MAAFS DNA Workshop

Introduction to Low Copy Number (LCN) DNA Testing Issues

John M. Butler, PhD
Richmond, VA
May 3, 2006

Presentation Outline

• LCN – what is it and why attempt it?
• DNA quantity in samples and qPCR
• Stochastic PCR amplification
• Caution & challenges with LCN
• Literature summary: DNA from fingerprints, single cells
• Consensus profiles and LCN interpretation rules
• Contamination and efforts to avoid it
• Secondary transfer and variable shedding
• Whole genome amplification – is it a solution to LCN?
• Other methods for higher sensitivity and signal enhancements
• Summary and final thoughts

Some Definitions of Low-Copy Number (LCN)

• Work with <100 pg genomic DNA (~15-17 diploid copies of nuclear DNA markers such as STRs)
• Below stochastic threshold level where PCR amplification is not as reliable (determined by each laboratory; typically 150-250 pg)
• Enhancing sensitivity of detection (34 cycles instead of 28 cycles)
• Too few copies of DNA template to ensure reliable PCR amplification
• Other terms for LCN:
  – Low-level DNA
  – Trace DNA
  – Touch DNA

LCN is dependent on the amount of DNA present NOT the number of PCR cycles performed; LCN conditions may exist with 28 or 34 cycles

LCN is not a “new” technique…

• 1996 – Taberlet et al. describe “reliable genotyping of samples with very low DNA quantities using PCR”
• 1997 - single cell STR analysis reported
• 1999 – Forensic Science Service begins LCN casework in UK (as an alternative to mtDNA)
• 2001 – Budowle and FBI co-authors urge caution with using LCN

Why attempt LCN? …

• Improved success rates with high sensitivity DNA testing vs. standard procedures
• Volume crime samples (burglary)
• Bone samples to provide improved matching statistics over mtDNA analysis

Amounts of DNA Required

<table>
<thead>
<tr>
<th>1985-1995</th>
<th>RFLP/VNTRs</th>
<th>50 ng – 1000 ng</th>
</tr>
</thead>
<tbody>
<tr>
<td>1991-present (kits since 1996)</td>
<td>PCR/STRs</td>
<td>0.5 – 2 ng</td>
</tr>
<tr>
<td>1999-present</td>
<td>LCN/STRs</td>
<td>&lt;0.1 ng</td>
</tr>
</tbody>
</table>

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

http://www.cstl.nist.gov/biotech/strbase/training.htm
**Introduction to LCN DNA Testing Issues**

**May 3, 2006**

**DNA quantity in samples**

**Calculation of the Quantity of DNA in a Cell**

1. **Molecular Weight of a DNA Base Pair** = 618 g/mol
   
   \[ A = 313 \text{ g/mol}; T = 304 \text{ g/mol}; \]
   \[ G = 329 \text{ g/mol}; C = 289 \text{ g/mol}; \]
   \[ A-T \text{ base pairs} = 617 \text{ g/mol}; \]
   \[ G-C \text{ base pairs} = 618 \text{ g/mol}; \]

2. **Molecular Weight of DNA** = 1.98 x 10^{12} g/mol

   There are \( 3.2 \text{ billion base pairs} \) in a haploid cell \( \sim 3.2 \times 10^9 \text{ bp} \)
   \[ (3.2 \times 10^9 \text{ bp}) \times (618 \text{ g/mol/bp}) = 1.98 \times 10^{12} \text{ g/mol} \]

3. **Quantity of DNA in a Haploid Cell** = 3 picograms

   \[ 3.3 \times 10^{-12} \text{ g} = 3.3 \text{ picograms (pg)} \]
   
   A diploid human cell contains \( \sim 6.6 \text{ pg} \) genomic DNA

4. **One ng of human DNA comes from \( \sim 152 \) diploid cells**

   \[ 1 \text{ ng genomic DNA} (1000 \text{ pg}) / 6.6 \text{ pg/cell} = \sim 152 \text{ copies of each locus} \]

   \( (2 \text{ per 152 diploid genomes}) \)

**Where does low copy number start?**

\(<100 \text{ pg template DNA}\)**


<table>
<thead>
<tr>
<th>Amount of DNA</th>
<th>~ # of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ng</td>
<td>152</td>
</tr>
<tr>
<td>0.5 ng</td>
<td>76</td>
</tr>
<tr>
<td>0.25 ng</td>
<td>38</td>
</tr>
<tr>
<td>0.125 ng</td>
<td>19</td>
</tr>
<tr>
<td>0.0625 ng</td>
<td>10</td>
</tr>
</tbody>
</table>

Robin Cotton, AAFS 2003 LCN Workshop

"Are we already doing low copy number (LCN) DNA analysis?"
Assume sample is from a single source:

<table>
<thead>
<tr>
<th>Amount of DNA</th>
<th>Total Cells in sample</th>
<th>~ # of copies of each allele if het.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ng</td>
<td>152</td>
<td>152</td>
</tr>
<tr>
<td>0.5 ng</td>
<td>76</td>
<td>76</td>
</tr>
<tr>
<td>0.25 ng</td>
<td>38</td>
<td>38</td>
</tr>
<tr>
<td>0.125 ng</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>0.0625 ng</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Assume sample is a 1:1 mixture of two sources:

<table>
<thead>
<tr>
<th>Amount of DNA</th>
<th>Total Cells in sample</th>
<th>~ # of cells from each component</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ng</td>
<td>152</td>
<td>76</td>
</tr>
<tr>
<td>0.5 ng</td>
<td>76</td>
<td>38</td>
</tr>
<tr>
<td>0.25 ng</td>
<td>38</td>
<td>19</td>
</tr>
<tr>
<td>0.125 ng</td>
<td>19</td>
<td>10</td>
</tr>
<tr>
<td>0.0625 ng</td>
<td>10</td>
<td>5</td>
</tr>
</tbody>
</table>

Assume sample is a 1:3 mixture of two sources:

<table>
<thead>
<tr>
<th>Amount of DNA</th>
<th>~ # of cells from major component</th>
<th>~ # of cells from minor component</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ng</td>
<td>114</td>
<td>38</td>
</tr>
<tr>
<td>0.5 ng</td>
<td>57</td>
<td>19</td>
</tr>
<tr>
<td>0.25 ng</td>
<td>28</td>
<td>10</td>
</tr>
<tr>
<td>0.125 ng</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>0.0625 ng</td>
<td>7</td>
<td>2</td>
</tr>
</tbody>
</table>

Assume sample is a 1:9 mixture of two sources:

<table>
<thead>
<tr>
<th>Amount of DNA</th>
<th>~ # of cells from major component</th>
<th>~ # of cells from minor component</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ng</td>
<td>137</td>
<td>15</td>
</tr>
<tr>
<td>0.5 ng</td>
<td>68</td>
<td>8</td>
</tr>
<tr>
<td>0.25 ng</td>
<td>34</td>
<td>4</td>
</tr>
<tr>
<td>0.125 ng</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>0.0625 ng</td>
<td>9</td>
<td>1</td>
</tr>
</tbody>
</table>

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.

Two different amplifications would be useful with a 1:9 mixture situation:
- Normal level of total DNA (e.g., 5 ng) so that major component is on-scale.
- High level of total DNA (e.g., 5 ng) so that minor (e.g., ~500 pg) is out of LCN realm – yes, the major component will be off-scale...

Impact of DNA Amount into Multiplex PCR Reaction

- We generally aim for 0.5-2 ng template.
- STR Kits Work Best in This Range
- Too much DNA
  - Off-scale peaks
  - Split peaks (+/-A)
  - Locus-to-locus imbalance
- Too little DNA
  - Heterozygote peak imbalance
  - Allele drop-out
  - Locus-to-locus imbalance

Stochastic effects when amplifying low levels of DNA can produce allele dropout.
Stochastic PCR amplification

Stochastic = random selection

Stochastic Fluctuation Effects

- Unequal sampling of the two alleles present in a heterozygous individual can occur when low levels of input DNA are used (results in allele drop-out)
- PCR reactions with <100 pg (~17 diploid copies)
- Walsh et al. (1992) – propose avoiding stochastic effect by adjusting the number of PCR cycles in an assay so that the sensitivity limit is around 20 or more copies of target DNA (i.e., a full profile is obtained with ~125 pg)


Stochastic Statistical Sampling

True amount

What might be sampled by the PCR reaction...

>20 copies vs allele 1

6 copies copies per allele (LCN)

Stochastic Effect

- Sometimes called “preferential amplification” – not really a correct term since either allele may be amplified if the other drops-out…not related to allele size
- Stutter product amounts may go up…
  - If in an early cycle of PCR, the stutter product is amplified more (due to sampling effect)
- Contaminating DNA can also be amplified giving rise to allele “drop-in” or a mixture

Comparison of STR Kit Amplification SOP with LCN Using the Same DNA Donor

Input DNA

SOP

1ng

50 μL PCR

PHR = 87%

8pg

5 μL PCR

PHR = 50%

Allele Drop Out

Allele Drop In

Heterozygote

Allele Imbalance

Balance of Assay Sensitivity and Potential for Stochastic Effects

- One of the ways that assays can be made more sensitive is by increasing the number of PCR amplification cycles
- Optimal cycle number will depend on desired assay sensitivity
- The number of PCR cycles was set to 28 for ABI STR kits to limit their sensitivity for generating full profiles to ~125 pg or 20 cells
- Sensitivity is a combination of fluorescent dye characteristics (relative to the instrument and laser excitation used) and PCR amplification conditions such as primer concentration and amount of polymerase used

Note that Promega STR kits use higher numbers of cycles to generate roughly equivalent sensitivity to ABI kits because they have less efficient dye labels and lower primer and polymerase concentrations

http://www.cstl.nist.gov/biotech/strbase/training.htm
Higher Sensitivity with More Polymerase and Cycle Numbers

![Higher Sensitivity with More Polymerase and Cycle Numbers](image)

Problems with Obtaining Correct Allele Calls at Low DNA Levels

![Problems with Obtaining Correct Allele Calls at Low DNA Levels](image)

What is a true peak (allele)?

![What is a true peak (allele)?](image)

Threshold Settings for the ABI 310/3100

- **Detection Limit**: $3 \times$ the standard deviation of the noise.
  - Estimated using $2 \times$ peak to peak noise, (approximately 35 - 50 RFUs)
- **Limit of Quantitation**: $10 \times$ the standard deviation of the noise
  - Estimated using $7 \times$ peak to peak noise (150-200 RFUs)
  - Below this point estimates of peak area or height are unreliable.
- **Dynamic Range**: The range of sample quantities that can be analyzed from the lowest to the highest (linear range is also important)
- **Stochastic Threshold**: Level of quantifiable DNA below which peaks can show severe imbalance (peak height ratios below 60%). Approximately 150 -200 RFUs. Enhanced stutter also occurs at these signal levels.

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

- This is fundamentally an issue of reliability
- For a peak intensity three times the standard deviation of the noise there is a limited chance that such a signal is the result of a random fluctuation
- This is because 99.7 percent of all noise signals fall below this value (from the definition of a Gaussian curve)
- Below this point the very real possibility exists that what you think is a peak is simply a statistical fluctuation in the baseline noise.

Sensitivity

- **Limit of detection (LOD)** – “the lowest content that can be measured with reasonable statistical certainty.”
- **Limit of quantitative measurement (LOQ)** – “the lowest concentration of an analyte that can be determined with acceptable precision (repeatability) and accuracy under the stated conditions of the test.”
- How low can you go?

Limit of Detection (LOD)

- Typically 3 times the signal-to-noise (based on standard deviation of the noise) or 2x Np-p

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

> 2 Np-p

2 x Np-p (baseline in a blank)

Types of Results at Low Signal Intensity

(Stochastic amplification potential)

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

Allelic Drop-out
- One or more sets of alleles do not amplify

TWGDAM validation of AmpFISTR Blue


- Minimum cycle # (27-30 cycles examined)
- Amplification adjusted to 28 cycles so that quantities of DNA below 35pg gave very low peaks or no peaks (below the analytical threshold!)
- 35 pg is approx 5 cells
- (but is 35pg the analytical threshold?) Determining this value might be a useful goal of a validation study

TWGDAM validation of AmpFISTR Blue


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” Why? Noise and stochastic fluctuation!

Sensitivity of Detection

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

- Different 310 instruments have different sensitivities; determination of stochastic threshold 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

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%
TWGDAM validation of AmpFISTR BluePCR
Wallin et al., JFS, 1998 43(4) 854-870

- In approximately 80 heterozygous loci in population samples:
  - Average peak height ratio was 92% for each locus – D3, vWA, FGA
  - Standard deviation was 7%
- Thus 99.7% of all samples should show a peak height ratio (PHR) above 71%
- Those that have a PHR of <70% may result from mixtures, low [DNA], inhibition, degradation or poor primer binding

Peak height ratios
Moretti et al., JFS 2001, 46(3) 647-660

- PP + Cofiler gave PHR >88% n= 230+ samples with a lower range PHR (~3sd) of 59%
- Suggest using 59% as a guide
- 2% of single source samples were below this value
- Many validation studies focus on 1ng input DNA. What happens with lower amounts?

Peak Height Ratio Guidelines
- One way to approach concentration dependence
  - Profiler Plus
    - 200 to 300 RFU: 55 to 60%
    - 300 to 1000 RFU: 60 to 65%
    - above 1000 RFU: 65 to 70%
  - Cofiler
    - 200 to 300 RFU: 60%
    - 300 to 1000 RFU: 65 to 65%
    - above 1000 RFU: 70% to 75%

http://www.cstl.nist.gov/biotech/strbase/training.htm
TWGDAM validation of AmpFISTR Blue
Wallin et al., JFS, 1998 43(4) 854-870

- Stutter increases with allele size:
- Greatest amount was 8.6% w/ sd of 0.6, thus 8.6 + 3(0.6) = 10.4 or approx. 11% is the stutter threshold
- Stutter increases at low copy 


<table>
<thead>
<tr>
<th>Peak height (D5S818)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Why do you want to be in the DNA quantitation “sweet spot”?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Higher quality data which results in easier data interpretation</td>
</tr>
<tr>
<td>- Better balance across loci,</td>
</tr>
<tr>
<td>- Peaks on-scale with no pull-up from dye bleedthrough</td>
</tr>
<tr>
<td>- No split peaks from partial adenylation</td>
</tr>
<tr>
<td>- No stochastic effects on amplification</td>
</tr>
<tr>
<td>• STR kits, especially those amplifying more loci, are optimized for a narrow range of input DNA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Issues with Data below the Stochastic threshold and above the analytical threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>• PCR artifacts and stutter become prevalent</td>
</tr>
<tr>
<td>• Low levels of bleed through are possible</td>
</tr>
<tr>
<td>• Instrument spikes are more numerous</td>
</tr>
<tr>
<td>• -A peaks may appear</td>
</tr>
<tr>
<td>• Dye blobs become more significant in overall e-gram</td>
</tr>
<tr>
<td>• Low level 2nd contributors may show peaks</td>
</tr>
</tbody>
</table>

Typically between 50 and 200 RFU – depends on validation studies

<table>
<thead>
<tr>
<th>Difference in DNA Quantitation Capability vs. STR Typing Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear DNA quantities</td>
</tr>
<tr>
<td>1 ng</td>
</tr>
<tr>
<td>STR typing (28 cycles) LOD</td>
</tr>
<tr>
<td>10 pg</td>
</tr>
<tr>
<td>LCN STR typing (34 cycles) LOD</td>
</tr>
<tr>
<td>1 pg (less than a single cell)</td>
</tr>
<tr>
<td>mtDNA possible due to higher copy #</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>qPCR for DNA Quantitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>– will it lead more labs into LCN?</td>
</tr>
</tbody>
</table>

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.

- What types of experiments might be done to satisfy you that “no result” from a qPCR assay is truly “no DNA”?

http://www.cstl.nist.gov/biotech/strbase/training.htm
DNA Quantitation with Real-Time qPCR

- RT-qPCR is a homogeneous PCR based method that enables human specific quantification
  - Is easily automated, provides electronic storage of data
  - SYBR green or targeted probes (e.g., TaqMan) can be used

- Results give quantity of amplifiable DNA – not necessarily overall quantity
  - PCR inhibition can be detected
  - Limited multiplexing can be performed

- Big advantages are speed and dynamic range

- Commercial kits are now available (e.g., Quantifiler)

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

Caution & Challenges with LCN

Challenges of LCN


- Increased chance for contamination (want a sterile lab environment to reduce staff contamination)
- Data interpretation is more complicated (due to stochastic variation during PCR amplification):
  - Allele drop-out
  - Allele drop-in
  - Increased stutter products
- May not be able to associate DNA profile with bloodstain or other visual evidence

Hierarch of Propositions


Premise that scientific evidence may only be interpreted if at least two competing propositions are considered

- Level III – Offense level
  - A) Suspect is the offender
  - B) Suspect is unconnected with the incident

- Level II – Activity level
  - A) Suspect broke the window at the scene
  - B) Suspect is unconnected with the incident

- Level I – Source level
  - A) Bloodstain came from the suspect
  - B) Bloodstain came from some unknown person

- Sub-level I proposition – LCN regime because there are additional uncertainties regarding source of DNA sampled ... scientist cannot express a strong opinion about how DNA arrived at the site where it was recovered
Time Line Showing the Potential for DNA Deposition/Transfer

<table>
<thead>
<tr>
<th>Time</th>
<th>Crime Event</th>
<th>Opportunity for Adventitious Transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Opportunity for DNA Transfer from Perpetrator</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Analysis completed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Laboratory analysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Investigators arrive, detect, and recover evidentiary material</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Discovery</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Potential to “Contaminate”</td>
</tr>
</tbody>
</table>

Adapted from Gill, P. (2002) BioTechniques 32(2): 366-385, Figure 5

Low Copy Number Limitations:

- Allele drop out
- Allele drop in – contamination
- Increased stutter
- Heterozygote imbalance
- No thresholds
- Kits have not been subjected to QC under LCN conditions
- Reproducibility


Low Copy Number Limitations (cont):

- 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 searches
- Statistics


Low Copy Number Philosophy:

- Cannot exclude
- Dilution for redundancy (no concentration)
- Reviewed within context of case
- Speculation
- Intelligence tool


Low Copy Number Application:

- Investigative tool
- Missing person/remains
- Samples that can be cleansed


Literature Summary

http://www.cstl.nist.gov/biotech/strbase/training.htm
### Early LCN Literature

- Early work on touched objects and single cells:

- Application to routine forensic casework was pioneered by the Forensic Science Service:

### Other Useful LCN Articles (1)


### Nature (1997) 387: 767 Article Summary

*DNA fingerprints from fingerprints*

Roland A.H. van Oorschot
Maxwell K. Jones
Victoria Police, Melbourne, Victoria, Australia

**Initial tests showed that they could readily obtain correct genetic profiles from swabs taken directly from the palm of a hand (13 of 13). DNA yields varied from 2 to 150 ng (average 48.6 ng). Dry hands and those that had been washed recently tended to provide the least DNA.**
Introduction to LCN DNA Testing Issues

**Nature** (1997) 389:555-556

**Article Summary**

- First time that single cells were typed using modern forensic techniques
- Used SGM assay (6 STR loci + amelogenin) with TaqGold and 34 cycles
- Analyzed 226 buccal cells from four different individuals, isolating each cell using micromanipulation procedures
- Amplified DNA in 91% (206/226) cells, obtaining a full DNA profile in 50% (114/226) and an acceptable profile (four or more STRs) in 64% of these cells (Table 1).

**Possible Reasons for Allele Dropout**

- Failure to transfer the cell when a portion of the extract is analyzed
- Target sequence is degraded or not present in DNA template
- PCR amplification problems

**Typical LCN 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)

**Replicate LCN Test Results from FSS**

<table>
<thead>
<tr>
<th>Allele</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
<th>TH0</th>
<th>VWA</th>
<th>D2I</th>
<th>FSE</th>
<th>D19</th>
<th>D22</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>3</td>
<td>K</td>
<td>18.1</td>
<td>18.1</td>
<td>15.0</td>
<td>7.9</td>
<td>9.4</td>
<td>19.1</td>
<td>29.2</td>
</tr>
<tr>
<td>Sample 1</td>
<td>1</td>
<td>10.0</td>
<td>15.0</td>
<td>15.0</td>
<td>20.0</td>
<td>20.0</td>
<td>15.0</td>
<td>9.3</td>
<td>12.1</td>
</tr>
<tr>
<td>Sample 2</td>
<td>1</td>
<td>10.0</td>
<td>15.0</td>
<td>15.0</td>
<td>20.0</td>
<td>20.0</td>
<td>15.0</td>
<td>9.3</td>
<td>12.1</td>
</tr>
<tr>
<td>Sample 3</td>
<td>1</td>
<td>10.0</td>
<td>15.0</td>
<td>15.0</td>
<td>20.0</td>
<td>20.0</td>
<td>15.0</td>
<td>9.3</td>
<td>12.1</td>
</tr>
<tr>
<td>Sample 4</td>
<td>1</td>
<td>10.0</td>
<td>15.0</td>
<td>15.0</td>
<td>20.0</td>
<td>20.0</td>
<td>15.0</td>
<td>9.3</td>
<td>12.1</td>
</tr>
<tr>
<td>Sample 5</td>
<td>1</td>
<td>10.0</td>
<td>15.0</td>
<td>15.0</td>
<td>20.0</td>
<td>20.0</td>
<td>15.0</td>
<td>9.3</td>
<td>12.1</td>
</tr>
<tr>
<td>Sample 6</td>
<td>1</td>
<td>10.0</td>
<td>15.0</td>
<td>15.0</td>
<td>20.0</td>
<td>20.0</td>
<td>15.0</td>
<td>9.3</td>
<td>12.1</td>
</tr>
</tbody>
</table>

**Consensus** 3 | 15.0 | 15.0 | 15.0 | 20.0 | 20.0 | 15.0 | 9.3 | 12.1 | 6.2 |

*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 analyzed then the risk of allele 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.

**New Interpretation Rules Required for LCN**

An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA

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Suggestions to Optimal Results with LCN

- At least two* PCR amplifications from the same DNA extract (if enough DNA is present to do more than 4-5 amplifications, then most likely a single aliquot would be run under standard STR typing conditions)
- 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
*five is better; results are investigative

Contamination and efforts to avoid it

Increased Possibility of Contamination

- While LCN methods increase the sensitivity, they also increase the background DNA…
- There is a greater need for vigilance to reduce potential contamination at the crime scene, in the consumables (e.g., pipet tips, PCR tubes, etc.), and in the lab.
- Protective clothing can be worn (Rutty et al. 2003)

Contamination

- Systematic
  - e.g., Contaminated water or PCR buffer
- Sporadic
  - e.g., individual PCR tube contamination
- To reduce risks of contamination:
  - Careful lab cleanliness
  - Constant monitoring of reagents and consumables
- Contaminants are more likely to show up in the low molecular weight STR loci because they amplify more efficiently (miniSTRs will have a greater chance of detecting contaminating DNA)
- A negative control can detect systematic contamination but may not detect sporadic contamination, such as could be found in a single PCR tube

Although Rare, Some PCR Tubes Have Been Observed to Possess Contaminant DNA

- FSS observation
  - 11 casework-contaminating profiles in testing >1M samples
  - Use of negative control log and staff elimination databases
- "Contaminant" database …

Impact of Contamination on Casework

- Use negative controls to predict the level of overall contamination in a lab
- Conclude that most likely outcome of a contamination event is a false exclusion if contaminating DNA is preferentially amplified over original LCN material
Secondary transfer and variable shedding

Potential DNA Transfer

• Crime scene investigator → Scene
• Scene 1 evidence → Scene 2 evidence
• Innocent “passerby” → Scene (background DNA)

Issues of Transference and Persistence

• Transference – how easy is a DNA molecule transferred from a source to a recipient?
• Persistence – how long with a DNA molecule “stick around” after being deposited?


• Good shedders vs. poor shedders

Need for staff and police elimination databases

Whole genome amplification – is it a solution to LCN?

http://www.cstl.nist.gov/biotech/strbase/training.htm
Whole Genome Amplification will also be subject to stochastic sampling when starting with LCN DNA templates


Whole genome amplification—the solution for a common problem in forensic casework?

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Allele Dropout Seen with WGA at LCN Amounts of 50 pg and 5 pg

Schneider et al. (2004) Progress in Forensic Genetics 10, ICS 1261: 24-26

Improving Sensitivity

- Improved recovery of biological material and DNA extraction
- Longer injection on CE
- Salt removal from CE sample – enhances electrokinetic injection
- Reduced volume PCR – concentrates amplicon
- Increase number of cycles in PCR and/or TaqGold concentration
- Use miniSTRs – shorter amplicons amplify better
- Use mtDNA – higher copy number per cell

Other methods for higher sensitivity and signal enhancements

Improving DNA Collection and Extraction Methods Will Help Recover More DNA

Different Extraction Methods

Van Oorschot et al. (2003) Progress in Forensic Genetics 9, ICS 1239: 803-807
Steps in DNA Analysis

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

**Steps in DNA Analysis**
- Usually 1-2 day process (a minimum of ~5 hours)

**Steps in STR Typing with ABI 310/3100**
- Argon ion LASER (488 nm)
- Color Separation
- Fluorescence
- ABI Prism spectrograph
- CCD Panel (with virtual filters)

**Modifications in DNA Analysis Process to Improve LCN Success Rates**

- **Collection** – better swabs for DNA recovery
- **DNA Extraction** – into smaller volumes
- **DNA Quantitation** – qPCR helps with low DNA amounts
- **PCR Amplification** – increased number of cycles
- **CE Detection** – longer electrokinetic injection; more sensitive fluorescent dyes
- **Interpretation** – composite profile from replicate analyses with at least duplicate results for each reported locus
- **Match** – is it even relevant to the case?

**Signal Enhancements**

- **Higher PCR cycles**
- **Lower PCR volume** (problems with inhibitors)
- **Brighter fluorescent dyes**
- **Longer CE injection**
  - 10 s @ 3 kV = 30
  - 5 s @ 2 kV = 10
- **Microcon cleanup** to remove salts that interfere with electrokinetic injection

**Higher Sensitivity**

- Raising the number of PCR cycles creates a higher potential of allele drop-in being detected (increased noise)
- Ideally an improved fluorescent dye could be used to improve detection sensitivity and thereby permit a lower number of PCR amplification cycles to be used (peak intensity does not always correlate with stochastic effect)

**Reduced Volume PCR**

- Possibility of lower volume PCR to effectively concentrate the amount of DNA in contact with the PCR reagents
- Can samples be concentrated or can extraction volume be reduced?

**Steps in STR Typing**

- Sample Injection
- Sample Separation
- Sample Detection
- Calculation of Match Probability

**ET dyes (example: LIZ) permit a 10-30X improvement in signal over non-ET dyes**

**High Sensitivity Energy Transfer Dye Labeling**

Ju et al., Nature Medicine 2, 394 (1996)

**Signal Enhancement**

- Higher PCR cycles
- Lower PCR volume (problems with inhibitors)
- Brighter fluorescent dyes
- Longer CE injection
- Microcon cleanup to remove salts that interfere with electrokinetic injection

**Steps in STR Typing**

- Sample Injection
- Sample Separation
- Sample Detection
- Calculation of Match Probability
FIG. 5—Effects of a reduction of PCR reaction volume and DNA template concentration on amplification of a casework sample with a minor profile representing 2% of the total mixture.

**LCN Summary**

- LCN often defined as <100-200 pg input DNA
- Typically involves increasing the number of PCR cycles when performing multiplex PCR to amplify DNA with conventional STR kits (e.g., 34 cycles instead of 28 cycles)
- Enables lower amounts of DNA to be detected with STR markers but is prone to contamination
- Cautious data interpretation rules must be adopted as allele drop-out and drop-in may occur due to stochastic amplification effects

**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...

**miniSTRs and LCN**

- miniSTR assays are typically more sensitive than conventional STR kits currently in use
- Labs will start “pushing the envelope” in order to try and get a result with more sensitive assays including future miniSTR assays and kits
- Labs may move into the LCN realm without realizing it or adopting the careful LCN interpretation rules such as replicate analyses with duplicate results prior to reporting alleles

**The Wisdom of Obi Wan Kenobi**

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!**

Legal Implications of Profiles

The presence of a suspect’s profile does not constitute evidence that the suspect was present at the crime scene.

The absence of a suspect’s profile does not constitute evidence of innocence.

DNA Profiles are An Investigative Tool

Finding DNA indicates contact.

Lack of a DNA profile is inconclusive.

LCN is analogous to a bigger, more powerful magnifying glass

Introduction to Remaining LCN Speakers

- Dr. Theresa Caragine
  - NYC Office of Chief Medical Examiner (OCME)
  - Years of validation experience with LCN
  - LCN casework since Jan 2006
- Dr. Peter Gill
  - Forensic Science Service (FSS)
  - Pioneered LCN technique
  - Developed interpretation rules used in LCN
  - Recent work has focused on simulation studies to understand allele dropout
  - Working to develop new LCN expert system

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Thank you for your attention…

Questions?

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

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