Setting Instrument Parameters and Thresholds

Bruce McCord FIU
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?
Amounts of DNA Required

1985-1995

RFLP/VNTRs

50 ng – 1000 ng

1991-present
(kits since 1996)

PCR/STRs

0.5 – 2 ng

1999-present

LCN/STRs

<0.1 ng

LCN extends the range of samples that may be attempted with DNA testing
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?

• How to deal with issue in a way to ensure a conservative result?
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*
**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.
Useful Range of an Analytical Method

- **LOL**: limit of linearity
- **LOD**: limit of detection
- **LOQ**: limit of quantitation
- **STO**: peak balance threshold

**Instrument Response**

- ~50 RFUs
- ~200 RFUs
- ~5-7000 RFUs

**Concentration of Sample**

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

• This is known as pulling data out of thin air.

Abracadabra! It’s an allele
Limit of Detection

• 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 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.

• 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

Copies of allele 1

Copies of allele 2

Resulting electropherogram

>20 copies per allele

True amount

What might be sampled by the PCR reaction...

6 copies per allele (LCN)

Allele imbalance

OR

Allele dropout
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 reamping the sample at higher input DNA.
Otherwise, straddle data makes locus inconclusive.

Straddle data may be caused by degradation, inhibition and low copy issues.
Stochastic Sampling can cause interpretational problems

This case resulted in a hung jury. Defense expert claimed low PHR indicated exclusion. Prosecution claimed inconclusive result.

Police Rule: loci must be above threshold and within 66% of each other to be included as major contributor. Defense confused this. Inconclusive ≠ Exclusion!

(12,13)  D13(12,14)  D7(9,12)
Other reasons to avoid interpreting low level data and setting thresholds

Spikes

Non Specific Amplification, -A,

Pull-up
Another problem is stutter

- Stutter increases at low copy #


![Graph showing peak height (D5S818) vs. % Stutter]
Extract of Pistol Grip and Trigger
Relatively low amount of amplified DNA?

Enhanced stutter or mixture?
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

Humic Acid Inhibited DNA Sample
Less predictable effects

Powerplex 16 9947A Positive Control
0.250 ng/12.5 ul

Bone Sample 2003.5.6
0.250 ng/12.5 ul

RFU

Degradation vs Inhibition

Degradation vs Inhibition

Degradation vs Inhibition

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Degradation vs Inhibition
The issue with low level data interpretation

Thompson, W.C. et al. The Champion, April 2003, pp 16-25
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 180RFUs.
Next determine the dynamic range

1. Perform a series of amplifications of 5 different samples with 5.00, 2.00, 1.00, 0.50, 0.25, 0.13, 0.06, 0.03 ng DNA

2. Use your laboratory quantification system, your thermal cycler, and your 310.

3. Determine the average and standard deviation of each set of samples

4. Your dynamic range is the range of concentrations that are not overloaded. Overload point is where peaks flat top. (this can be checked by ex
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 CCD camera saturates leading to flat-topped peaks.

![Diagram showing off-scale peaks](image)

Off-scale peaks

Stutter and noise may be artificially enhanced.
Determination of Minimum Sample

• Goal: avoid situations where peak imbalance results in only one detectable allele from a heterozygous pair.

• Perform serial dilution (1ng- 8pg) of 2 control samples which were heterozygous at all 3 loci
  – Samples above 125pg had peak height RFUs above 150
  – Below 125pg peak heights were not significantly above background
  – At 31 pg peaks were very low or undetectable

• “Peaks below 150 RFU should be interpreted with caution”
Sensitivity of Detection

Moretti et al, JFS, 2001, 46(3), 661-676

• Different 310 instruments have different sensitivities; determination of analytical thresholds should be performed following in-house studies
  – Variations in quantitation systems
  – Variations in amplification systems
  – Variations in instrument sensitivity

• Peaks with heights below the threshold should be interpreted with caution
  – Caution should be used before modification of
    • Amplification cycles
    • Electrophoretic conditions

• Peak heights are also a function of sample condition/PCR, extraction, inhibition, degradation
Observation: Peak height variation increases with concentration
Therefore: its difficult to assess the quantity of DNA solely by peak height
Next set the stochastic threshold

- Stochastic Threshold – the signal intensity at which a particular quantity of DNA can no longer reliably be detected
  - Reliability can be defined by an increase in the standard deviation of peak height intensity or an increase in the standard deviation of signal intensity or both.
  - The stochastic threshold should be greater than the limit of quantitation
Stochastic 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
Heterozygote Peak Height Ratios

Observation: Peak height ratio variation inversely proportional to input DNA.

<table>
<thead>
<tr>
<th>PHR</th>
<th>50–150</th>
<th>150–300</th>
<th>300–1000</th>
<th>&gt;1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed Minimum</td>
<td>48%</td>
<td>57%</td>
<td>58%</td>
<td>78%</td>
</tr>
<tr>
<td>Theoretical Minimum (Mean—3 SdDev)</td>
<td>40%</td>
<td>54%</td>
<td>63%</td>
<td>75%</td>
</tr>
<tr>
<td>Mean</td>
<td>80%</td>
<td>85%</td>
<td>88%</td>
<td>92%</td>
</tr>
<tr>
<td>N</td>
<td>145</td>
<td>140</td>
<td>233</td>
<td>209</td>
</tr>
</tbody>
</table>

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).

Mixture Example
The proper setting of thresholds underlies rules for mixture interpretation.

List the genotypes?

\[
\frac{1909}{2229} = 85\%
\]

\[
\frac{2173}{3137} = 70\%
\]

\[
\frac{410}{1420} = 30\%
\]

\[
\frac{284}{410} = 70\%
\]

Peak height ratios and thresholds control interpretation
Excessive variation in PHR makes this data uninterpretable
Alternative Procedure

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
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.

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4. In this way you define straddle data as at the point 50% of your alleles will be above this mark.
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 and/or adjustment of qPCR results.
**310 vs 3100**

**Sample**
- 310 1.5uL in 24 uL formamide
- 31xx 1uL in 10uL formamide

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

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

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

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 unless proper validations are performed.
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 LCN data.
MiniSTRs and LCN.

MiniSTRs were developed to access degraded DNA.

They do not solve the inherent low copy limitation of the PCR.

Instead because of their sensitivity, they complicate it.
1. Short PCR primers amplify better.
2. Better amplification means laboratories can access extremely low levels of DNA
3. At such levels (1-20 cells) a scientist cannot express a strong opinion about how DNA arrived at the site where it was recovered.
4. This DNA could just as easily come from pre or post deposition as it could come from the suspect.
MiniStrs show the same effect
In spite of the improved sensitivity, peak balance is poor at low template concentration.
This sample needs MiniSTRs

These do not:

Numerous problems with allele dropout

Lab interpretation

threshold = 125 RFU

Below threshold allele

Hair Sample

victim

suspect

No Size Data
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
• The ABI 310 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.
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
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 2\textsuperscript{nd} 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

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

Input DNA

SOP

1ng

Donor C ... L rxn) - 52 92 Yellow Donor C 1ng (50 µL rxn)

PHR = 87%

50 µL PCR

Allele Drop Out

LCN

8pg

Donor C ... L rxn) - 74 74 Yellow Donor C 0.0078ng (5 µL rxn)

PHR = 50%

5 µL PCR

Allele Drop In

Heterozygote Allele Imbalance
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?”
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
Typical LCN Procedure

Extract DNA from stain

Perform 3 Separate PCR Amplifications

Quantify Amount of DNA Present

Interpret Alleles Present

Develop a Consensus Profile
(based on replicate consistent results)
## Replicate LCN Test Results from FSS


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

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<thead>
<tr>
<th></th>
<th>Amel</th>
<th>D19</th>
<th>D3</th>
<th>D8</th>
<th>THO</th>
<th>VWA</th>
<th>D21</th>
<th>FGA</th>
<th>D16</th>
<th>D18</th>
<th>D2</th>
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<tr>
<td><strong>CONTROL</strong></td>
<td>X</td>
<td>X</td>
<td>14,14</td>
<td>18,18</td>
<td>15,15</td>
<td>7.9.3</td>
<td>19,19</td>
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<tr>
<td>2</td>
<td>X F’</td>
<td>15 F’</td>
<td>18 F’</td>
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<td>28</td>
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<td>3</td>
<td>X F’</td>
<td>15 F’</td>
<td>18 F’</td>
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<td>4</td>
<td>X F’</td>
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<td><strong>Consensus</strong></td>
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<td></td>
<td>28</td>
<td>32.2</td>
<td>20 F’</td>
<td></td>
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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”)

Replicate analysis of 100pg samples
28 cycles standard conditions

Note allele dropout and drop-in at the stutter position
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?
Interesting effects with low copy data

Table 1 Details of analysis

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

*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.

Straddle data may be low copy!
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

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.

Adapted from Gill, P. (2002) *BioTechniques* 32(2): 366-385, Figure 5
Low Copy Number Limitations

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

Issues:

Was evidence collected with LCN in mind?

Is LCN evidence reliable?

Does the obtained profile result from the evidence?
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 massive issues with stochastic effects.
Various methods to push sensitivity
(where did the alleles go?)

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

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

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

- Increased amplification from 28 to 32 cycles
Overall results indicate some pretty weird stuff.

Table 5
Shows the total number of possible donor peaks, the number of donor peaks duplicated between replicate PCRs, the number of non-donor peaks which were not duplicated between replicates PCRs and the number of missing donor peaks.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total possible number of donor peaks</th>
<th>Number of donor peaks duplicated between replicate PCRs</th>
<th>Number of donor peaks not duplicated between replicate PCRs</th>
<th>Number of donor peaks missing from either PCR</th>
<th>Percentage of observed donor peaks which are consistent</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>796</td>
<td>12</td>
<td>64</td>
<td>708</td>
<td>27</td>
</tr>
<tr>
<td>B</td>
<td>796</td>
<td>140</td>
<td>168</td>
<td>348</td>
<td>63</td>
</tr>
<tr>
<td>C</td>
<td>796</td>
<td>188</td>
<td>140</td>
<td>280</td>
<td>73</td>
</tr>
<tr>
<td>D</td>
<td>796</td>
<td>178</td>
<td>166</td>
<td>274</td>
<td>68</td>
</tr>
</tbody>
</table>

NB for donor peaks duplicated between PCR replicates a duplicated peak is counted once.

Table 9
Shows the summary data from the conditions examined.

<table>
<thead>
<tr>
<th>No significant difference between conditions C and D</th>
<th>Criteria where condition C is superior to D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples with ≥1 allelic peak</td>
<td>Mean stutter peak ratio</td>
</tr>
<tr>
<td>Mean number of peaks</td>
<td>0</td>
</tr>
<tr>
<td>Average peak area ratio</td>
<td>0.71a</td>
</tr>
<tr>
<td>% of heterozygous loci showing peak area ratios of &lt;0.5</td>
<td>18a</td>
</tr>
<tr>
<td>Allelic peaks in controls</td>
<td>2</td>
</tr>
</tbody>
</table>

a There is no significant difference between the asterisked conditions.

Direct comparison of post-28-cycle PCR purification and modified capillary electrophoresis methods with the 34-cycle “low copy number” (LCN) method for analysis of trace forensic DNA samples.

Luke Forster, Jim Thomson, Stefan Kutanov
LGC Forensics, Queens Road, Teddington, Middlesex TW11 9LY, UK
Received 1 February 2008; received in revised form 28 March 2008; accepted 9 April 2008
qPCR for DNA Quantitation

– will it lead more labs into LCN?

or

Are we already there and about to find out?

When properly used real time PCR can provide clearer information on absolute quantities of DNA
Why?

- Most laboratories will use amplification results to provide information on DNA thresholds.
- But: The reason qPCR was developed is that using endpoint detection to determine quantity is notoriously imprecise.
- Depending on amplification conditions, it is possible to produce full profiles from a single cell - 8pg.
- So wouldn’t it be better to use qPCR to do so?
qPCR Assays Are Also Impacted by Stochastic Sampling in the LCN Region

Note the larger spread in these dilution series points for the LCN samples (16 pg) because of stochastic sampling.

Remember that DNA quantitation assays are also impacted by stochastic problems and may not be extremely reproducible on the low end, i.e., <100 pg... This is especially true for Y as there is ½ as much DNA for taqman.

Figure 6-4 Sensitivity using the Quantifiler Y kit
Proceeding with Testing when “No DNA” Detected

If the qPCR results indicate that there is no detectable DNA, will you stop testing or will you proceed with attempting STR typing?

• The practice of proceeding even with a “no result” Quantiblot was because the STR typing assay was more sensitive than the quantification method.

• Real time PCR should have solved this problem.
Challenge with Being Able to Go Lower In DNA Quantitation Measurements

• Multi-copy marker (e.g., Alu assay) will be better than a single copy (e.g., Quantifiler) with qPCR of low quantity DNA samples

• qPCR enables measurement of lower amounts of DNA but...

• Going into the low copy number realm introduces new challenges
  – Interpretation of mixtures
  – Defining thresholds for different dyes and amplification systems
  – Defining the difference between investigative data and reliable “court-worthy” data
Difference in DNA Quantitation Capability vs. STR Typing Sensitivity

Quantiblot Limit of Detection (LOD) → 1 ng

STR typing (28 cycles) LOD → 100 pg

LCN STR typing (34 cycles) LOD

- Real-time qPCR LOD → 40 pg
- Multicopy qPCR LOD → 23 pg
- Multicopy qPCR LOD → 6 pg  
  roughly a single cell.

Because you could not be sure of the low level of DNA if LOD was 100 pg

Nuclear DNA quantities

Low Copy Number Realm

A typical amplification uses 10µL thus lowest quantity detected by qPCR that can be amplified with certainty is 230 pg single copy or 80pg multicopy.
TECHNICAL NOTE

Catherine M. Cupples,¹ M.S.; Jarrod R. Champagne,² M.S.; Kristen E. Lewis,³ M.S.;
and Tracey Dawson Cruz,⁴ Ph.D.

STR Profiles from DNA Samples with “Undetected” or Low Quantifiler™ Results

ABSTRACT: Screening methods capable of identifying DNA samples that will not yield short tandem repeat (STR) profiles are desired. In the past, quantitation methods have not been sensitive enough for this purpose. In this study, low level DNA samples were used to assess whether Quantifiler™ has a minimum quantitation value below which STR profiles would consistently fail to be detected. Buccal swabs were obtained and the DNA extracted, quantified, and serially diluted to concentrations ranging from 0.002 to 0.250 ng/µL. Samples were analyzed once with Quantifiler™, followed by Profiler Plus™ amplification and capillary electrophoresis analysis. An absolute minimum value below which STR results were unobtainable could not be defined. From the 96 low level samples tested, STR loci (including one full profile) were successfully amplified and detected from 27% of the samples “undetected” by Quantifiler™. However, no STR alleles were detected in 73% of these “undetected” samples, indicating that Quantifiler™ data may be useful for predicting STR typing success.

KEYWORDS: forensic science, DNA typing, quantitative polymerase chain reaction, Quantifiler™, DNA quantitation, low level DNA

27% of negative quantifiler results produced typable STR loci
QPCR is a better way to assess quantity but be careful!.

Sample quantity is outside the calibration. The number has no meaning. 
### The Issue of calibration

**ie: where exactly is 7 pg?**

<table>
<thead>
<tr>
<th>Quantification Standard Dilution (ng/μL)</th>
<th>Human</th>
<th>Male</th>
<th>IPC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$C_T$ (Mean)</td>
<td>Standard Deviation</td>
<td>$C_T$ (Mean)</td>
</tr>
<tr>
<td>-----------------------------------------</td>
<td>--------</td>
<td>-------------------</td>
<td>--------</td>
</tr>
<tr>
<td>50</td>
<td>23.36</td>
<td>0.26</td>
<td>23.92</td>
</tr>
<tr>
<td>16.7</td>
<td>24.98</td>
<td>0.23</td>
<td>25.55</td>
</tr>
<tr>
<td>5.56</td>
<td>26.62</td>
<td>0.28</td>
<td>27.22</td>
</tr>
<tr>
<td>1.85</td>
<td>28.26</td>
<td>0.23</td>
<td>28.88</td>
</tr>
<tr>
<td>0.62</td>
<td>29.79</td>
<td>0.29</td>
<td>30.44</td>
</tr>
<tr>
<td>0.21</td>
<td>31.32</td>
<td>0.34</td>
<td>32.01</td>
</tr>
<tr>
<td>0.068</td>
<td>32.83</td>
<td>0.32</td>
<td>33.61</td>
</tr>
<tr>
<td>0.023</td>
<td>34.48</td>
<td>0.58</td>
<td>35.33</td>
</tr>
</tbody>
</table>

*Figure 6-28, Figure 6-29, and Figure 6-30 provide the human, human male, and IPC $C_T$ mean values obtained using the Quantifiler® Duo DNA Quantification Kit.*

At levels below 23pg/μL, you are below the calibration line and making it impossible to determine a true value. What is more, your lowest datapoint varies 2X as much.
Outside the calibration curve, a 5-fold difference in the estimate is a reasonable possibility simply due to uncertainty in the slope of the line. The software reports the number as 6.83 pg/μL. How can it be accurate to three places? 6.83 pg/μL?????? It could be 11 just as easily as it could be 2.
How to improve LCN analysis by QPCR?

**Alu Sequence**

- Family of repetitive elements amplified immensely during primate evolution
- 500,000 to 1,000,000 copies in the human genome (6-13%)
- Consensus sequence is ~280bp in length
- Large number of copies in the human genome make *Alu* an excellent target or marker for human DNA

**Sybr Green**

- Binds at multiple locations along the amplified product
- Strong fluorescence enhancement following DNA binding
SYBR Green with multicopy Alu Loci - Nicklas and Buel

Reproducibility over 5 experiments (1 week)

R² = 0.9934

ng of input DNA

Ct

0.001  0.01  0.1  1  10  100

Genome equivalent of a single cell

R² = 0.9934
The difference between detection of amplification and reliability of result
Multilocus probes.

The instrument reports 1.9pg/μL the analyst puts in 10 μL the result? An
amplification of 20pg – Surely LCN. And the results show a partial profile.
With only 6% of the total alleles recovered.
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