Capillary electrophoresis: analysis and related interpretational issues

Outline

• Overview of STR analysis by CE
  • Separation
  • Injection
  • Detection

• Setting thresholds
  • Analytical figures of merit
  • Sensitivity studies
  • Interpretation of low level DNA
With 23 pairs of Chromosomes you can get Specific

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**Genotype**

The Random Match Probability for this profile in the FBI Caucasian population is 1 in 1.56 quadrillion (10^{15})
But How To Process All This Data?

Hundreds of thousands of samples?

Silver Stained Slab Gel?

Lab Floors like a Darkroom!

Fingers stained like Some strange election
Capillary Electrophoresis and Daubert: Time for Admission
Capillary Electrophoresis System

Capillary filled with entangled Polymer

Argon Ion Laser

Buffer (Sample)

Buffer

Deconvoluted Result

Fast, automated injection and analysis

Capillary

15 kV
Process Involved in CE Analysis

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

- **Separation**
  - Capillary – 50um fused silica, 47 cm (36 cm to detector)
  - POP-4 polymer – Polydimethyl acrylamide
  - Buffer – TAPS pH 8.0
  - Denaturants – urea, pyrolidinone

- **Detection**
  - fluorescent dyes with excitation and emission traits
  - CCD with defined virtual filters produced by assigning certain pixels
The development of refillable capillaries revolutionized DNA analysis and lead to the early sequencing of the human genome.
“Ok here’s my recipe idea called the electric pickle. Attach the hot lead to a screw and shove it in. The neutral lead goes in the other end. Turn out the lights and plug it in. It glows and sizzles. The juicy ones work best”

www.voltnet.com/cook

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

Pickle cooks

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

Ions move through pickle faster at high voltage

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

Small ions with high charge move fastest
DNA and Electrophoresis

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

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

small ions with high charge move fastest

As size increases so does charge!
Separation Mechanism

Electrophoretic flow

Electroosmotic flow

Electroosmotic flow is a bulk flow that is created by the build up of charge on a capillary wall. POP polymer minimizes this effect.

\[ \text{SiOH} \rightarrow \text{SiO}^- + \text{H}^+ \]
So what are sieving buffers?

They are gels - very similar to polyacrylamide.

They are not gels - they flow.

Actually these are known as entangled linear polymers and there are many common applications.
Transient Pores Are Formed Above the Entanglement Threshold.

\[ C < C^* \quad C = C^* \quad C > C^* \]
Ogston Sieving: $\mu \sim \mu_0 e^{-NC}$

Reptation: $\mu \sim 1/N$

Entanglement: $\mu \sim f(1/CN)$
Synthesis of DNA Sieving Buffers
Effect of Concentration and Molecular Weight on Resolution

4% PDMA (100K), Taps buffer

7.3% PDMA (1M), Taps buffer

3.5% PVP(1M)/HEC(250K), Taps buffer

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

Follow the dancing DNA
Resolution vs Field Strength

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

1% HEC 100 bp Ladder
Separation Issues

- **Electrophoresis buffer** –
  - Urea for denaturing and viscosity
  - Buffer for consistent pH
  - Pyrolidinone for denaturing DNA
  - EDTA for DNA stability and chelating metals

- **Polymer solution** –
  - Entangled to separate DNA
  - High molecular weight for good resolution
  - Minimum concentration/viscosity for easy refilling (POP4, POP6, etc.)
  - Coats capillary wall to minimize adsorption

- **Run temperature** –
  - 60 °C helps reduce secondary structure improves precision.
  - (Temperature control affects DNA sizing)

- **Electric field** –
  - affects orientation and diffusion of DNA
Injection

Injection is actually improved in CE through dilution of the sample.
Injection Methods for CE


Stacking Effects

(a) Stacking with Low Ionic Strength

(b) Regular EK Injection
Ion Mobility Effects

The Injection of DNA by voltage is described by

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

However this equation assumes no interfering ions are present. 

$\text{Cl}^-$ ions and other interferents will compete with DNA

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

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

![Graph showing DNA concentration with values: 208 uS, 338 uS, 408 uS, 1180 uS]
Golden Gate Effect
Attributed to poor formamide
Effect of bad Formamide

-Note broad peaks and extra bands

Problem disappears if sample is dissolved in pure water instead of formamide and denatured. This indicates degraded formamide
Shadow peaks

Shadow peaks result from bad formamide, incomplete denaturation or from rehybridization.

dsDNA migrates faster than ssDNA and the extra peaks appear ahead of the main peaks.

They are most visible in the size standard but can appear in other dye lanes.

In sample - shadow peaks appearing to left of allelic peaks
Measuring Formamide Conductivity

(not this way)

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

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

Another way to check for bad formamide is to run a heat denatured sample in distilled water. If it looks different the formamide is contaminated.
Detection

Laser induced fluorescence provides exquisite sensitivity and specificity
Matrix calculations

\[ I_{540} = bx_b + gy_b + yz_b + rw_b \]
\[ I_{560} = bx_g + gy_g + yz_g + rw_g \]
\[ I_{580} = bx_y + gy_y + yz_y + rw_y \]
\[ I_{610} = bx_r + gy_r + yz_r + yw_r \]

Intensity of blue
Intensity of green
Intensity of yellow
Intensity of red

Where

- \( b \) is the \% blue labeled DNA
- \( g \) is the \% green labeled DNA, etc.
- \( x, y, z, w \) are the numbers in the matrix (sensitivity to each color)

If you solve \( xyzw \) for each dye individually
Then you can determine dye contribution for any mixture
Uncorrected Raw Data
(AFLP of a Marijuana Sample)
AFLP Analysis of Plant DNA

Corrected Result

AFLP of Marijuana
Issues with the Optical System

- Argon Ion lasers outgas and eventually lose intensity; **take note of laser power and monitor it over time**

- Fluorescence expression:
  \[ I_f = I_0 k \varepsilon d C \phi \]
  - changes in input intensity: \( I_0 \)
  - changes in capillary diameter: \( d \)
  - cleanliness of capillary, optics: \( k \)

  All these things directly affect peak RFUs, however, baseline noise is more affected by detector.

- A good QC step is to monitor the ladder intensity
Part II  Setting Thresholds

Now that we know how it works,

Where to go next?

How to find the bottom?
Setting thresholds for the ABI 310/3100

(Determining a true allele)

- Every laboratory needs to set an analytical threshold, a stochastic threshold, a limit of linearity and minimum peak height threshold.

- Can these values be set globally for the entire lab or are they instrument dependent?

- How do these values affect detection, stutter, pull-up, mixture interpretation, low copy DNA?
Fundamental parameters for allele detection by CE

Detection Limit (analytical threshold): 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

Stochastic Threshold: Level of DNA below which a significant chance of allele dropout can occur. Set high enough that a heterozygous peak will produce its companion allele in the grey zone between stochastic and analytical threshold. (150-200 RFUs)

Limit of linearity: The level of DNA above which enhanced pull-up, flat top peaks and elevated stutter occurs. Determined by examining the relationship between input DNA and fluorescence signal varies (~4500 RFUs for ABI 310, ~3500 for a 3100, >20,000 for a 3500)

Heterozygous peak ratio: The minimum peak height ratio expected for a clean, single source DNA sample at a particular concentration (typically 60-70%)
Useful Range of an Analytical Method

- **Instrument Response**
  - LOD: limit of detection
  - STO: peak balance threshold
  - LOL: pull-up/mixture threshold (loss of linearity)

- **Concentration of Sample**
  - LOD = 3x SD of blank
  - STO = peak balance threshold
  - LOL = pull-up/mixture threshold (loss of linearity)
Visual representations

- **Analytical threshold** – peaks below 3x the baseline can’t be distinguished from noise with scientific certainty.

- **Stochastic threshold** – peak height ratios below 60% may yield false homozygotes. Grey zone permits detection of 2nd homozygous peak.

- **Limit of Linearity** – relationship between fluorescence and quantity breaks down. Pull-up appears and peaks become flat topped.
The Scientific Reasoning behind the Concept of an Analytical Threshold/limit of detection

• 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

• You want to be sure to avoid labeling noise!

• LOD = 2x $N_{pp}$ or 3x $SD_n$

• Levels are typically set high to avoid constantly resetting thresholds

Abracadabra! It’s an allele
How thresholds are currently set

- **Analytical threshold** – Set to 3 times the baseline noise plus an additional amount to be conservative.

- **Stochastic threshold** – Set to avoid the presence of peak height ratios below 60%.

- **Grey zone** between two threshold permits detection of 2\(^{nd}\) homozygous peak,
Observation: Peak height variation increases with concentration
Therefore: it's difficult to assess the quantity of DNA solely by peak height
The Scientific Reasoning behind the LOQ/Stochastic threshold

• With peak intensity below the LOQ, you have significant variation in height from one sample to the next.

• Similarly due to stochastic fluctuation in peak height ratios, interpreting data below the stochastic threshold presents the real problem of allele dropout due to variation.

• You rely on peak heights to detect major and minor profiles and **you need to be certain when calculating statistics that you do not have a dropped heterozygous allele.**

How low can you go?
Stochastic Statistical Sampling

Copies of allele 1

Copies of allele 2

Resulting electropherogram

True amount

What might be sampled by the PCR reaction...

>20 copies per allele

6 copies copies per allele (LCN)

Allele imbalance

Allele dropout

John Butler
Heterozygote Peak Height Ratios

Identifiler STR Kit Developmental Validation

Template inputs
250 pg - 3 ng

60 %

Outliers

Heterozygote peak height ratios vary with DNA template. Above 250 pg, the effect is very rare. At 31 pg severe dropout occurs. Even with this issue, full profiles can be seen even at the lowest levels. The problem is one of decreasing reliability.
The grey zone is predicated on a minimum peak height ratio (PHR)

Above the stochastic limit a peak at 60% RFU will show is partner allele in the grey zone.

A single peak in the grey zone is considered unreliable as it may be heterozygous and its partner allele dropped in the noise.

Statistically how will you calculate the result?

One allele peak above the detection threshold and one below

Stochastic limit

160RFUs

150 RFUs

Detection limit

96 RFUs

50 RFUs

Inconclusive result due to present of potential allele in between two thresholds.

Stochastic limit

80 RFUs

150 RFUs

Detection limit

50 RFUs

Unusable result: (60% of 80 RFU is 48 and below threshold)
Alternative Procedure
(Mass State Police)

1. Since most estimates for LCN show up from 100-250pg DNA, select a low level—say 150pg 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.
The issue with low level data interpretation

Thompson, W.C. et al. The Champion, April 2003, pp 16-25
Sensitivity issues

- Improved STR multiplexes have better buffers, more mini STRs and increased sensitivity

- What is wrong with that?

- The fundamental problem with the PCR is stochastic amplification. It exists regardless of sensitivity of detection, and manifests itself as peak imbalance, enhanced stutter and peak dropout.

- These new kits and instruments detect better, but the PCR hasn’t changed!!! New thresholds need to be set.

Powerplex Fusion-24 loci multiplex
Previously suggested thresholds ABI310 vs 3100

Threshold (ABI)
310   50 RFUs
31xx   30 RFUs

Stochastic
310   150 RFUs
31xx   90 RFU

Dynamic Range
310            4500
311 31xx   3500

Bottom line: 310 will appear more sensitive with a wider dynamic range. 3100 detects more samples with its array but has a more limited dynamic range.

However, new kits and new instruments may change these values!
The ABI 3500
a new paradigm?

This new instrument has 6 dye lanes and an improved laser, but the dynamic range of the system is much different-

RFU signals can range from 0 to over 20,000 RFU.
This compares to approximately 0-4500 RFU for a standard 310
What does this mean in practical terms?

1. The old threshold values for the 310 and 3100 can not be used, new ones must be calculated

2. 100pg of DNA produces heterozygous peaks of 750 or more indicating a stochastic threshold of 750 RFU – a very high value for labs used to 310 or 3100.

3. With the potential for pullup of up to 2 %, or more, laboratories may expect artifact peaks up to 400 RFU.

4. Signal to noise is quite low and caution must be taken when setting the analytical threshold particularly given the issue of artifacts– How to deal with this???
# Peak Balance ABI-3500 ID+

Major problems with peak balance below 125pg. This indicates strong potential for false homozygous peaks.

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<td>64.73%</td>
<td>77.21%</td>
<td>97.94%</td>
<td>87.29%</td>
<td>54.87%</td>
<td>69.53%</td>
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<tr>
<td></td>
<td>3</td>
<td>51.22%</td>
<td>84.62%</td>
<td>89.61%</td>
<td>66.98%</td>
<td>86.42%</td>
<td>70.40%</td>
<td>62.41%</td>
<td>80.90%</td>
<td>54.90%</td>
<td>69.53%</td>
<td>100.00%</td>
</tr>
</tbody>
</table>
Setting an analytical threshold based on method, not instrument

Many times there is more to the analytical method than just doing a reaction or submitting it to direct analysis. For example it might be necessary to heat a sample that is to be analyzed for a particular metal with the addition of acid first (this is called digestion). The sample may also be diluted or concentrated prior to analysis on an instrument. Additional steps in an analysis add additional opportunities for error.

Since detection limits are defined in terms of error, this will naturally increase the measured detection limit. This detection limit (with all steps of the analysis included) is called the MDL. The practical method for determining the MDL is to analyze 7 samples of concentration near the expected limit of detection. The standard deviation is then determined. The one-sided t-distribution is determined and multiplied versus the determined standard deviation. For seven samples (with six degrees of freedom) the t value for a 99% confidence interval is 3.14 (Wikipedia)

Using a Method Limit of Detection may be a more realistic way to set an Analytical Threshold than an LOD as it takes more into account.
10 replicate amplifications of a low level sample indicating SD of 113 RFU.

<table>
<thead>
<tr>
<th>Dye</th>
<th>FAM</th>
<th>Locus</th>
<th>Sample</th>
<th>Allele 1</th>
<th>Height (RFU)</th>
<th>Allele 2</th>
<th>Height (RFU)</th>
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<td></td>
<td>FAM</td>
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<td>JP20.1</td>
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<td>464</td>
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<td>321</td>
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<tr>
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<td>JP20.10</td>
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<td>202</td>
<td>15</td>
<td>353</td>
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<tr>
<td></td>
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<td>Average Peak Height:</td>
<td></td>
<td></td>
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<td></td>
<td>323.2</td>
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<tr>
<td></td>
<td>stdev</td>
<td></td>
<td></td>
<td></td>
<td>115.1271761</td>
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<td>0</td>
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Indicates a method limit of detection for an ABI 3500 of 3 x 113 ≈ 350 RFUs
3500 pull-up study
(for determining limit of linearity)

Figure 1

Pull-up (Red) vs peak height (Green)

Pull-up becomes excessive above 20,000 RFU

indicates limit of linearity for a 3500 around 20,000-25,000 RFU
Thresholds for the 3500

For 10 samples independently amplified and run on a 3500, the pooled standard deviation is approximately 113. Multiplied by 3 this gives an analytical threshold of approximately 350.

At 100 pg the average peak intensity for a 3500 was measured at approximately 750 RFU this provides an estimate of the stochastic threshold.

Thus for the 3500 one could set a stochastic threshold at 750 RFU and an analytical threshold based on the MDL of 350.

The Limit of linearity is around 20,000 RFU and was set by examining the effect of pullup vs input DNA quantity.
What else can go wrong?

• 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.

Yarr, Take care mates!
Degradation vs Inhibition

Degraded DNA Sample
Ski slope effect

Powerplex 16 9947A Positive Control 0.250 ng/ 12.5 ul

Bone Sample 2003.5.6 0.250 ng/ 12.5 ul

Humic Acid Inhibited DNA Sample
Less predictable effects

Degradation vs Inhibition
The bottom line:

1. Low signal levels are bad because:
   a. They may indicate low copy # DNA = inconsistent or confounding 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
Fuzzy Logic in Data Interpretation

- Capillary Electrophoresis is a dynamic process

- 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.

- In addition, the interpretation and its significance cannot be dissociated from the overall facts of the case.
So why examine low level data at all?

• Touch DNA can be a powerful lead in a criminal investigation

• 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

• So How to Make it Reliable?
Extract of touch DNA

If the major profile is the victim, can we find the suspects alleles?

Analyst must consider allele dropout, drop-in, stutter and masking of peaks. A very complex problem.
The problem occurs when you have to interpret a low level contributor or a single source profile along with these stochastic artifacts.
Reproducibility over the long term

Control

LCN amp 1

LCN amp 2

LCN amp 3


How to score this?
D3 16, Z ; vWA 14, Z ; FGA 19, 25 where Z indicates a potential partner allele

1. Allele dropouts everywhere but are not reproducible,
2. Allele dropins are mainly in the stutter position.
3. You need to run replicates to interpret this kind of data.

This kind of data is why we have thresholds and why these thresholds should be conservative.
Meatloaf Principle

- I want you
  I need you
  But -- there aint no way ‘Im ever gonna love you
  Now don’t be sad
  cause two out of three aint bad
  – Meatloaf

Catch 22 Principle

- The Catch 22 is that to do all these runs you end up wasting precious sample, further reducing the quality of results.

- It might be better to use more and amplify less – but is it scientific?
Interesting effects with low copy data

Table 1 Details of analysis

<table>
<thead>
<tr>
<th>Description</th>
<th>Count</th>
<th>Percentage</th>
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</thead>
<tbody>
<tr>
<td>Number of single cells analyzed</td>
<td>226</td>
<td></td>
</tr>
<tr>
<td>Results obtained</td>
<td>206</td>
<td>(91%)</td>
</tr>
<tr>
<td>Amplification failure</td>
<td>20</td>
<td>(9%)</td>
</tr>
<tr>
<td>Full STR profile</td>
<td>114</td>
<td>(50%)</td>
</tr>
<tr>
<td>Acceptable profile (amelogenin, &gt;4 STRs)</td>
<td>144</td>
<td>(64%)</td>
</tr>
<tr>
<td>Partial profile (1-4 STRs)</td>
<td>62</td>
<td>(27%)</td>
</tr>
<tr>
<td>Surplus alleles*</td>
<td>28</td>
<td>(12%)</td>
</tr>
<tr>
<td>False alleles**</td>
<td>11</td>
<td>(5%)</td>
</tr>
<tr>
<td>Allele dropout</td>
<td>88</td>
<td>(39%)</td>
</tr>
</tbody>
</table>

*Additional allele present in conjunction with true alleles.
**Additional allele in place of true allele.

So with one cell 64% of the time you get a usable profile and 27% a partial profile =91 % OK!

How would you know had a problem?

The danger is clearly false exclusion and the potential presence of low level exogenous DNA.

Findley et al. (1997) Nature article
Post PCR manipulation

• Why not just cleanup after PCR?
  – 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 massive issues with stochastic effects.
Various methods to increase the sensitivity of a low level sample. Post PCR cleanup $\approx$ Extra Cycles  (different peaks, same sample)

- **Standard method**
  - 1uL input DNA

- **Post PCR cleanup**
  - 2uL input DNA

- **Post PCR cleanup**
  - 2uL Input DNA 30s inj.

- **Increasing the number of Amplification cycles from 28 to 32**

Does stochastic amplification occur with Y STRs? -YES!

Single source thresholds might involve a minimum number of alleles to insure a single individual is present.

Mixtures, a minimum peak height to eliminate the potential for switching between major and minor contributors.

Imbalance between loci
The problem with LCN DNA is that you can’t be sure if it is from the case or if it is from some other event.

Higher sensitivity techniques are most likely to pick up previously deposited (background) DNA

Opportunity for Adventitious Transfer

Opportunity for DNA Transfer from Perpetrator

Crime Event

Potential to “Contaminate”

Laboratory analysis

Investigators arrive, detect, and recover evidentiary material

Discovery

Analysis completed

Adapted from Gill, P. (2002) BioTechniques 32(2): 366-385, Figure 5
Guidelines for use with low template/low copy DNA

- Low copy DNA is not just more cycles, stochastic effects can occur anytime levels of input DNA are low.
  (effects begin below 250pg DNA, commonly set at 100pg)

- An allele should not be scored (considered real) unless it is present at least twice in replicate samples, usually 3 replicates are performed.

- Extremely sterile environments are required for PCR setup to avoid contamination from laboratory personnel or other sources. Personnel must be typed for contamination events.

- The potential for contamination from DNA not related to the events in the case must always be considered.

- Guidelines for minimum number of heterozygous alleles should be considered.
A Precautionary Tale

When we analyze lower amounts of DNA...
It is critical to understand the importance of setting thresholds and knowing input levels.

WHEN ARE SUCH SAMPLES SUBJECT TO LCN ISSUES?
### Murder of a Witness by Rifle (recovered)

<table>
<thead>
<tr>
<th>ub #</th>
<th>ITEM</th>
<th>FGA</th>
<th>TPOX</th>
<th>D8S1179</th>
<th>vWA</th>
<th>Amelo</th>
<th>Penta E</th>
<th>D18S51</th>
<th>D21S11</th>
<th>TH01</th>
<th>D3S1358</th>
<th>Penta D</th>
<th>CSF1PO</th>
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<tbody>
<tr>
<td>2</td>
<td>Suspect</td>
<td>19</td>
<td>30.2</td>
<td>8</td>
<td>9</td>
<td>14</td>
<td>15</td>
<td>17</td>
<td>X</td>
<td>Y</td>
<td>9.12</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>Accomplice</td>
<td>23</td>
<td>22</td>
<td>14</td>
<td>17</td>
<td>X</td>
<td>7</td>
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<td>14</td>
<td>18</td>
<td>15</td>
<td>29</td>
<td>33.2</td>
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**State Lab Private Lab**

**Private Lab**

**State Lab**

**Private Lab**

**T MINIFILER DATA:**

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<tr>
<th>ITEM</th>
<th>STATE LAB</th>
<th>PRIVATE LAB</th>
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<tbody>
<tr>
<td>19(25)</td>
<td>8</td>
<td>14</td>
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<tr>
<td>(26)</td>
<td>12</td>
<td>(14)</td>
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<td>21</td>
<td>PA = 25</td>
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</tbody>
</table>

**T MINIFILER DATA:**

<table>
<thead>
<tr>
<th>ITEM</th>
<th>STATE LAB</th>
<th>PRIVATE LAB</th>
</tr>
</thead>
<tbody>
<tr>
<td>19(23.2)</td>
<td>8</td>
<td>13</td>
</tr>
</tbody>
</table>

**Recovered profile from laboratory matches suspect and accomplice**

**Second lab analyzes remainder of swab and gets a completely different result - minifiler**

**Would you exclude a suspect based on this data?**
Look closer:
Quantitation indicated no DNA for the second sample.....
Even worse, the lab reported quantitative data below the lowest calibrator! (23pg/uL) A very bad practice....

Defendant was declared guilty. It helped that there was a video of him buying the rifle....
If you are going to look at low level samples at least measure them by multicopy qPCR!

Current single copy method

![Graph showing current single copy method with low detection limits and high fluctuation.]

- Cannot detect low level DNA
- Too much fluctuation

MultiCopy Alu based Method

![Graph showing MultiCopy Alu based method with higher detection limits and lower fluctuation.]

- Genome equivalent of a single cell

Why isn't everyone using multicopy techniques?

1. Conclusions

1. CE based DNA analyses are complex

   a. Separations are affected by polymer length, concentration and field strength
   b. Injections vary greatly with salt content and PCR product quality
   c. Detectors require careful monitoring for pull-up and intensity

2. There are a multiplicity of instrument thresholds
   a. Analytical thresholds are based on standard deviation of noise.
   b. Stochastic thresholds are based on fundamental aspects of the PCR reaction which result in peak imbalances
   c. Limits of linearity affect ultimate peak height, stutter and pullup.
   d. Remember however, thresholds are guidelines- not rules be smart and keep things within the context of the case.
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