Amplification Variation and Stochastic Effects

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Outline: Amplification Variation and Stochastic Effects

• PRINCIPLES
  – Sources of variation
  – Sampling
  – Probability of allele drop out
• PROTOCOLS
  – Approaches to characterizing variation and recognizing when stochastic effects are likely to occur
  – Use of this data in protocol development
• PRACTICE
  – How do we choose a threshold
  – Impact of that choice
  – SWGDAM Guidelines and ISFG Recommendations

Ideally

• Begin with extracted DNA

• If all were perfect there would be an equal number of copies of each allele at each locus and all copies would amplify equally

Even in a mixture, amplification would be proportional to the number of alleles present.

• 2000 cells from person A who is 13, 19
• 760 cells from person B who is 13, 16
• 2760 copies of 13
• 2000 copies of 19
• 760 copies of 16

Life is Beautiful!!

Interpretation is Easy

But there are storm clouds!

“Easy” is hard to come by.

• why?

• Multiple sources of variation
  – Different analysts (hands)
  – Different samples
  – Different pipettes
  – Different kit lots
  – Different instruments

http://www.cstl.nist.gov/biotech/strbase/training.htm
Module 5: Amplification Variation & Stochastic Effects

How do we increase consistency i.e. (reduce variation)?

1. One person does all the work
2. Quality assurance procedures
3. Protocols
4. Cute uniforms for DNA Analysts
5. Answers 2 and 3

Where do you think most of the amplification variation comes from?

1. Pipetting
2. Amplification chemistry
3. Injection and detection
4. Too much coffee before work

Variation Summary

From the DNA Extract
- Pipetting from extract
- Sampling during amplification chemistry

Amplification
- Kit lots:
  - Component concentrations
  - Enzyme activity
  - Manufacturing processes
  - Company QC

Detection
- Instrument changes over time:
  - Laser
  - CCD camera

Sample preparation for instrument; pipetting injection electrophoresis

Analysis
- Protocol changes

See example in reference section under Peak Height Ratio: Debernardi et al. 2010

How can we characterize variation?

- Look at total amount of variation at end of process
  - Follow the positive control over time
- Experimentally break process into components and characterize using appropriate statistics
  - E.g. Separate amplification variation from injection variation
- Analyze existing or new validation data, training sample data, SRM data, kit QC data
- Use casework data
  - E.g. Variation between knowns and matching single source evidence profiles

Typical places we characterize variation associated with amplification

- % stutter
- Peak height
- Peak height ratio
- Calculated mixture ratio

Example 1: Characterization of Variation in Positive Control

- From: “One year variability of peak heights, heterozygous balance and inter-locus balance for the DNA positive control of AmpFlSTR Identifiler STR Kit”
- This article looks at a compilation of data obtained at the end of the testing process
- Experimental design
  - 205 positive controls
    - Peak height
    - Heterozygous balance
    - Inter-locus balance


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Module 5: Amplification Variation & Stochastic Effects

Distribution of Peak height ratio

50% Pk. Ht. ratio cut off used by laboratory.

Data from the positive control showed:

- High peak heights predicted other peak heights would be high.
- Peak heights and inter-locus balance varied more than peak height ratio.
- The two Genetic Analyzers (3130s) had different sensitivity in the blue channel.
- Heterozygous balance has no correlation with locus and “behaves as a random variable”... as “would be expected for a purely stochastic process...”

Example 2: Design experiment to have minimum variation in amplification

- Make single large master mix for each mass of DNA tested
  - DNA and master mix components added once and divided into multiple reaction tubes for amplification
- Therefore,
  - DNA concentrations are constant
  - master mix component concentrations are constant

Amplification replicates of 1 ng from single master mix

Little Variation across multiple amps with sufficient template

Amplification replicates of 125 pg from single master mix

Four samples - 0.5 ng (500 pg)
Four replicate amplifications
2, 5 and 10 second injections

Average PMR at Each Locus

http://www.cstl.nist.gov/biotech/strbase/training.htm
Four samples- 0.0625 ng (62 pg)  
Each sample injected at 2, 5 and 10 seconds

In normal sample processing cannot avoid:
- Pipetting variation affect on:
  - Amount of template
  - Concentration of master mix components
- These concentration differences have a more profound affect when working with low template amounts

“*The use of bounds applied to data that show continuous variation* is common in forensic science and is often a pragmatic decision. However it should be borne in mind that applying such bounds has arbitrary elements to it and that there will be cases where the data lie outside these bounds.”

*From: Bright, Turkington and Buckleton, FSI Genetics, 2010*

• Peak height ratio
  - One value above which two alleles can be considered to constitute a genotype
  - Two or more values used based on DNA mass
• Stochastic threshold
  - One threshold
  - Multiple thresholds reflecting different DNA amount
  - Incorporate probability of drop out into likelihood ratio

http://www.cstl.nist.gov/biotech/strbase/training.htm
**Module 5: Amplification Variation & Stochastic Effects**

**Problem**
- Occurs in single source samples and mixtures
- Analyst is unable to distinguish complete allele drop from a homozygous state

**PRINCIPLES**

<table>
<thead>
<tr>
<th>Slight</th>
<th>Moderate</th>
<th>Extreme</th>
<th>No detectable amplification</th>
</tr>
</thead>
</table>

**SWGDAM Definition of Stochastic**

**SWGDAM Guidelines glossary:**

- **Stochastic effects:** the observation of intra-locus peak imbalance and/or allele drop-out resulting from random, disproportionate amplification of alleles in low-quantity template

**Broader Definition of Stochastic**

- Stochastic is synonymous with "random." The word is of Greek origin and means "pertaining to chance." ... Stochastic is often used as counterpart of the word "deterministic," which means that random phenomena are not involved. Therefore, stochastic models are based on random trials, while deterministic models always produce the same output for a given starting condition.


**We cannot avoid stochastic effects and allele or locus drop out.**

**Why?**

**WE DO NOT KNOW THE NUMBER OF CONTRIBUTORS TO A SAMPLE!**

**PRINCIPLES**

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>1ng</td>
<td>107</td>
<td>36</td>
</tr>
<tr>
<td>0.5ng</td>
<td>53</td>
<td>18</td>
</tr>
<tr>
<td>0.25ng</td>
<td>27</td>
<td>9</td>
</tr>
<tr>
<td>0.125ng</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>0.0625ng</td>
<td>7</td>
<td>2</td>
</tr>
</tbody>
</table>

**PRINCIPLES**

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>1ng</td>
<td>129</td>
<td>14</td>
</tr>
<tr>
<td>0.5ng</td>
<td>64</td>
<td>7</td>
</tr>
<tr>
<td>0.25ng</td>
<td>32</td>
<td>4</td>
</tr>
<tr>
<td>0.125ng</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>0.0625ng</td>
<td>8</td>
<td>1</td>
</tr>
</tbody>
</table>

http://www.cstl.nist.gov/biotech/strbase/training.htm
An overview of sampling:

(Modified outline from Gill et al., NAR 2005)

Evidence has \( N \) cells

\[ \downarrow \]

Extraction recovers \( n \) genome copies

\[ \downarrow \]

DNA sample for PCR removes \( n_0 \) target copies

\[ \downarrow \]

\( n_1 \) amplicons of an allele are produced

\[ \downarrow \]

\( n_2 \) amplicons are added to HiDi-LIZ sample prep

\[ \downarrow \]

\( n_3 \) amplicons are injected & contribute to RFU


**Implications: Possible template amounts with replicate sampling**

**PROTOCOLS**

Sampling during amplification occurs by imperfect success of making copies

1\(^{st}\) amp.

- 1 copy of 7
- 3 copies of 9
- 4 copies of 11

Begin

- 2 copies of 8
- 2 copies of 11

2\(^{nd}\) amp.

- 2 copies of 7
- 6 copies of 9
- 1 copies of 10
- 7 copies of 11

Efficiency is not 100%

Some copies are stutter

Allele 8

Stutter, 7

Allele 11

Stutter, 10

http://www.cstl.nist.gov/biotech/strbase/training.htm
**What is Drop Out?**

- **Scientifically**
  - **Failure to detect** an allele within a sample or failure to amplify an allele during PCR. *From SWGDAM Guidelines, 2010*
  - Note that: **Failure to detect ≠ failure to amplify**
- **Operationally**
  - Setting a threshold(s) or creating a process, based on validation data and information in the literature, which allows assessment of the likelihood of drop out of an allele or a locus.

**Developing data to make informed choice for stochastic threshold(s) determination**

- Assuming some amplification of both peaks
  - Whether one or both peaks are observed will depend on:
    - the amount of amplified product injected
    - the analytical threshold used
- **Look at different experimental approaches**

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**Data from NIST: Experimental Design**

- **Pristine DNA Samples**
  - 2 single-source samples
  - heterozygous for all loci tested (permits peak height ratio studies)
- **Low DNA Temple Amounts**
  - Dilutions made after DNA quantitation against NIST SRM 2372
  - $100 \text{ pg, } 30 \text{ pg, and } 10 \text{ pg}$ (1 ng tested for comparison purposes)
  - Replicates
    - 10 separate PCR reactions for each sample
- **STR Kits**
  - **Identifier and PowerPlex 16 HS** (half-reactions)
  - **Increased Cycle Number**
    - Identifier (31 cycles, 28 for 1 ng)
    - PowerPlex 16 HS (31 cycles and 34 cycles, 30 for 1 ng)
  - **NIST data:**
    - PowerPlex 16 HS
      - 31 cycles
      - 10 pg → 10 multiple profile targets
      - 30 pg
        - 2 with 0 allele drop outs
        - 2 with 1 allele drop outs
        - 2 with 3 allele drop outs
        - 1 with 4 allele drop outs
      - 100 pg → 10 profiles with 0 allele drop outs

**Drop Out Probability as a Function of Present Allele Height**

From Gill et al., 2008 FSI Genetics

![Drop Out Probability as a Function of Present Allele Height](attachment:image.png)


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Data represents a probability distribution: The smaller rfu of the observed peak the greater probability that drop out has occurred.

Use data to:
- Develop a single threshold
- Develop multiple thresholds for different applications

With computer assistance or expert systems can use the probability distribution.

Can use some types of casework samples to look at drop out:
- Provides examples of drop out observed in sample which you are likely to encounter
- Provides additional data to consider when setting thresholds

Can examine casework data from known heterozygous loci using:
- Profiles from sexual assault cases having
  - two person mixtures
  - one component is consistent with a known victim
  - loci with 4 alleles

Looking for Lost Alleles:
- Casework samples exhibiting drop-out at a peak-calling threshold of 60 rfu were reanalyzed at 30 rfu to determine:
  - when no measurable amplification was truly occurring
  - when the dropped peak was simply below the 60 rfu peak-calling threshold.
- If an allele was not detected at 30 rfu = no amplification

Allelic dropout at peak-calling threshold of 60 rfu

Allelic dropout at peak-calling threshold of 30 rfu

http://www.cstl.nist.gov/biotech/strbase/training.htm
Comparison of data with rfu threshold set at 60 and 30 rfu:

- 72% of peaks lost using a threshold of 60 rfu were observed between 60 and 30 rfu.
- These peaks represent uneven amplification but not complete absence of amplification.

Dropout percentages by larger minor peak height intensity (threshold: 60 rfu)

<table>
<thead>
<tr>
<th>RFU Range</th>
<th>Dropout</th>
<th>% Smaller Allele</th>
<th>RFU Range</th>
<th>Dropout</th>
<th>% Smaller Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>60-100</td>
<td>18</td>
<td>30</td>
<td>60-100</td>
<td>29</td>
<td>61</td>
</tr>
<tr>
<td>101-150</td>
<td>28</td>
<td>21.4%</td>
<td>101-150</td>
<td>74</td>
<td>17.6%</td>
</tr>
<tr>
<td>151-200</td>
<td>16</td>
<td>18.8%</td>
<td>151-200</td>
<td>64</td>
<td>6.3%</td>
</tr>
<tr>
<td>201-250</td>
<td>12</td>
<td>8.3%</td>
<td>201-250</td>
<td>46</td>
<td>2.2%</td>
</tr>
<tr>
<td>251-300</td>
<td>11</td>
<td>0.0%</td>
<td>251-300</td>
<td>42</td>
<td>0.0%</td>
</tr>
<tr>
<td>301-350</td>
<td>11</td>
<td>9.1%</td>
<td>301-350</td>
<td>30</td>
<td>3.3%</td>
</tr>
<tr>
<td>350-400</td>
<td>3</td>
<td>0.0%</td>
<td>350-400</td>
<td>19</td>
<td>0.0%</td>
</tr>
<tr>
<td>&gt;400</td>
<td>0</td>
<td>0.0%</td>
<td>&gt;400</td>
<td>47</td>
<td>0.0%</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td></td>
<td>48</td>
<td>383</td>
<td></td>
</tr>
</tbody>
</table>

Considering this data...

Dropout probability as a function of present-allele height

What value will you choose for today’s stochastic threshold?

1. 100 RFU
2. 150 RFU
3. 200 RFU
4. 250 RFU
5. None of the above