Scientific Issues with Analysis of Low Amounts of DNA

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Presentation Overview

• **Stochastic Effects** during PCR Amplification
  – A fundamental physical law of PCR

• **Consensus Profiles** from Replicate Testing
  – Efforts to improve overall result reliability

• **Validation**
  – Example sensitivity studies
New Section of STRBase on This Issue

- Plan to launch it within a few weeks
  - Low-template DNA = LTDNA (not LCN!)

- What will be included:
  - My presentations from today and Becky Hill’s from the Technical Leader's meeting this afternoon (and any of the other speakers willing to provide their slides)
  - Validation data from our 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

Framing the Issues

- Forensic science methods often **must work close to the edge** of a technique due to the limited nature of the evidence
  - perpetrators are usually not willing to go back and add more biological material to a crime scene...

- **Validation studies** are performed in order to define the limits of a technique
  - sensitivity studies to determine at what point a lab cannot obtain reliable results anymore

We would always like improved sensitivity to enable results wherever possible
“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

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?)
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

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**)

- *Walsh et al.* (1992) – proposed 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 (Random) Effects with Low [DNA]**
When Combined with Higher Sensitivity Techniques

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

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

- **Heterozygote Peak Imbalance**
- **Allelic Drop-out**
- **Higher Stutter**
- **Allelic Drop-in**

In conjunction with interpretation rules, duplication of observed alleles in replicates was shown to correctly define the original sample.

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**Early Work on Replicate Testing with Low Levels of DNA**

Reliable genotyping of samples with very low DNA quantities using PCR

Pierre Taberlet*, Sally Griffin, Benoit Goossens, Sophie Questiau, Valerie Manceau, Nathalie Escaravage, Lisette P. Waits and Jean Bouvet

Laboratoire de Biologie des Populations d'Altitude, CNRS UMR 5553, Université Joseph Fourier, BP 53, 38041 Grenoble Cedex 9, France

Received May 1, 1999; Revised and Accepted July 2, 1999

Replicate testing introduced (up to 7 times) to account for allele drop-out and avoid miscalling allele drop-in.
Replicate Testing and Consensus Profiles

Extract DNA from stain

Quantify Amount of DNA Present

Perform 2 or 3 Separate PCR Amplifications

Interpret Alleles Present

Develop a Consensus Profile
(based on replicate consistent results)

10 pg template DNA with 31 cycles of PCR - triplicates

Replicate #1
14,19
7,9.3
12,13
11,13
18,24

Replicate #2
High stutter

Replicate #3

Consensus Profile (2 out of 3)

D3S1358 (14,19) correct
TH01 (7,9.3) correct
D13S317 (12,13) correct
D16S539 (11,13) correct
D2S1338 (24,Z) partial

Allele PHR imbalance

Allele dropout
Impact of “Unreliable” Results

- Allele drop-out can be dealt with using moderate stringency searches in CODIS algorithms
  - a homozygote “14” would hit to a heterozygote “11,14”

- Allele drop-in is most problematic for DNA database searches
  - this can be corrected for with replicate testing and consensus profiles to eliminate incorrect alleles

Comparison of Approaches

<table>
<thead>
<tr>
<th>Replicate Amplification with Consensus Profile</th>
<th>Single Amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low amount of DNA examined</td>
<td>Low amount of DNA examined</td>
</tr>
<tr>
<td>Stochastic effects</td>
<td>Stochastic effects</td>
</tr>
<tr>
<td>Amplification #1</td>
<td>Amplification #1</td>
</tr>
<tr>
<td>Amplification #2</td>
<td>(only a single test)</td>
</tr>
<tr>
<td>Amplification #3</td>
<td></td>
</tr>
<tr>
<td>Consensus Profile Developed</td>
<td>Result can be</td>
</tr>
<tr>
<td>(from repeated alleles observed)</td>
<td>Unreliable</td>
</tr>
<tr>
<td>Interpretation Rules Applied</td>
<td>(based on validation</td>
</tr>
<tr>
<td>(based on validation experience)</td>
<td>experience)</td>
</tr>
<tr>
<td>e.g., specific loci may dropout more</td>
<td></td>
</tr>
<tr>
<td>Result can be and usually is</td>
<td>Individual results</td>
</tr>
<tr>
<td>Reliable &amp; Reproducible</td>
<td>may vary but a</td>
</tr>
<tr>
<td></td>
<td>consensus profile is</td>
</tr>
<tr>
<td></td>
<td>reproducible</td>
</tr>
<tr>
<td></td>
<td>(based on our experience with sensitivity studies and replicate amplifications)</td>
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</table>
Experimental Design to Study LCN Issues

- Pristine DNA Samples
  - 2 single-source samples (and mixtures created from these)
  - heterozygous for all loci tested (permits peak height ratio studies)

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

- Replicates
  - 10 separate PCR reactions for each sample

- STR Kits
  - Identifiler and PowerPlex 16 HS (half-reactions)

- Increased Cycle Number
  - Identifiler (31 cycles; 28 for 1 ng)
  - PowerPlex 16 HS (31 cycles and 34 cycles; 30 for 1 ng)

PowerPlex 16 HS (½ Reaction)
1 ng @ 30 cycles

High signal, balanced peak heights (>0.80), no artifacts, low stutter

A Fully Heterozygous Sample (2 alleles for each locus)
Sensitivity & Performance

PowerPlex 16 HS

Results broken down by locus

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

Full (correct) profiles observed in all replicates at 100 pg

<table>
<thead>
<tr>
<th>Locus</th>
<th>10 pg</th>
<th>30 pg</th>
<th>100 pg</th>
</tr>
</thead>
<tbody>
<tr>
<td>31 Cycles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34 Cycles</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Sensitivity Comparison

Tested sample is heterozygous (possesses 2 alleles) at every locus, which permits an examination of allele dropout

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

10 replicates of each [DNA]

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

A single profile slice
A replicate slice

34 Cycles
A Few Observations:

- Locus-dependent performance
- Calling alleles as only those above detection threshold of 50 RFU
- Heterozygote balance is many times <60%
- From any grouping of three, the correct consensus profile of 14,17 would be made
FGA Replicates
PowerPlex 16 HS (10 pg @ 34 cycles)

FGA is more prone to Allele Drop-in from stutter

Summary of Data Observed at NIST

• Increasing the cycle number creates a higher number of full profiles (note: at both 31 and 34 cycles, 100 pg results were all correct with PowerPlex 16 HS)

• Across any grouping of 3 replicates, there was never an instance of an incorrect allele being called when two of three replicates matched

• Certain loci are more prone to allele and locus drop-out (depends on kit and PCR product sizes)

KNOW YOUR SYSTEM THROUGH VALIDATION STUDIES!
Kary Mullis – Inventor of PCR

“If it works, fine; if it works again, even better!”

- DTRA Talk 9/30/09

My Responses to the Panel Questions

#1 What is LCN?

- low amounts of DNA being tested often with “enhanced interrogation” techniques (such as higher cycle numbers)
  - It is not a pre-set DNA quantitation threshold (e.g., 200 pg) because quantitation does not always match PCR amplification performance
  - It is not a pre-set cycle number as each STR kit has a different sensitivity

- I agree with Peter Gill in the name change to “Low Template DNA”
My Responses to the Panel Questions

#2 LCN use in non-forensic areas

• Yes
  • Ancient DNA studies
  • Medical field with single cell analysis
    – following collection with laser capture microdissection

My Responses to the Panel Questions

#3 Biggest limitation with LCN?

• Relevance of result
  – Obtaining such a small amount of DNA from an evidentiary item … is it meaningful (probative)?
  – Will of course depend on the context of the specific case
My Responses to the Panel Questions

#4 Can single source samples be accurately interpreted at low levels?

• Yes, absolutely
  • But requires replicate testing and consensus profiles with cautious interpretation rules

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

Single Amplification
- Low amount of DNA examined
- Stochastic effects
- Amplification #1 (only a single test)
- Result can be Unreliable

Individual results may vary but a consensus profile is reproducible (based on our experience with sensitivity studies and replicate amplifications)
My Responses to the Panel Questions

#5 Advice to scientists on potential LCN

- Validate your system and understand the limitations of the protocol you are using

- Be cautious especially with the next generation STR typing kits that are “turbo-charged engines” and capable of much higher sensitivity
  - stochastic thresholds will need to be raised and/or replicate testing and consensus profiles introduced

My Responses to the Panel Questions

#6 Consume with single amp or perform replicate testing on smaller amounts

- Replication is better (depends on amount of DNA)

- Consensus profiles increase confidence that correct results are being obtained (repeated allele calls help avoid allele drop-out and allele drop-in problems)
My Responses to the Panel Questions

#7 Where next with LCN?

• Need further work on improved recovery of DNA
  – Direct PCR amplification (with better buffer systems) should help avoid extraction losses

• Make more validation data available
  – Advocated by John Buckleton (FSI Genetics 2009, 3:255-260)
    • “Validation issues around DNA typing of low level DNA”
  – Invite submissions to the STRBase Validation page

NIST will set the example by including all of our data for Identifiler 31 cycles and PowerPlex 16 HS 34 cycles with 100 pg, 30 pg, and 10 pg DNA samples

The Value and Relevance of Scientific Writing

Lesser value

• Website blogs and opinion pieces
• Non-peer reviewed articles
  – Conference proceedings
  – Letters to the editor
  – Many review articles

Greater value

• Peer-reviewed research articles – with data!
  • Highly cited scientific articles
    – Shows support from other scientists over time
    – Truly a measure of “scientific acceptance”
Is LCN Typing “Scientifically Acceptable” (as deemed by citation in the literature)?

For context:
- Budowle et al. (1999) JFS 13 STRs population data
  - 82 citations
- Chakraborty et al. (1999) Electrophoresis STR utility
  - 71 citations
- Moretti et al. (2001) JFS FBI validation of STRs
  - 61 citations
- Budowle et al. (2001) FSI STR primer concordance
  - 38 citations

Consensus profile approaches work with low template DNA

There Are Some Things I Agree with in the Budowle et al. (2009) CMJ Review Article

LCN testing should not be used for exculpatory purposes such as post-conviction testing due to potential of the LCN profile not being relevant to the case due to contamination