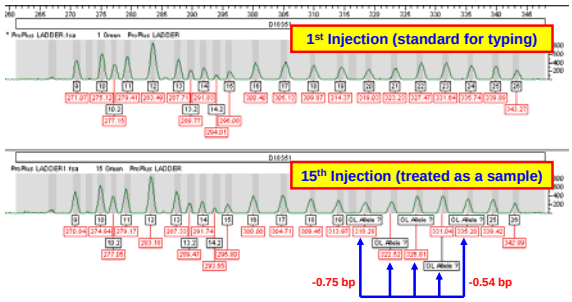


What to do if calibration is lost?

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

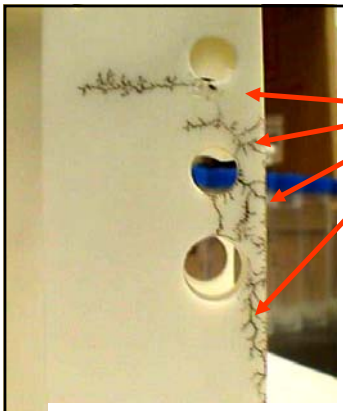
Use of Second Allelic Ladder to Monitor Potential Match Criteria Problems



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

Cleanliness

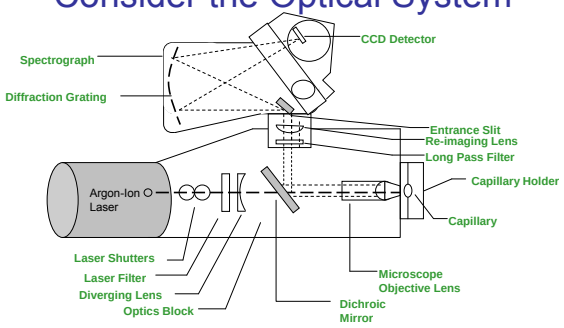
- Urea sublimates and breaks down to ionic components - these find a path to ground
- Similarly wet buffer under a vial creates paths to ground
- Capillary windows must be clear or matrix effects will occur
- Laser will often assist in this process
- Vial caps will transfer low levels of DNA to capillary



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

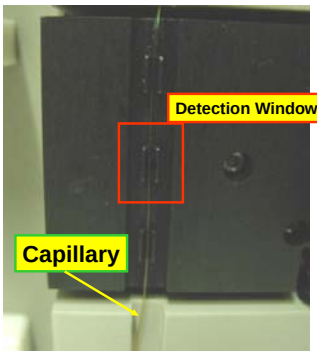


Watts, D. Genotyping STR Loci using an automated DNA Sequencer. In *Forensic DNA Profiling Protocols;* Lincoln, P.J.; Thomson, J. Eds.; Humana Press Inc.: Totowa, NJ, 1998; Vol. 48, pp 193-208.

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.**

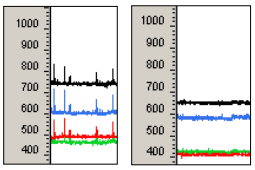
The Detection Window



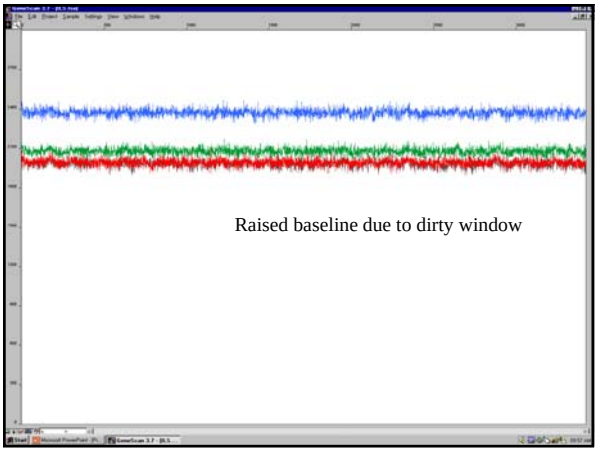
Make sure that the capillary window is lined up (if it is not, then no peaks will be seen)

Window may need to be cleaned with ethanol or methanol

Review Start of Raw Data Collection



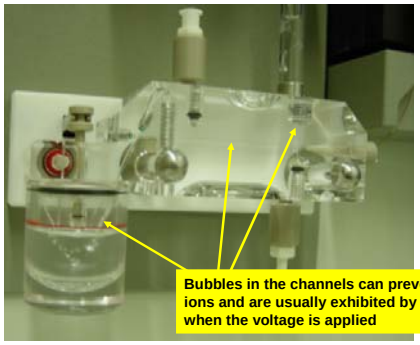
Little spikes indicate need to change buffer… check current

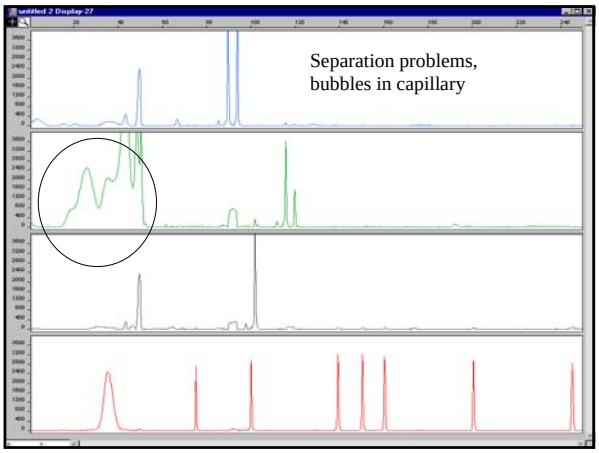


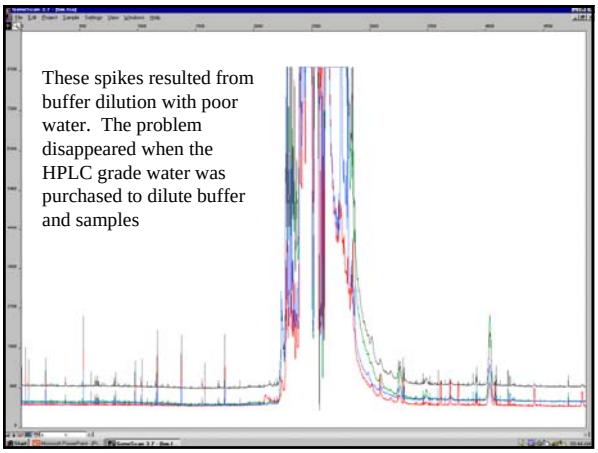
Buffer Issues

- 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

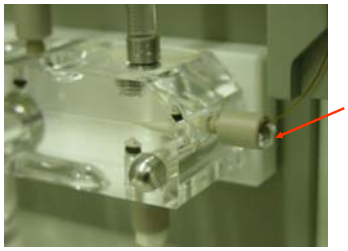
Remove all bubbles from the channels







Beware of Urea Crystals



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

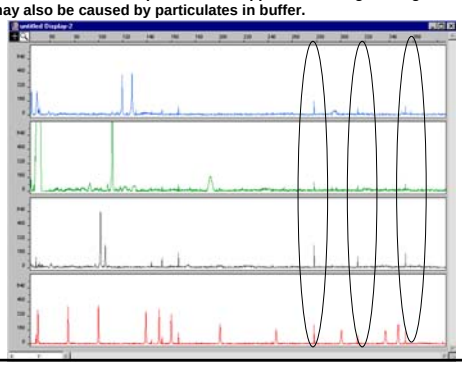
Urea sublimates and can evaporate to appear elsewhere

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

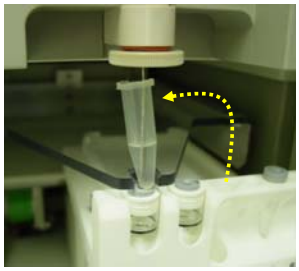
Pump block should be well cleaned to avoid problems with urea crystal formation

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

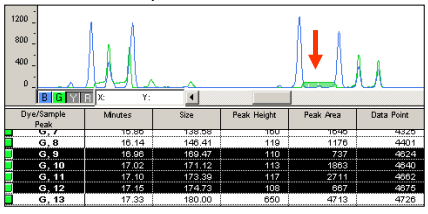


- Keep inlet of capillary in water…if it dries out then urea crystals from the polymer will clog the opening
- The waste vial (normally in position 3) can be moved into position
- A special device can be purchased from Suppelco to rinse the capillary off-line
- Store in distilled water
- Note that the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

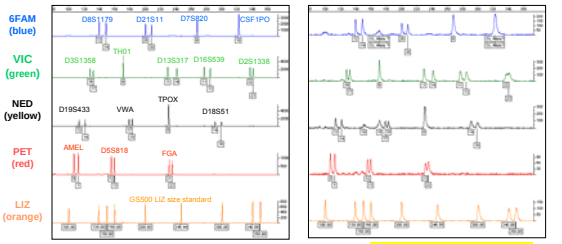
- A poor matrix can lead to raised baseline and therefore calling of too many peaks
- Larger sized alleles will not be identified as peaks because the GeneScan table for a particular dye color has filled up



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
G, 7	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

(A) Good resolution (B) Poor resolution **Identifiler data**



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.

NEW SLIDE

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.

NEW SLIDE

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:

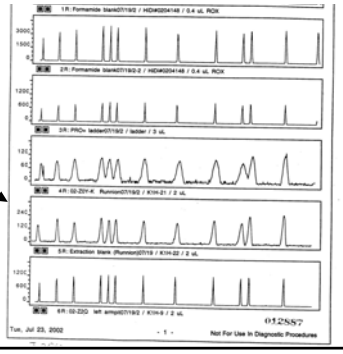
- Flush the system 10 to 15 times with warm (40°C) deionized system water wash prior to array/polymer replacement. Using a high purity bottled water source may help to eliminate water as a potential contributing factor.
- The warm water wash should be followed immediately by replacement of the capillary 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

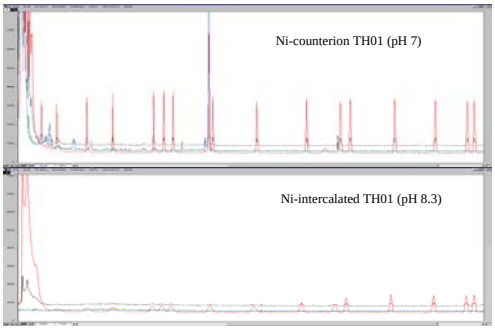
Contamination results in problems in subsequent analyses

Effect is transitory



Metal Ions in the Sample

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



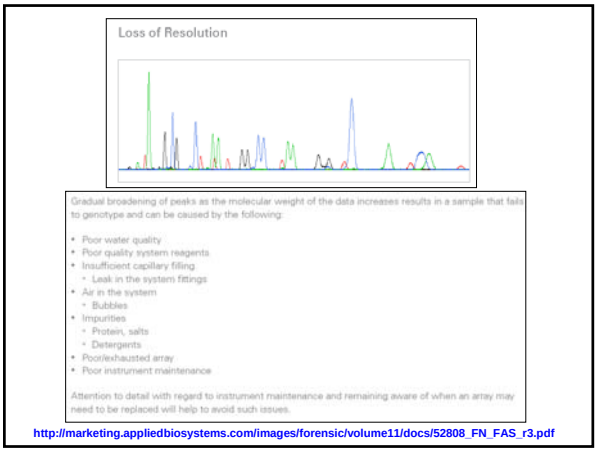
1 µl TH01 added to 10 µl of 3.0 mM $NiCl_2$ in 10 mM Tris, pH 7 or pH 8.3. Sample allowed to interact for 1 hr and then 1 µl added to ROX/formamide.

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)

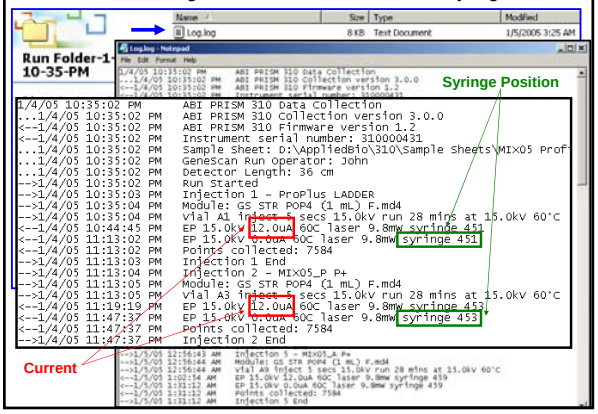
Measurement of Current

- 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

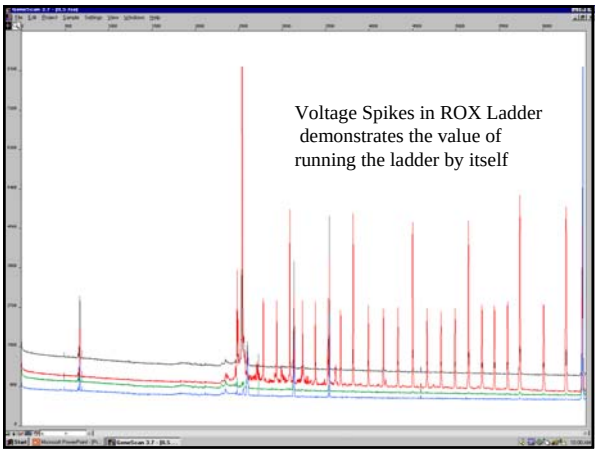
- The ABI 310 instrument also keeps track of the position of the syringe (in the log file)
- Depending on the resistance to flow, the syringe will travel different lengths
- Syringe leaks may be reflected in a longer distance traveled prior to each injection
- These leaks occur around the barrel of the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

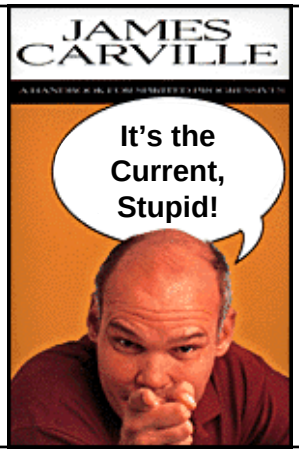
Conclusion:

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

Just Remember



Review of Some Specific Problems

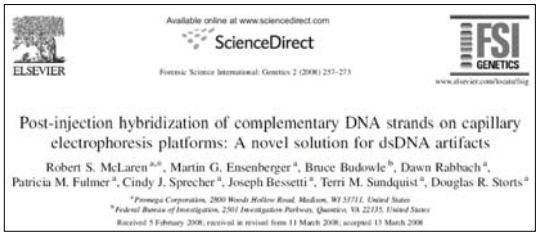
John & Bruce

Questions from Workshop Attendees

Specific Problems

- Post-PCR oligo hybridization to eliminate vWA artifacts with PowerPlex 16 results
- Allele dropout with MiniFiler D16S539 (vs Identifiler)
- Resolution loss due to 3130xl pump failure
- Signal loss (data gap) from 3100 laser shutter sticking
- Some variant and tri-alleles

Recent Promega Solution to Eliminating vWA Artifacts in PowerPlex 16 Results



Available online at www.sciencedirect.com
ScienceDirect
Forensic Science International: Genetics 2 (2008) 257–273
ELSEVIER
FSI GENETICS
www.elsevier.com/locate/fsig

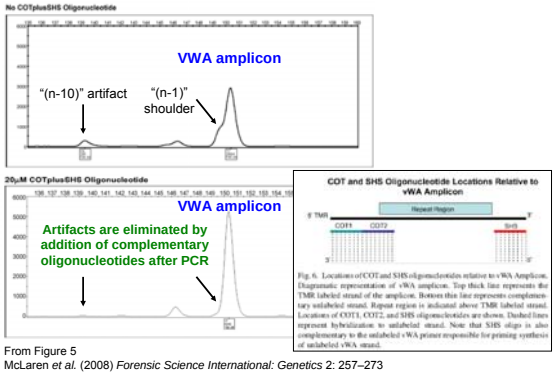
Post-injection hybridization of complementary DNA strands on capillary electrophoresis platforms: A novel solution for dsDNA artifacts

Robert S. McLaren [a,*], Martin G. Ensenberger [a], Bruce Budowle [b], Dawn Rabbach [a], Patricia M. Fulmer [a], Cindy J. Sprecher [a], Joseph Bessetti [a], Terri M. Sundquist [a], Douglas R. Storts [a]

[a] Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711, United States
[b] Federal Bureau of Investigation, 2501 Investigation Parkway, Quantico, VA 22135, United States
Received 5 February 2008; received in revised form 11 March 2008; accepted 13 March 2008

Several laboratories have reported the occurrence of a split or n -1 peak at the vWA locus in PowerPlex 16… The root cause of this artifact is post-PCR reannealing of the unlabeled, unincorporated vWA primer to the 30-end of the tetramethylrhodamine (TMR)-labeled strand of the vWA amplicon. **This reannealing occurs in the capillary post-electrokinetic injection**. The split peak is eliminated by incorporation into the loading cocktail of a sacrificial hybridization sequence (SHS) oligonucleotide that is complementary to the vWA primer. The SHS preferentially anneals to the primer instead of the TMR-labeled strand of the vWA amplicon…

Impact of Added Oligos to vWA Amplicon Peaks



From Figure 5
McLaren *et al.* (2008) *Forensic Science International: Genetics* 2: 257–273

Different Genetic Tests Can Give Different Results Based on PCR Primer Positions

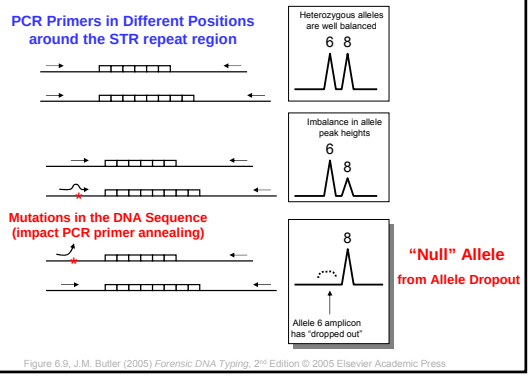
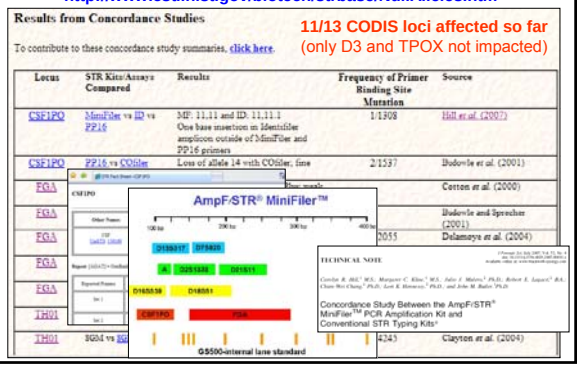
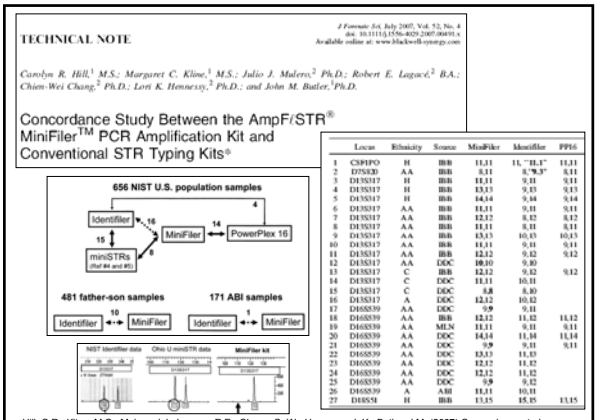


Figure 6.9, J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition © 2005 Elsevier Academic Press

New Null Allele Section on STRBase

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



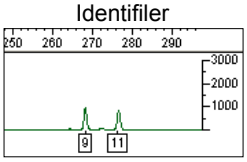


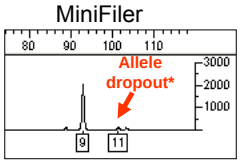
Hill, C.R., Kline, M.C., Mulero, J.J., Lagace, R.E., Chang, C.-W., Hennessy, L.K., Butler, J.M. (2007) Concordance study between the AmpFISTR MiniFiler PCR Amplification Kit and conventional STR typing kits. *J. Forensic Sci.* 52(4): 870-873.

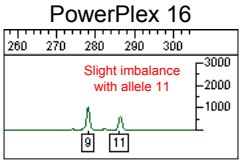
MiniFiler Discordance

SRM 2391b Genomic 8 with D16S539

All allele calls with MiniFiler for CSF1PO, D7S820, D13S317, D18S51, D21S11, FGA, and D16S539 (with the exception noted below) ***match previously certified values.***



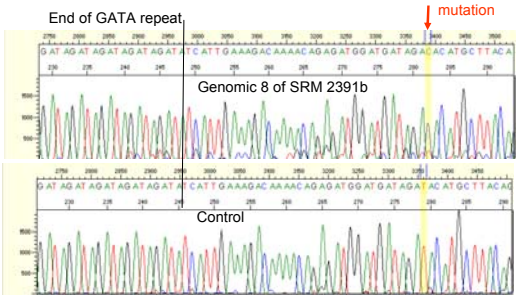




Due to primer binding site mutation

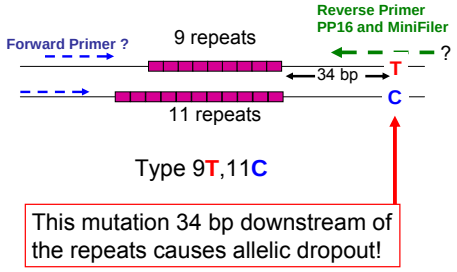
D16S539 SRM 2391b Genomic 8

T→C mutation 34 bp downstream of the repeat



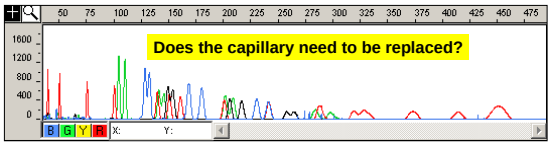
Position of the T→C probably affects the reverse primer of Minifiler and is the 3rd base found the 5'end of the Reverse PP16 primer. This could explain the imbalance of the allele seen when using PP16.

D16S539 SRM 2391b Genomic 8

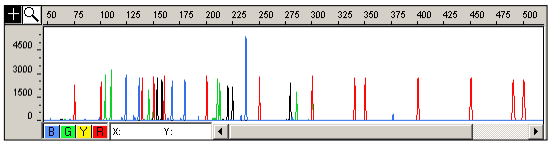


What we call "melt downs"…

probably due to an incompletely filled capillary

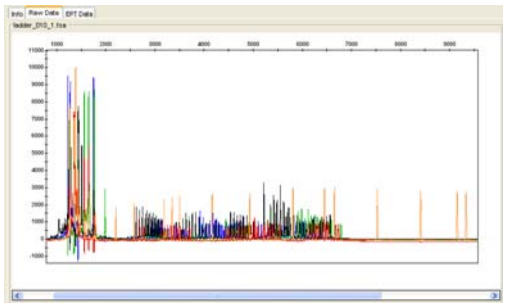


No! The next injection looks fine…



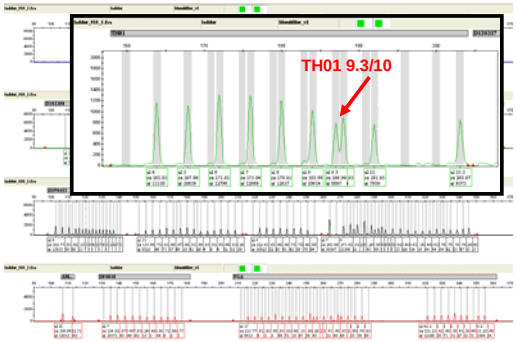
ABI 310 Data from Margaret Kline (NIST)

Identifiler Allelic Ladder March 14, 2007



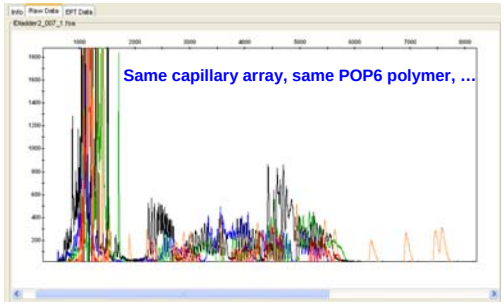
Data from Becky Hill (NIST)

Identifiler Allelic Ladder March 14, 2007



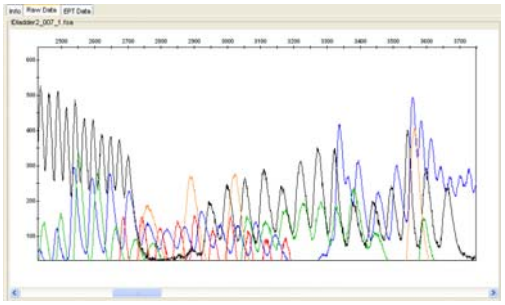
Data from Becky Hill (NIST)

Identifiler Allelic Ladder March 23, 2007



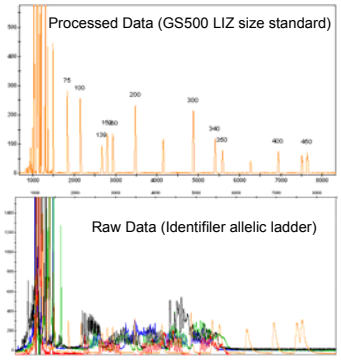
Data from Becky Hill (NIST)

Examination of Resolution in TH01 Region

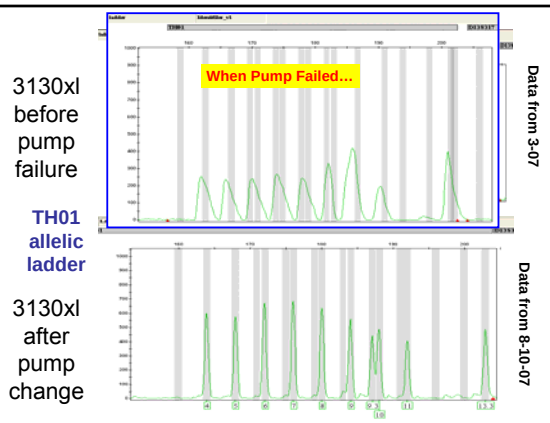


Data from Becky Hill (NIST)

Examine the Size Standard…

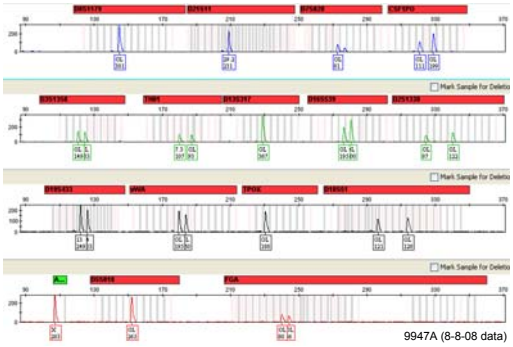


Data from Becky Hill (NIST)



Data from Amy Decker (NIST)

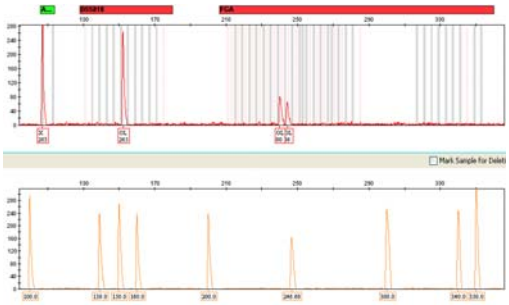
Poor Resolution and Peak Tailing



9947A (8-8-08 data)

Data from Amy Decker (NIST)

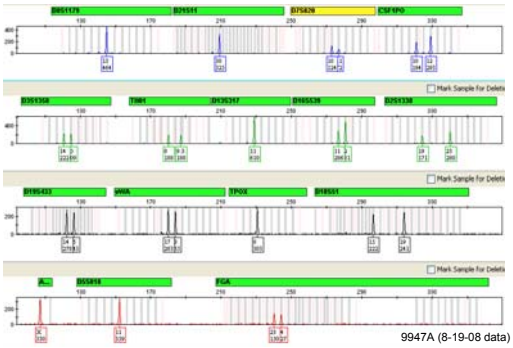
Identifiler PET channel and GS 500 LIZ Size Standard



9947A (8-8-08 data)

Data from Amy Decker (NIST)

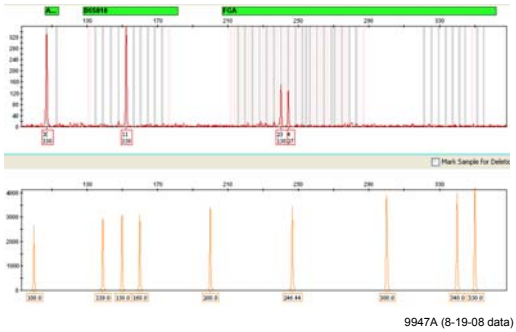
Diluted Sample in Fresh Hi-Di Formamide



9947A (8-19-08 data)

Data from Amy Decker (NIST)

Identifiler PET channel and GS 500 LIZ Size Standard

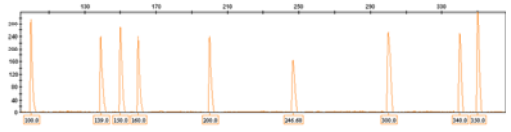


9947A (8-19-08 data)

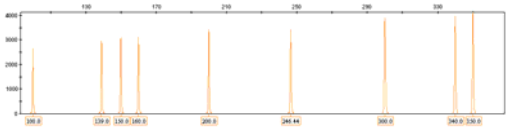
Data from Amy Decker (NIST)

Comparison of GS500 LIZ Size Standard

Sample diluted in **Old Hi-Di Formamide**



Sample diluted in **Fresh Hi-Di Formamide**

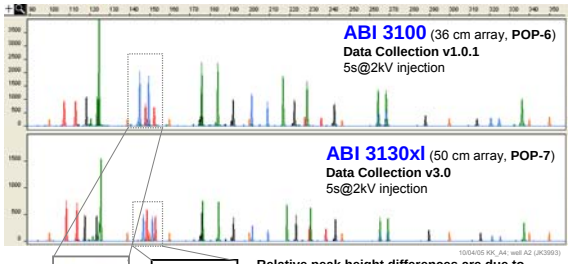


Data from Amy Decker (NIST)

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.

3100 phenomena

what happened on one instrument during the last year...(Sept 2006-2007)

Data from Walter Parson's Lab (Innsbruck, Austria)

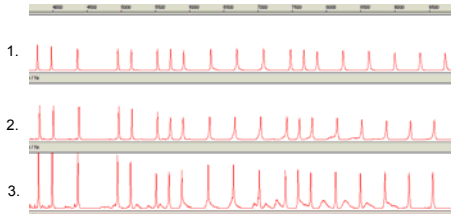
3100 phenomena

- **"Septa" – phenomenon**
  - buffer and water reservoir septa
  - septa of the 96-well plates
- **"Shift" – phenomenon**
  - slow runs
  - fast runs
- **"Shining through" – phenomenon**
- **"Carry-over" – phenomenon**
- **"Data gap" - phenomenon**

Data from Walter Parson's Lab (Innsbruck, Austria)

"Septa" – phenomenon

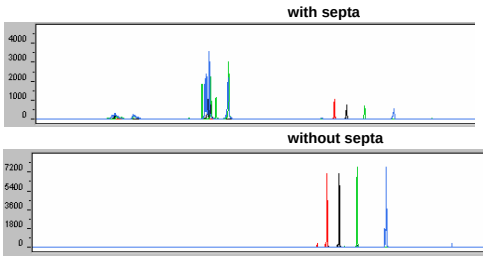
- with buffer and water reservoir septa
  - bad CE after about three injections



Data from Walter Parson's Lab (Innsbruck, Austria)

"Septa" – phenomenon

- septum of the 96-well plate + buffer and water reservoir septa: bad matrix runs (spectral calibration)

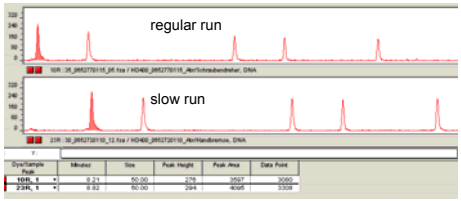


Data from Walter Parson's Lab (Innsbruck, Austria)

"Shift" – phenomenon

slow runs

- Shifted runs each 3. and/or 5. run (up to 400 data points measured at the 50bp-Std)
- Problem: "off ladder alleles"
- possible cause: bad POP6 Lot



Data from Walter Parson's Lab (Innsbruck, Austria)

"Shift" – phenomenon

slow runs

- 1. run after starting the 3100 are shifted (up to 400 data points measured at the 50bp-Std)
- Problem: "off ladder alleles"
- cause: not explained yet – but no temperature problem

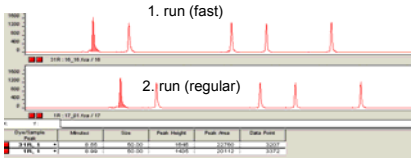
Data from Walter Parson's Lab (Innsbruck, Austria)

"Shift" – phenomenon

fast runs

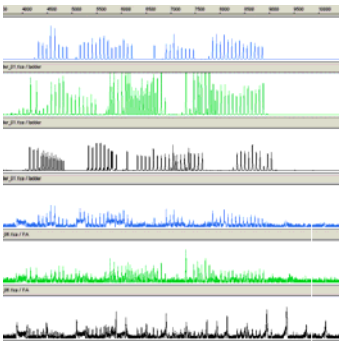
- 1. run after starting the 3100 are shifted (up to 400 data points measured at the 50bp-Std)
- Problem: "off ladder alleles"

cause: Capillary fill not possible – sources of errors: syringes, check valve, capillary sleeve are leaking but no error message by the software.



Data from Walter Parson's Lab (Innsbruck, Austria)

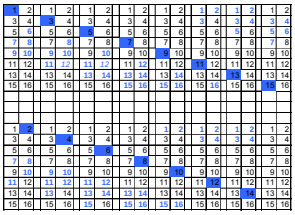
"Shining through" phenomenon



Data from Walter Parson's Lab (Innsbruck, Austria)

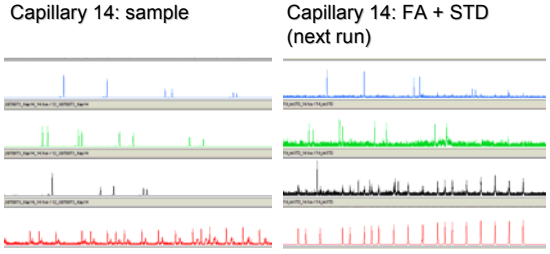
"Shining through" phenomenon

the samples shine through into other capillaries systematically:
blue box: sample (ca 8000rfu)
blue letters: numbers of capillaries that show the profile "shining through"
cause: the laser shutter opens again wrongly for a short time after closing



Data from Walter Parson's Lab (Innsbruck, Austria)

"Carry-over" – phenomenon



Data from Walter Parson's Lab (Innsbruck, Austria)

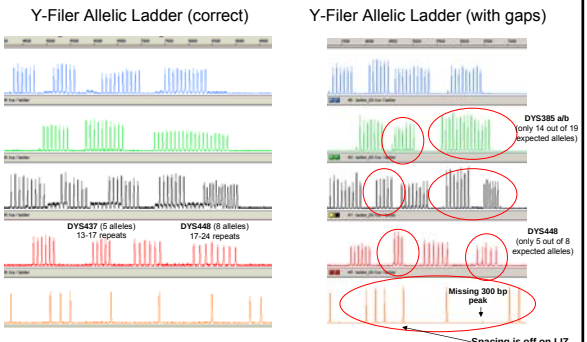
"Carry-over" – phenomenon

cause: repeated injections using the same 96-well plate septum;
other causes possibly involved (e.g. no array filling because of defective syringes)

Data from Walter Parson's Lab (Innsbruck, Austria)

"Data gap" - phenomenon

STRs

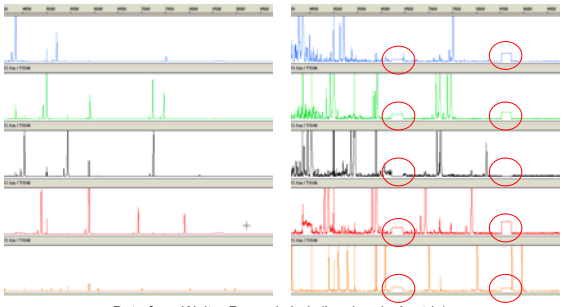


Data from Walter Parson's Lab (Innsbruck, Austria)

"Data gap" - phenomenon
STRs

Y-filer sample with "invisible" gap

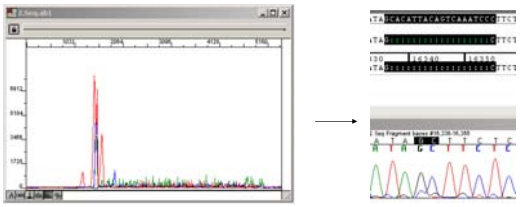
Y-filer sample with "detectable" gaps (zoomed in)



Data from Walter Parson's Lab (Innsbruck, Austria)

"Data gap" - phenomenon
sequences

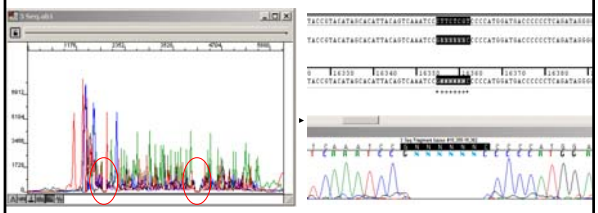
"invisible" gap



Data from Walter Parson's Lab (Innsbruck, Austria)

"Data gap" - phenomenon
sequences

"detectable" gap



Data from Walter Parson's Lab (Innsbruck, Austria)

"Data gap" - phenomenon

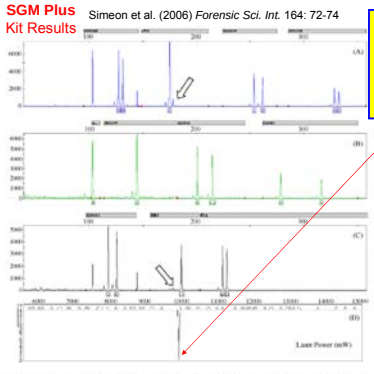
There are gaps in the data records
cause: the laser shutter is closed too long
these gaps can be detectable in the profile or are concealed by the software ("invisible" gaps)

Data from Walter Parson's Lab (Innsbruck, Austria)

Signal Loss Due to Laser Power Supply Shutdown

SGM Plus Kit Results

Simeon et al. (2006) *Forensic Sci. Int.* 164: 72-74



A double discordance discovered?

SGM Plus	PowerPlex 16
vWA 16,16	vWA 16,17
TH01 9.3,9.3	TH01 8,9.3

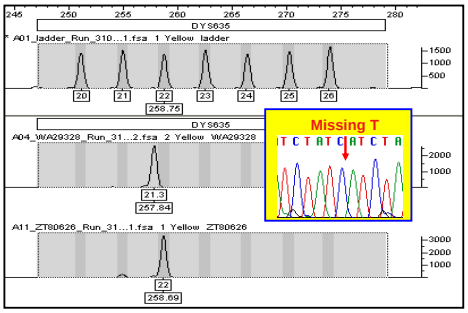
The laser power turned off for ~1 sec at the precise time that the vWA allele 17 and TH01 allele 8 migrated past the detector. **The data collection software spliced together the .fsa file without any warning messages to the user.**

ABI 3100 *Avant* with GeneMapper*ID* software

Variant Alleles

- "Off-ladder" Alleles within a locus
- "Off-ladder" Alleles between loci
- Tri-Allelic Patterns

An Example of an "Off-Ladder" Microvariant at the Yfiler Locus DYS635

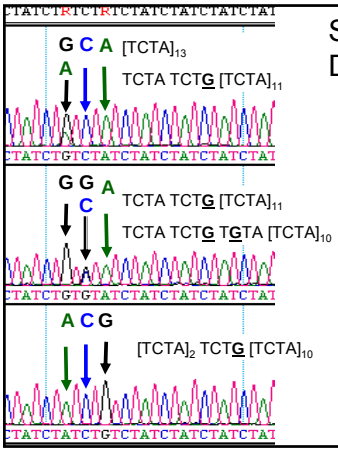


Allele 22 bin
258.75 +/- 0.5 = 258.25 to 259.25

Allele 21.3
257.84
(-0.41 from bin)

$[TCTA]_4(TGTA)_2[TCTA]_2(TGTA)_2[TCTA]_2(TGTA)_2[TCTA]_5$ TC-A $[TCTA]_2$

SNPs within the D8S1179 repeat



Repeat is TCTA
Three NIST samples have genotypes 13,13.

Analysis by Mass Spec indicates the presence of SNPs (Tom Hall, IBIS)
Confirmation of the Mass Spec by sequencing at NIST indicates:

There are **4** different 13 alleles in these 3 samples.

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

Lab Resources and Tools

- Addresses for scientists working with STRs
- Training Materials
- STR Allele Sequencing

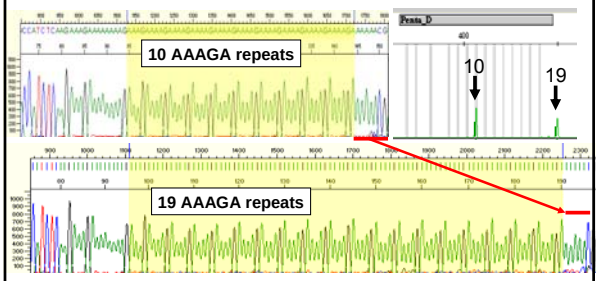
STRbase has a summary of alleles that have been submitted and sequenced, **if** the submitting agency agrees to share the information.

We require a minimum of 10 ng for the sequencing.
We request copies of the electropherograms demonstrating the variant allele.
The more information we have up front the better.
Please have patience we will get to your samples!

Sample Submissions

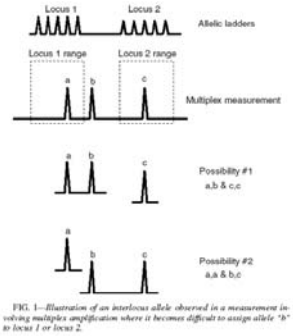
- For those that desire more assurances of confidentiality we can have MOUs signed.
- We generally re-type the samples at NIST prior to starting sequencing.
- We may run a monoplex assay (single locus).
- We return results as PowerPoint slides.
- We thank all of those agencies that have used this free service (thanks to NIJ)!
- Contact Margaret Kline: **margaret.kline@nist.gov**

Penta D 10, Variant Allele 19



All sequenced bases align before and after the repeat region.
The 19 allele has been previously reported in STRBase.
The Penta D ladder has Alleles 2.2, 3.2, 5, 7 – 17 represented.

Characterizing a Variant Allele That Occurs Between Two Loci

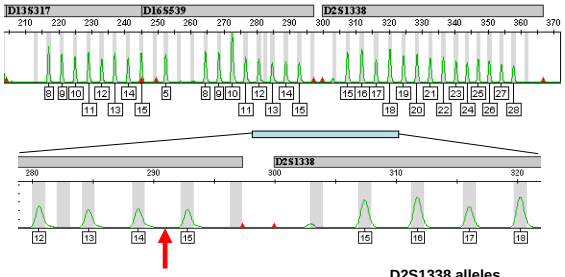


- Use a different multiplex STR kit with different locus combinations
- Test singleplex for each putative locus
- Example: Identifiler D16S539 and D2S1338

Butler, J.M. (2006) Genetics and genomics of core STR loci used in human identity testing. *J. Forensic Sci.* 51(2): 253-265

Steps to Detection
of Which Locus an Out-of-Range Allele Belongs With…

- Consider locus heterozygosities – heterozygote is likely from locus with higher heterozygosity (e.g., D16 = 0.766 while D2 = 0.882)
- Remember that tri-allelic patterns and homozygotes are less common than heterozygotes – thus two heterozygotes are more likely than a homozygote next to a tri-allelic pattern
- Check STRBase for variant alleles reported previously by other labs (e.g., D16 has no >16 alleles while D2 has several <15 alleles)
- Consider genotype frequencies observed for the various possible combinations (e.g., D16 11,11 = 10.7% while D2 20,20 = 0.92%)



D16S539
"14.2" = 291 bp

A state lab submitted to STRBase a new tri-allele:
D16S539 10, 12, 14.2 (Identifiler)

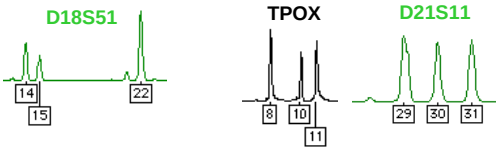
D2S1338 alleles
11 = 291 bp
12 = 295 bp
13 = 299 bp
14 = 303 bp
15 = 307 bp

Likely a D2S1338 allele 11

SWGDAM July 2007 (Doug Hares): search of NDIS for D16 tri-alleles with single D2 alleles found **25 profiles**

Three-Peak Patterns

Clayton *et al.* (2004) A genetic basis for anomalous band patterns encountered during DNA STR profiling. *J Forensic Sci.* 49(6):1207-1214



"Type 1"

Sum of heights of two of the peaks is equal to the third

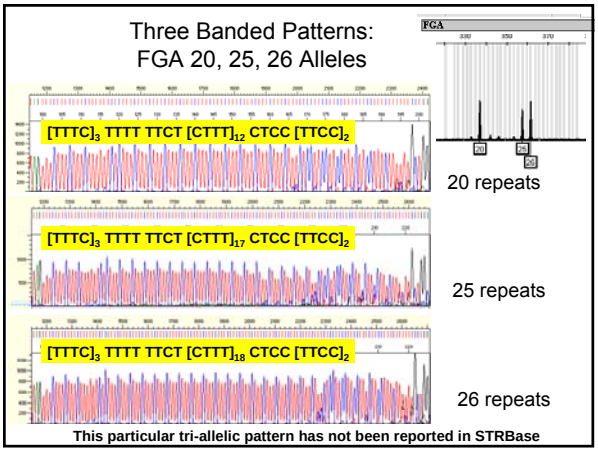
Most common in D18S51 and .....

"Type 2"

Balanced peak heights

Most common in TPOX and D21S11

Three Banded Patterns:
FGA 20, 25, 26 Alleles



This particular tri-allelic pattern has not been reported in STRBase

TPOX Tri-Allelic Patterns

FSI Genetics 2008; 2(2): 134–137

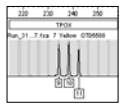
Available online at www.sciencedirect.com
ScienceDirect

The nature of tri-allelic TPOX genotypes in African populations

A.B. Lane

Approximately 2.4% of indigenous South Africans have three rather than two TPOX alleles. Data collected during routine paternity testing revealed that **the extra allele is almost always allele 10** and that it segregates independently of those at the main TPOX locus. Approximately twice as many females as males have tri-allelic genotypes which suggested that **the extra allele is on an X chromosome.**

TPOX Tri-Allelic Patterns Reported on STRBase



http://www.cstl.nist.gov/biotech/strbase/var_TPOX.htm#Tri

- 6,8,**10** (4x)
- 6,9,**10** (5x)
- 6,**10**,11 (4x)
- 6,**10**,12 (1x)
- 7,8,**10** (2x)
- 7,9,**10** (1x)
- 7,**10**,11 (2x)
- 8,9,**10** (14x)
- **8,9,11 (1x)**
- 8,**10**,11 (19x)
- 8,**10**,12 (4x)
- **8,11,12 (3x)**
- 9,**10**,11 (11x)
- 9,**10**,12 (2x)
- **10**,**10**,11 (1x)
- **10**,11,12 (4x)

TPOX 10 freq
In NIST U.S. pop
Af Am 8.9%
Cau 5.6%
Hisp 3.2%

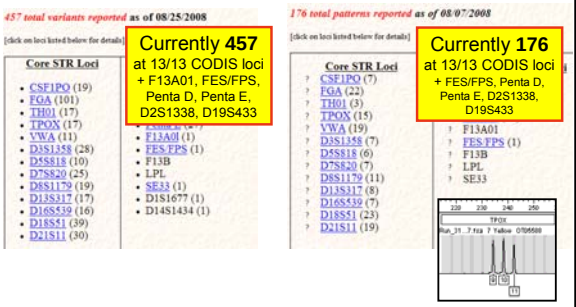
In 78 observations of 16 different TPOX tri-allelic patterns, only 4 times (5%) is allele "10" not present

Variant Alleles Cataloged in STRBase

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

Off-Ladder Alleles

Tri-Allelic Patterns



Thank you for your attention!

Contact Information

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- Fax: 301-975-8505
- Email: **john.butler@nist.gov**
- Website: http://www.cstl.nist.gov/biotech/strbase/

Bruce McCord

- FIU (Miami, FL)
- Phone: 305-348-7543
- Fax: 305-348-3772
- Email: **mccordb@fiu.edu**
- Website: http://www.fiu.edu/~mccordb/

NIST

FLORIDA INTERNATIONAL UNIVERSITY