Challenges with Low-Level DNA and Mixture Interpretation

John M. Butler

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Low-Level DNA

Data from Becky Hill (NIST)
Name Change…

LT DNA
low template
DNA
Some Definitions of Low Template (LT) DNA

- Working with <100-200 pg genomic DNA

- Considered to be data below stochastic threshold level where PCR amplification is not as reliable (determined by each laboratory; typically 150-250 RFUs)

- Enhancing the sensitivity of detection (increasing PCR cycles, PCR product clean-up, increasing CE injection/voltage)

- Having too few copies of DNA template to ensure reliable PCR amplification (allelic or full locus drop-out)
Low Template DNA Testing

• **Every lab faces samples with low template DNA**
  – Do you choose to attempt an “enhanced interrogation technique” such as increasing the cycle number, desalting samples, etc.?
  – **Next generation kits coming from manufacturers are capable of greater sensitivity** – will they be misused without appropriate caution and validation?

• **At what point do you draw a line and not attempt to analyze data below this line?**
  – A certain amount of input DNA (based on what data?)
  – A pre-determined stochastic threshold (based on what data?)
How low can you go?
Should you just say no?

Since we began meeting “halfway in between” in San Mateo a few years ago, the food scene seems to have picked up. Taking a break from our usual “office” at Astaria, we had a couple of wonderful meals at Capellini’s over the winter. For this meeting, we decide to try a new location, Aquapazza. We
“Enhanced Interrogation” Techniques to Improve Sensitivity

- **Increased PCR cycle number**
  
  With 100% efficiency:
  - 28 cycles = 67 million copies
  - 31 cycles = 1 billion copies (x16)
  - 34 cycles = 4 billion copies (x64)

- Reduced volume PCR
- Sample desalting (e.g., MinElute) prior to CE
- Extended CE injections

Are you “waterboarding” your DNA trying to get more information from the sample?

Requires validation to determine appropriate thresholds for reliability
Illustration of Potential Results at a Heterozygous Locus

**DNA amount**

( log scale)

- 10 ng
- 1 ng
- 0.1 ng
  - (100 pg)
- 0.01 ng
  - (10 pg)

**Detection Sensitivity**

<table>
<thead>
<tr>
<th>DNA amount (log scale)</th>
<th>28 cycles</th>
<th>31 cycles</th>
<th>34 cycles</th>
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<tbody>
<tr>
<td>10 ng</td>
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<td>0.1 ng (100 pg)</td>
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<tr>
<td>0.01 ng (10 pg)</td>
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**Off-scale data (leads to artifacts)**

- **Optimal data**
- **Allele drop-out**
- **Locus drop-out**

**Allele imbalance**

**Allele drop-in**
Profiles in DNA Article on Low Level DNA

Scientific Issues with Analysis of Low Amounts of DNA

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Faced with limited evidence that yield low amounts of DNA, forensic analysts will continually have to confront the question of how far to push DNA-testing techniques. Low copy number (LCN) analysis, also known as low template DNA (LT-DNA) testing, involves enhancing detection sensitivity usually through increasing the number of PCR cycles. Stochastic effects inherent with analysis of low amounts of DNA yield allele or locus drop-out. Additionally, increasing detection sensitivity can result in a greater potential for contamination or allele drop-in. Validation studies with replicate testing of low amounts of DNA were performed to assess the level of allele and locus drop-out and allele drop-in using 10, 30 and 100 picograms with several commercially available STR-typing kits under both standard and increased number of PCR cycles. The results with pristine, fully heterozygous samples demonstrate that a replicate testing approach can produce reliable information with single-source samples when consensus profiles are created.
**Stochastic (Random) Effects with LT-DNA When Combined with Higher Sensitivity Techniques**

**Loss of True Signal (False Negative)**

- Severe Peak Imbalance
- Allelic Drop-out
- High Stutter
- Allelic Drop-in

**Gain of False Signal (False Positive)**

- Identifiler, 30 pg DNA, 31 cycles
- Identifiler, 10 pg DNA, 31 cycles

**Correct genotype:**

- 10,11
- 12,14
- 12,13
- 18,19

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- 12,13
- 18,19
High copy number
>20 copies per allele

What is sampled is consistent with the true amount present in the sample

Resulting electropherogram

Complete (and correct) genotype

Low copy number
6 copies per allele

What might be sampled by the PCR reaction…

OR

Extreme allele imbalance

Copies of allele 1

Copies of allele 2

True amount

Allele imbalance

Allele dropout

Allele imbalance
Replicate #1

Replicate #2

Replicate #3

Consensus Profile: 14,19, 7,9.3, 12,13, 11,13, 24,Z

Correct Profile: 14,19, 7,9.3, 12,13, 11,13, 18,24
Suggestions for Optimal Results with LT-DNA

• Typically at least 2 – 3 PCR amplifications from the same DNA extract are performed to obtain consensus profiles

• 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 LT-DNA Analysis Procedure

1. Extract DNA from stain
2. Perform 3 Separate PCR Amplifications
3. Quantify Amount of DNA Present
4. Interpret Alleles Present
5. Develop a Consensus Profile (based on replicate consistent results)
Comparison of Approaches

Replicate Amplification with Consensus Profile

- Low amount of DNA examined
- Stochastic effects

- Amplification #1
- Amplification #2
- Amplification #3

Consensus Profile Developed (from repeated alleles observed)

Interpretation Rules Applied (based on validation experience)
  e.g., specific loci may dropout more

Result can be and usually is Reliable & Reproducible

Individual results may vary but a consensus profile is reproducible (based on our experience with sensitivity studies and replicate amplifications)

Single Amplification

- Low amount of DNA examined
- Stochastic effects

- Amplification #1 (only a single test)

Result can be Unreliable

What “LCN Labs” Are Doing
Experimental Design to Study LT-DNA Issues

• Pristine DNA Samples
  – 2 single-source samples
  – \textit{heterozygous for all loci tested} (permits peak height ratio studies)

• Low DNA Template Amounts
  – Dilutions made after DNA quantitation against NIST SRM 2372
  – 100 pg, 30 pg, and 10 pg (1 ng tested for comparison purposes)

• Replicates
  – 5 separate PCR reactions for each sample

• STR Multiplex Kits
  – \textit{Identifiler Plus} and \textit{PowerPlex 16 HS} (half-reactions)

• Increased Cycle Number
  – Identifiler Plus (\textbf{29 cycles and 32 cycles}; 28 for 1 ng)
  – PowerPlex 16 HS (\textbf{31 cycles and 34 cycles}; 30 for 1 ng)
Tested sample is heterozygous (possesses 2 alleles) at every locus, which permits an examination of allele dropout.

Results broken down by locus.

Green = full (correct) type
Yellow = allele dropout
Red = locus dropout
Black = drop-in

A single profile slice

A replicate slice

5 replicates of each [DNA]

5 replicates of each [DNA]

5 replicates of each [DNA]

10 pg (~2 cells)
30 pg (~6 cells)
100 pg (~18 cells)

5 replicates of each [DNA]

32 Cycles

10 pg
30 pg
100 pg

MT97150

D
8 D D C
S 2 7 S
1 1 S F 1 T S S 1
1 S 8 1 3 H 3 5 3
7 1 2 P 5 0 1 3 3
9 1 0 O 8 1 7 9 8

D
D
D
D

D
D
D

D
D
1
9
S
T

8 A S
4 v P S
3 W O 5
3 A X 1
L 8 A
Identifiler Plus (10 pg @ 32 cycles)

- Locus drop-out
- Allele drop-in
- Allele dropout
- Imbalance

- Allele 11,15
- Allele 29,31
- Allele 8,12
- Allele 12,13
- Allele 14,19
- Allele 7,9.3
- Allele 12,13
- Allele 11,13
- Allele 18,24
- Allele 12,14
- Allele 10,11
- Allele 22,25

9 = high stutter
17 = high stutter
# Impact of Three More PCR Cycles

## Identifier Plus

*Full type = both alleles above 50 RFU (does not account for peak imbalance)*

![Color Legend]

- Green = full (correct) type
- Yellow = allele dropout
- Red = locus dropout
- Black = drop-in

<table>
<thead>
<tr>
<th>Cycle</th>
<th>MT97150 D 8 D D C S 2 7 S 1 1 S F 1 S 8 1 7 1 2 P 9 1 0 O</th>
<th>MT97150 D 8 D D C S 2 7 S 1 1 S F 1 S 8 1 7 1 2 P 9 1 0 O</th>
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**MT97150**

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<th>MT97150 D 3 D D 3 9 8 1 7 9 8 3 A X 1 L 8 A</th>
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## Comparison

- **29 Cycles**: 33% vs. 53% full profiles
- **32 Cycles**: 38% improvement with 3 extra cycles
Sensitivity & Performance

PowerPlex 16 HS

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Green = full (correct) type
Yellow = allele dropout
Red = locus dropout
Black = drop-in

31 Cycles

16% vs. 60% full profiles

73% improvement with 3 extra cycles

34 Cycles
Summary of LT-DNA Testing

• More and more labs are “pushing the envelope” and attempting LT-DNA testing.

• LT-DNA testing has been “generally accepted as reliable” in many recent court cases.

• Our results demonstrate that replicate testing can produce reliable information with single source samples at low levels of DNA when consensus profiles are created.
MinElute PCR Purification Kit

Identifiler Plus, 29 cycles, 10 pg

- No MinElute
- MinElute

*5 extra peaks were called

Signal Improvement: ~66% ~67%

*96 well plates with vacuum protocol used
Comments on DNA Quantitation

• qPCR has enabled lower amounts of DNA to be quantified in recent years – providing in some cases a false sense of confidence in accuracy at these low levels

• Remember that qPCR is also subject to stochastic effects and thus DNA quantitation will be less accurate and exhibit more variation at the low end…

• Next generation STR kits with their greater sensitivity and ability to overcome inhibition have the potential to make the current qPCR DNA quantitation kits obsolete as an appropriate gatekeeper to whether or not to continue with a low level, compromised DNA sample
Important Role of DNA Quantitation

Extract DNA

Quantitation

Too much DNA
Dilute sample (to normalize)

Optimal range

Too little DNA
Concentrate sample (to normalize)

PCR Amplification

Low template DNA approaches (e.g., more PCR cycles)
3,068 casework samples

EZ1 DNA extraction (no inhibitors seen)

DNA quantitation

Quantifiler (performed twice and results averaged)

STR amplification

Nanoplex\textsuperscript{QS} and SEfiler (with up to 500 pg DNA added)

Group 1
0-5 pg/µL
1564 samples

No results 96%
Full profile 3%
Partial profile 1%

Group 2
5-10 pg/µL
279 samples

Group 3
10-30 pg/µL
371 samples

Group 4
>30 pg/µL
854 samples

Kremser et al. 2009
<table>
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<th>0,0</th>
<th>0,&gt;0</th>
<th>&gt;0,&gt;0</th>
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<tbody>
<tr>
<td>Number of Samples</td>
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<td>478</td>
<td>336</td>
</tr>
<tr>
<td>Positive results</td>
<td>0%</td>
<td>7%</td>
<td>27%</td>
</tr>
<tr>
<td>Negative results</td>
<td>100%</td>
<td>93%</td>
<td>63%</td>
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</tbody>
</table>

When both Quantifiler results were zero, then all subsequent STR testing failed to obtain a result

Kremser et al. 2009
The 2009 LCN Debates

ISFG Session - September 2009
Promega LCN Panel - October 2009
UK Court Decision – December 2009
ISFG LCN Session – September 18, 2009

Adrian Linacre (UK)  Bruce Budowle (US)  Peter Gill (UK)


LCN Panel

Articles planned for publication in March 2010 issue of Promega’s Profiles in DNA; freely available at http://www.promega.com/profiles/
Questions Addressed:
(1) How do you define or use the term “LCN”? – Theresa and Bruce
(2) Has PCR testing of small amounts of DNA been appropriately validated and accepted in non-forensic DNA testing? – Gillian and Angela
(3) What do you see as the biggest scientific challenge with “LCN” testing? – Bruce and Theresa
(4) Can single-source DNA samples with low amounts of DNA be interpreted reliably? – Bruce and Gillian
(5) What advice do you have to offer to forensic scientists working with attorneys on cases that may be considered “LCN” cases? – Brad, Theresa, Bruce
(6) Is it better to consume a sample with a single amplification vs. replicate amplifications? – Angela and Gillian
(7) Where do we go next with “LCN” testing? – Bruce, Theresa, Brad, John, Angela, Gillian
Some LT-DNA Court Rulings

• “…a challenge to the validity of the method of analysing Low Template DNA by the LCN process should no longer be permitted at trials where the quantity of DNA analysed is above the stochastic threshold of 100-200 picograms…”
  – United Kingdom: Crown vs. Reed & Reed, Dec. 21, 2009

• LT-DNA testing is “…generally accepted as reliable in the forensic scientific community under the standard enunciated in Frye…”
  – NYC OCME: People vs. Megnath, Feb. 8, 2010

• “LCN DNA evidence is not inherently unreliable.”
  – New Zealand: Crown vs. Wallace, Mar. 3, 2010

The judge in the Wallace case quotes from John Butler’s *Fundamentals of Forensic DNA Typing* in drawing the court’s conclusion.
Literature Debates

• A number of letters to the editor went on-line in *FSI Genetics* with back and forth arguments between Peter Gill & John Buckleton and Bruce Budowle

• These contentious opinion articles were terminated with a January 2011 editorial in FSI Genetics when the letters were all published in a single issue
LT-DNA Section of STRBase

- Launched October 30, 2009
  - Low-template DNA = LTDNA (not LCN!)

- Includes:
  - Presentations from Promega 2009 LCN Panel and Technical Leader’s meeting
  - Validation data from NIST sensitivity studies to illustrate problems and consensus profile solution to low levels of DNA testing
  - Literature listing of pertinent articles to help explain the issues involved in this topic
New STRBase Website on LT-DNA (LCN)

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

**Information on Low Template / Low Copy Number DNA Testing**

Sessions were held at several recent conferences, which is widely referred to as low copy number DNA. The intent is to help readers better understand this topic.

*At the International Society for Forensic Genetics (ISFG) annual meeting, member of the Caddy report, Bruce Budowle (FBI DNA Science Service). At the International Symposium on Forensic Genetics (ISFG) annual meeting, member of the Caddy report, Bruce Budowle (FBI DNA Science Service). At the International Symposium on Forensic Genetics (ISFG) annual meeting, member of the Caddy report, Bruce Budowle (FBI DNA Science Service).*

**Scientific Issues with Analysis of Low Amounts of DNA**

**Presentations on LTDNA**

- John Butler - ISHI (Promega)
- Becky Hill - ISHI (Promega)
- Theresa Caragine - ISHI (Promega)

**LTDNA Validation Data**

Labs having validation data on the LT-DNA Panel can be found at:

john.butter@nist.gov

**NIST Sensitivity Data with low level DNA**

10 replicate amplifications for each condition

NEW
Complete Set of NIST Sensitivity Data Available on New LT-DNA Website

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

NIST Sensitivity Data with low level DNA templates
10 replicate amplifications for each condition with two fully heterozygous, single-source samples

<table>
<thead>
<tr>
<th>STR kit - PCR conditions</th>
<th>Sample 1</th>
<th>Sample 2</th>
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<tbody>
<tr>
<td>Identifiler - 28 cycles</td>
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PowerPlex 16 HS – 34 cycles

Sample #1 (MT97150)

Sample #2 (PT84411)

MT97150 - 10 pg, amp #1
Literature Listing on LT-DNA (LCN)

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

Subdivided into categories

- Peer-reviewed literature (containing data)
- Reports (evaluating the methodology)
- Review articles (commenting on other's data)
- Non-peer reviewed literature (representing the authors' opinions only)

Links to papers when freely available

**LTDNA References**

**Peer-reviewed literature (containing data)**

Is LCN Effort Worthwhile?

Thoughts to Consider…

• Success rates are often low
• Requires dedicated “clean” facilities and extreme care to avoid limit contamination
• Complex interpretation procedure – requires more experienced analysts to do
• Significance of a DNA match?? – intelligence information but likely not to be probative due to unknown time when sample may have been deposited…
The Wisdom of Obi Wan Kenobi

http://www.starwars.com/kids/explore/lore/img/news20000902_1.jpg

Just before entering the Mos Eisley spaceport in Episode IV, Ben (Obi Wan) Kenobi warned Luke Skywalker, "You will never find a more wretched hive of scum and villainy… WE MUST BE CAUTIOUS!"
Mixtures

All TrueAllele data from Mike Coble (NIST)
Stochastic threshold 3500 data from Erica Butts (NIST)
“If you show 10 colleagues a mixture, you will probably end up with 10 different answers.”
- Dr. Peter Gill

“Don’t do mixture interpretation unless you have to”
- Dr. Peter Gill (1998)
Mixture Interpretation Protocols Build on Single-Source Sample Information

The Steps of Data Interpretation

Peak (vs. noise)  Allele (vs. artifact)  Genotype (allele pairing)  Profile (genotype combining)

Next step: Examine feasible genotypes to deduce possible contributor profiles

Moving from individual locus genotypes to profiles of potential contributors to the mixture is dependent on mixture ratios and numbers of contributors.
If your laboratory uses a stochastic threshold (ST), it is:

1. Same value as our analytical threshold (we don’t use a ST)
2. About twice as high as our AT (e.g., AT = 50 and ST = 100 RFU)
3. Less than twice as high as our AT
4. Greater than twice as high as our AT
5. I don’t know!

Responses from 140 participants in ISHI 2011 Workshop
Stochastic and Analytical Thresholds
Impact Lowest Expected Peak Height Ratio

Lowest Expected Peak Height Ratio

ST

AT
Setting a Stochastic Threshold is Essentially Establishing a Risk Assessment

“Currently, most laboratories use an arbitrary stochastic threshold. When a protocol is changed, especially if it is made more sensitive to low-level DNA, then the stochastic threshold must also change.”


The position and shape of this curve may change based on anything that can impact peak detection (e.g., CE injection time, PCR cycle number, post-PCR cleanup).


Drop Out Probability as a Function of Surviving Sister Allele Peak Height

With a single peak at 75 RFU, there is approximately a 22% chance of a sister heterozygous allele having dropped out (being below the analytical threshold).

With a single peak at 100 RFU, there is approximately a 7% chance of a sister heterozygous allele having dropped out (being below the analytical threshold).
Acknowledgments

• For additional information, see http://www.cstl.nist.gov/biotech/strbase/mixture.htm

• NIJ Funding to our NIST Group through NIST OLES interagency agreement 2008-DN-R-121

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