STRs, CE, and Mixtures

Florida Statewide DNA Training

Indian Rocks Beach (Largo), FL
May 12-13, 2008

Dr. John M. Butler
National Institute of Standards and Technology

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Introductions

Dr. John M. Butler
National Institute of Standards and Technology
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Topics and Techniques for Forensic DNA Analysis

Florida Statewide Training Meeting
Indian Rocks Beach, FL
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NIST History and Mission

• National Institute of Standards and Technology (NIST) was created in 1901 as the National Bureau of Standards (NBS). The name was changed to NIST in 1988.
• NIST is part of the U.S. Department of Commerce with a mission to develop and promote measurement, standards, and technology to enhance productivity, facilitate trade, and improve the quality of life.
• NIST supplies over 1,300 Standard Reference Materials (SRMs) for industry, academia, and government use in calibration of measurements.
• NIST defines time for the U.S.

NIST Human Identity Project Team

John Butler
Margaret Appleby
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Former Project Team Members

Mike Coble
Chris DeAngelis
Jill Appleby
Rich Scholke
Amy Decker
Dennis Reeder

Retired/ABI

Location of NIST

Washington, D.C.
FBI Lab

Richmond, VA

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Current Areas of NIST Effort with Forensic DNA

• Standards
  – Standard Reference Materials
  – Standard Information Resources (STRBase website)
  – Interlaboratory Studies
• Technology
  – Research programs in SNPs, miniSTRs, Y-STRs, mtDNA, qPCR
  – Assay and software development, expert system review
• Training Materials
  – Review articles and workshops on STRs, CE, validation
  – Powerpoint and pdf files available for download
    http://www.cstl.nist.gov/biotech/strbase/NIJprojects.htm

Training Materials Available on STRBase

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

http://www.cstl.nist.gov/biotech/strbase/training.htm
Contributors to These Workshop Slides

- Bruce McCord
- Mike Coble
- Angie Dolph

Florida International University
AFDIL
Marshall U./NIST

CE
miniSTRs
mixtures

Primary Sources for Material Covered in this Workshop

- NIST STRBase website: http://www.cstl.nist.gov/biotech/strbase/

These workshop materials will be made available at http://www.cstl.nist.gov/biotech/strbase/training.htm

Outline for Workshop

Day 1
- STRs and Artifacts
- miniSTRs
- CE Troubleshooting

LUNCH

Day 2
- Mixture Interpretation
- Mixture Examples
- Mixture Stats
- Interlab Studies
- Company presentations

Understanding the Audience Here

- STR kits in use?
  - Profiler Plus/COFlter
  - Identifier
  - PowerPlex 16
  - Y-STRs?
- Instrumentation is use?
  - ABI 310
  - ABI 3100/3130xl
  - Other?
- Software in use?
  - GeneScan/Genotyper
  - GeneMapperID
  - Other?

NIST and NIJ Disclaimer

Funding: Interagency Agreement 2003-JJ-R-029 between the National Institute of Justice and NIST Office of Law Enforcement Standards

Points of view are mine and do not necessarily represent the official position or policies of the US Department of Justice or the National Institute of Standards and Technology.

Certain commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose.

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

http://www.cstl.nist.gov/biotech/strbase/training.htm
Topics and Techniques for Forensic DNA Analysis

STRs and Molecular Biology Artifacts

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Advantages for STR Markers

• Small product sizes are generally compatible with degraded DNA and PCR enables recovery of information from small amounts of material
• Multiplex amplification with fluorescence detection enables high power of discrimination in a single test
• Commercially available in an easy to use kit format
• Uniform set of core STR loci provide capability for national and international sharing of criminal DNA profiles

Value of STR Kits

Advantages
• Quality control of materials is in the hands of the manufacturer (saves time for the end-user)
• Improves consistency in results across laboratories – same allelic ladders used
• Common loci and PCR conditions used – aids DNA databasing efforts
• Simpler for the user to obtain results

Disadvantages
• Contents may not be completely known to the user (e.g., primer sequences)
• Higher cost to obtain results
How many STRs in the human genome?

- The efforts of the Human Genome Project have increased knowledge regarding the human genome, and hence there are many more STR loci available now than there were 10 years ago when the 13 CODIS core loci were selected.

- More than 20,000 tetrancleotide STR loci have been characterized in the human genome (Collins et al. An exhaustive DNA microsatellite map of the human genome using high performance computing. Genomics 2003;82:10-19)

- There may be more than a million STR loci present depending on how they are counted (Ellegren H. Microsatellites: simple sequences with complex evolution. Nature Rev Genet 2004;5:435-445).


Categories for STR Markers

<table>
<thead>
<tr>
<th>Category</th>
<th>Example Repeat Structure</th>
<th>13 CODIS Loci</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple repeats - contain units of identical length and sequence</td>
<td>(GATA)(GATA)(GATA)</td>
<td>TPOX, CSF1PO, D5S818, D13S317, D16S539</td>
</tr>
<tr>
<td>Simple repeats with non-consensus alleles (e.g., TH01 9.3)</td>
<td>(GATA)(GAT)(GATA)</td>
<td>TH01, D18S51, D7S820</td>
</tr>
<tr>
<td>Compound repeats - complex loci or more adjacent simple repeats</td>
<td>(GATA)(GATA)(GACA)</td>
<td>vWA, FGA, D3S1358, D8S1179</td>
</tr>
<tr>
<td>Complex repeats - contain several repeat blocks of variable unit length</td>
<td>(GATA)(GACA)(GATA)(GACA)</td>
<td>D2S1311</td>
</tr>
</tbody>
</table>

These categories were first described by Urquhart et al. (1994) Int. J. Legal Med. 107:13-20
Biological "Artifacts" of STR Markers

- Stutter Products
- Non-template nucleotide addition
- Microvariants
- Tri-allelic patterns
- Null alleles
- Mutations

Chapter 6 covers these topics in detail.

Stutter Products

- Peaks that show up primarily one repeat less than the true allele as a result of strand slippage during DNA synthesis
- Stutter is less pronounced with larger repeat unit sizes (dinucleotides > tri- > tetra- > penta-)
- Longer repeat regions generate more stutter
- Each successive stutter product is less intense (allele > repeat-1 > repeat-2)
- Stutter peaks make mixture analysis more difficult

STR Alleles with Stutter Products

![STR Alleles with Stutter Products](image)

Measured Stutter Percentages Variable by Allele Length and Composition

![Measured Stutter Percentages](image)

Stutter Product Formation

![Stutter Product Formation](image)

N+4 Stutter Evaluation Summaries

- Mass State Police DNA Lab
- Trying to collect data from as many laboratories as possible to characterize N + 4 stutter percentages in various platforms.
- Please email information to rebecca.post@pol.state.ma.us

http://www.cstl.nist.gov/biotech/strbase/validation/N+4_stutter_spreadsheet.xls

http://www.cstl.nist.gov/biotech/strbase/training.htm
Non-Template Addition

- Taq polymerase will often add an extra nucleotide to the end of a PCR product; most often an "A" (termed "adenylation")
- Dependent on 5'-end of the reverse primer; a "G" can be put at the end of a primer to promote non-template addition
- Can be enhanced with extension soak at the end of the PCR cycle (e.g., 15-45 min @ 60 or 72 °C) – to give polymerase more time
- Excess amounts of DNA template in the PCR reaction can result in incomplete adenylation (not enough polymerase to go around)

Excess template

Best if there is NOT a mixture of "+/- A" peaks (desirable to have full adenylation to avoid split peaks)

Incomplete adenylation

Impact of the 5’ Nucleotide on Non-Template Addition

D8S1179

Promega includes an ATT sequence on the 5'-end of many of their unlabeled PP16 primers to promote adenylation


Higher Levels of DNA Lead to Incomplete Adenylation

DNA Size (bp)

Relative Fluorescence (RFUs)

10 ng template (overloaded)

2 ng template (suggested level)

Identifiler – Rapid PCR (36 min total time)

with 1 min 60 °C adenylation soak (using different polymerases)

Result from Peter Vallone (NIST)

Microvariant “Off-Ladder” Alleles

- Defined as alleles that are not exact multiples of the basic repeat motif or sequence variants of the repeat motif or both
- Alleles with partial repeat units are designated by the number of full repeats and then a decimal point followed by the number of bases in the partial repeat (Bar et al. Int. J. Legal Med. 1994, 107:159-160)
- Example: TH01 9.3 allele: [TCAT]₄-CAT [TCAT]₅

http://www.cstl.nist.gov/biotech/strbase/training.htm
An Example of an “Off-Ladder” Microvariant at the Yfiler Locus DYS635

SNPs within the D8S1179 repeat

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

Lab Resources and Tools

- Addresses for scientists working with STRs
- Training Materials
- STR Allele Sequencing

STRbase has a summary of alleles that have been submitted and sequenced, if the submitting agency agrees to share the information.

We require a minimum of 10 ng for the sequencing.

We request copies of the electropherograms demonstrating the variant allele. The more information we have up front the better. Please have patience we will get to your samples!

Sample Submissions

- For those that desire more assurances of confidentiality we can have MOUs signed.
- We generally re-type the samples at NIST prior to starting sequencing.
- We may run a monoplex assay (single locus).
- We return results as PowerPoint slides.
- We thank all of those agencies that have used this free service (thanks to NIJ)!

Contact Margaret Kline: margaret.kline@nist.gov

Characterizing a Variant Allele That Occurs Between Two Loci

- Use a different multiplex STR kit with different locus combinations
- Test singleplex for each putative locus
- Example: Identifier D16S539 and D2S1338


http://www.cstl.nist.gov/biotech/strbase/training.htm
Steps to Detection of Which Locus an Out-of-Range Allele Belongs With...

- Consider locus heterozygosities – heterozygote is likely from locus with higher heterozygosity (e.g., D16 = 0.766 while D2 = 0.882)
- Remember that tri-allelic patterns and homozygotes are less common than heterozygotes – thus two heterozygotes are more likely than a homozygote next to a tri-allelic pattern
- Check STRBase for variant alleles reported previously by other labs (e.g., D16 has no >16 alleles while D2 has several <15 alleles)
- Consider genotype frequencies observed for the various possible combinations (e.g., D16 11,11 = 10.7% while D2 20,20 = 0.92%)

Three-Peak Patterns


D18S51

TPOX

D21S11

“Type 1”

Sum of heights of two of the peaks is equal to the third

Most common in D18S51 and TPOX and D21S11

“Type 2”

Balanced peak heights

Most common in TPOX

TPOX Tri-Allelic Patterns

FSI Genetics 2008; 2(2): 134–137

The nature of tri-allelic TPOX genotypes in African populations

A. B. Lane*

*Department of Human Genetics, University of Cape Town and the DNA Forensics Reference Laboratory, Cape Town, South Africa

Approximately 2.4% of indigenous South Africans have three rather than two TPOX alleles. Data collected during routine paternity testing revealed that the extra allele is almost always allele 10 and that it segregates independently of those at the main TPOX locus. Approximately twice as many females as males have tri-allelic genotypes which suggested that the extra allele is on an X chromosome.

http://www.cstl.nist.gov/biotech/strbase/training.htm
Variant Alleles Cataloged in STRBase

Off-Ladder Alleles

Tri-Alelic Patterns

Currently 439 at 13/13 CODIS loci

+ F13A01, FES/FPS, Penta D, Penta E, D2S1338, D19S433

Currently 170 at 13/13 CODIS loci

+ FES/FPS, Penta D, Penta E, D2S1338, D19S433

Is this an FGA - Tri-allelic pattern identified using Identifiler?

PK HT Ratio
12/10 - 0.48

D5S818

PK HT Ratio
19/24 - 0.55
25/24 - 0.89

Or is this a D13S317 - Tri-allelic pattern identified using Powerplex 16?

PK HT Ratio
12/10 - 0.48

D13S317

PK HT Ratio
13/11 - 0.83
14.3/11 - 0.42

It’s really a D5S818 Tri-allelic pattern identified using multiple STR Kits

D5S818

PK HT Ratio
12/10 - 0.48
12+29/10 - 0.86

D13S317 (IDfiler)

PP16

D5S818

D13S317

PP16 (PP16)

D5S818 Apparent 29 Allele Sequencing Results

PP16 Forward Primer

12 repeats

4 base deletion

5 repeat insertion

Complete PP16 Reverse Primer

There is a 4 bp deletion, the last 4 bases of the PP16 reverse primer binding site, followed by an insertion of 5 repeats. The 10 and 12 alleles of this sample have been sequenced and have the expected sequences.

D5S818 monoplex results

The 68 bp size difference between the 12 allele and the variant allele sizing as an “apparent 29” allele.
Are there other large D5S818 alleles?

- STRBase Tri-allelic reports for FGA for 19,*,* patterns with AB amplification kits.
  - 5 reports:
    - 19,20,21; 19,20,23; 19,20,24; 19,22,23; 19,24,25
  - But there we have sequenced true tri-allelic FGA samples
- STRBase Tri-allelic reports for D13S317 for *,*, OL patterns with PP16 amplification kits.
  - NO tri-allelic patterns with Off-Ladder alleles reported

Null Alleles

- Allele is present in the DNA sample but fails to be amplified due to a nucleotide change in a primer binding site
- Allele dropout is a problem because a heterozygous sample appears falsely as a homozygote
- Two PCR primer sets can yield different results on samples originating from the same source
- This phenomenon impacts DNA databases
- Large concordance studies are typically performed prior to use of new STR kits


Concordance between STR primer sets is important for DNA databases

- PowerPlex 16
- Profiler Plus
- DNA Database
- Allele Dropout
  - Reduced match stringency is a common solution

Impact of DNA Sequence Variation in the PCR Primer Binding Site

- No mutation
- Mutation in middle of primer binding site
- Mutation at 3’-end of primer binding site

vWA Primer Position Comparisons

- PowerPlex® 16
- Promega STR Kit
- PowerPlex® 16
- ABI STR Kit
- vWA Primer Position Comparisons
- T→A
- Reduced match stringency is a common solution

D18S51 Null Allele from Kuwait Samples with ABI Primers

- 172 bp downstream of STR repeat (G–AG)
- 10 nucleotides from 3’-end of ABI D18-R primer (PowerPlex 16 primers are not impacted)


D13S317 Flanking Region Deletion
A 4 bp deletion outside the miniSTR primers causes the commercial kit produced allele to appear one repeat smaller. Sequence analysis identified two regions where 4 bp deletions occur to cause this 1 repeat variation.

Ohio U miniSTR data

D13S317

 Mutation Observed in Family Trio

<table>
<thead>
<tr>
<th>STR Locus</th>
<th>Mutation</th>
<th>Normal Transmission of Alleles</th>
<th>Paternal Mutation</th>
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<tbody>
<tr>
<td>13S317</td>
<td></td>
<td>father mother son</td>
<td></td>
</tr>
<tr>
<td>15.17</td>
<td></td>
<td>14.18</td>
<td></td>
</tr>
<tr>
<td>15.18</td>
<td></td>
<td>24.18</td>
<td></td>
</tr>
<tr>
<td>15.17</td>
<td></td>
<td>15.18</td>
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Summary of STR Mutations
Mutations impact paternity testing and missing persons investigations but not forensic direct evidence-suspect matches...

- Mutations happen and need to be considered
- Usually 1 in ~1000 meioses
- Paternally normally higher than maternal
- VWA, FGA, and D18S51 have highest levels
- TH01, TPOX, and D16S539 have lowest levels


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

Apparent Null Alleles Observed During Concordance Studies

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<th>Null Allele Frequencies</th>
<th>Paternal Mutation</th>
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<td>330/51,940</td>
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New Section of STRBase (launched to track MiniFiler discordance and allele dropout frequency):
http://www.cstl.nist.gov/biotech/strbase/NullAlleles.htm

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http://www.cstl.nist.gov/biotech/strbase/training.htm
PCR Primer Quality Control

- UV Spec to determine concentration
- HPLC to evaluate purity
- TOF-MS to confirm correct sequence
- CE (ABI 310) to determine presence of residual dye molecules (“dye blobs”)


Problems with Dye Artifacts from Fluorescent Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>No Filtering (Straight from PCR)</th>
<th>Filtered with Edge columns</th>
</tr>
</thead>
<tbody>
<tr>
<td>THO1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPOX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSF1PO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D21S11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D7S820</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGA</td>
<td></td>
<td></td>
</tr>
<tr>
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STRBase
Short Tandem Repeat DNA Internet Database
http://www.cstl.nist.gov/biotech/strbase

- General Information
  - Intro to STRs (downloadable PowerPoint)
  - STR Fact Sheets
  - Sequence Information
  - Multiplex STR Kits
  - Variant Allele Reports
  - Training Slides

- Forensic Interest Data
  - FBI CODIS Core Loci
  - DAB Standards
  - NIST SRMs 2391
  - Published PCR Primers
  - Y-Chromosome STRs
  - Population Data
  - Validation Studies
  - miniSTRs

- Supplemental Info
  - Reference List
  - Technology Review
  - Addresses for Scientists
  - Links to Other Web Sites
  - DNA Quantitation
  - mtDNA
  - New STRs

- New information is added regularly…

Thank you for your attention…
Funding from the National Institute of Justice (NIJ) through NIST Office of Law Enforcement Standards

http://www.cstl.nist.gov/biotech/strbase
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301-975-4049

miniSTR Collaborators
- Bruce McCord (FIU)
- Mike Coble (AFDIL)

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**Topics and Techniques for Forensic DNA Analysis**

**miniSTRs**

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**Current Areas of NIST Effort with Forensic DNA**

- **Standards**
  - Standard Reference Materials
  - Standard Information Resources (STRBase website)
  - Interlaboratory Studies
- **Technology**
  - Research programs in SNPs, miniSTRs, Y-STRs, mtDNA, qPCR
  - Assay and software development, expert system review
- **Training Materials**
  - Review articles and workshops on STRs, CE, validation
  - PowerPoint and pdf files available for download

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

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**Technology: Research Programs**

- **miniSTRs**
- Y-chromosome STRs
- mtDNA
- SNPs
- qPCR for DNA quantitation
- DNA stability studies
- Variant allele characterization and sequencing
- Software tools
- Expert System review
- Assay development with collaborators

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**Timeline for miniSTRs**

- 1994 – FSS finds that smaller STR loci work best with burned bone and tissue from Branch Davidian fire
- 1997 – New primers developed for time-of-flight mass spectrometry to make small STR amplicons
- 2001 – Work at NIST and OhioU with CODIS STRs; BodePlexes used in WTC investigation starting 2002
- 2004 – Work at NIST with non-CODIS (NC) miniSTRs
- 2007 – Applied Biosystems releases 9plex MiniFiler

http://www.cstl.nist.gov/biotech/strbase/miniSTR/timeline.htm

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**miniSTR Overview Article**


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http://www.cstl.nist.gov/biotech/strbase/training.htm
Miniplexes improve detection of degraded DNA

Comparison of PCR Amplification Success Rates with Commercial Kit vs. miniSTR Assays

AmpFISTR® Identifiler™

miniSTR Allelic Ladders (Beta-test materials)
Summary of Samples Typed with ABI MiniFiler kit at NIST and ABI

- Primarily only population samples examined – no extensive sensitivity or degraded DNA tests were performed
- 656 NIST U.S. population samples
  - 260 Caucasian, 253 African American, 140 Hispanic, 3 Asian
  - Previously examined with Identifier; also with PowerPlex 16
  - Also tested with Butler et al. (2003) published miniSTR primers
- 481 father-son pairs
  - 184 Caucasian, 196 African American, 101 Asian samples
    - Previously examined with Identifiler
- 171 samples from Applied Biosystems
  - 1,308 samples Allele concordance = 10,437/10,464 = 99.7%

Concordance Conducted at NIST

27 Discordant Calls

656 NIST U.S. population samples

- Identifiler - 700
- ABI MiniFiler
- PowerPlex 16
- miniSTRs - 532

656 NIST U.S. population samples
- 0.26% discordance (primarily D13, D16)

481 father-son samples

- 10,464 genotype comparisons (1,308 samples x 8 loci)

Concordance Studies Reveal Potential Primer Binding Site Mutations with Different Primer Sets

- D16S539

Identification

NIST miniSTR data
Ohio U miniSTR data
AB miniSTR beta-test

Examination of D13S317 Concordance:

African American sample ZT79305

Full MiniFiler Profile for NIST Sample with D13S317 Allele Dropout

- Note the Relative D13 Peak Heights (Suggests Allele Dropout)

- A true homozygous allele is taller than other heterozygous alleles

http://www.cstl.nist.gov/biotech/strbase/training.htm
More Loci are Useful in Situations Involving Relatives

- **Missing Persons** and Disaster Victim Identification (kinship analysis)
- Immigration Testing (often limited references)
  - Recommendations for 25 STR loci
- Deficient Parentage Testing
  - Often needed if only one parent and child are tested

Relationship testing labs are being pushed to answer more difficult genetic questions...and we want to make sure the right tools are in place.

Why Go Beyond the CODIS Loci?

1. **Large Allele Ranges** (e.g. FGA)
2. "Unclean" Flanking Sequences (e.g. D7S820)

Locations of Focus for New miniSTR Loci (relative to CODIS 13 STRs)

Characterization of New miniSTR Loci

**"Computer Work"**

- Candidate STR marker selection
- Test primers for Multiplex-ability
- Order primers from commercial source

**"Laboratory Work"**

- Test polymers on Population samples
- Sequence Alleles in distinctive allele area
- Build Mazes for Genotyping
- Contrast Allele Ladders

New miniSTR Non-CODIS (NC) Loci

- 32 STR loci tested on NIST 665 U.S. population samples
- 26 STR loci with allele sizes below 140 bp and good heterozygosities (above TPOX level)
- All new STR loci are **physically unlinked** to the 13 CODIS core loci
- Submitted articles regarding primer sequences and locus characterization including population statistics
- SRM 2391b components are being certified through sequencing for D10S1248, D2S441, D22S1045; for reference purposes, genotypes for standard samples (9947A, 9948, 007, K562) will be made available on STRBase

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

http://www.cstl.nist.gov/biotech/strbase/training.htm
Initial Testing Results with Potential miniSTR Loci

26 new miniSTRs (NC01-NC09)

20 additional loci characterized across U.S. population groups

Miniplex "NC01"

PCR Product Size (bp)

D10S1248
D14S1434
D22S1045
D2SS1045
D4S1434
EFAM (blue)
VIC (green)
NED (yellow)

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

“Autoplex” (26plex)

See Hill et al. AAFS 2006 talk (Washington, DC) and poster PP50 at DNA in Forensics 2006 meeting (Ancona)

European Labs Have Adopted the NIST-Developed NC miniSTRs

F31 (2006) 156(2): 242-244

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

- Reduced size amplicons improve success rates with degraded DNA or samples possessing PCR-inhibitors – European leaders view miniSTRs as “the way forward”

- MiniFiler concordance testing performed

- New miniSTR loci are being characterized at NIST – 26 loci developed

Thank you for your attention…

Funding from the National Institute of Justice (NIJ) through NIST Office of Law Enforcement Standards

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

john.butler@nist.gov

301-975-4049

Margaret Kline
Becky Hill

http://www.cstl.nist.gov/biotech/strbase/training.htm
Topics and Techniques for Forensic DNA Analysis

**Capillary Electrophoresis Fundamentals and Troubleshooting**

Dr. John M. Butler
National Institute of Standards and Technology
john.butler@nist.gov

**Florida Statewide Training Meeting**
Indian Rocks Beach, FL
May 12-13, 2008

**Questions?**

- What are your biggest challenges with keeping your ABI 310/3100/3130xl running?
- What kind of signal intensity variation are you seeing between your different instruments?
- Have anyone seen uneven injection across a sample plate? (We believe this to be an autosampler calibration issue...e.g., position G10 or H12 does not inject properly)

**Planned Promega 2008 Meeting**

**Troubleshooting Workshop**

- **Title:** “Principles of Interpretation and Troubleshooting of Forensic DNA Typing Systems”
- **Instructors:** John Butler (NIST) and Bruce McCord (FIU)
- **Date:** October 16, 2008 with Promega Int. Symp. Human ID

The workshop will consist of three parts:

1. a through examination of theoretical issues with capillary electrophoresis PCR amplification of short tandem repeat markers
2. a discussion of how to properly set instrument parameters to interpret data (including mixtures), and
3. a review of specific problems seen by labs submitting problematic data and commentary on possible troubleshooting solutions.

**Identifiler Allelic Ladder**
March 14, 2007

**Identifiler Allelic Ladder**
March 23, 2007

http://www.cstl.nist.gov/biotech/strbase/training.htm
Examination of Resolution in TH01 Region

Examine the Size Standard...

The Size Standard Provides an Excellent Indicator of Performance on Every Sample

Genotype Results with Profiler Plus™ kit

Review Article on STRs and CE

Genotyping

PCR Product Size (bp)

PCR Product Size (bp)

Relative Fluorescence Units

PCR Product Size (bp)
Analytical Requirements for STR Typing


- Fluorescent dyes must be spectrally resolved in order to distinguish different dye labels on PCR products
- PCR products must be spectrally resolved – desirable to have single base resolution out to >350 bp in order to distinguish variant alleles
- High run-to-run precision – an internal sizing standard is used to calibrate each run in order to compare data over time

Detection with Multiple Capillaries

(Irradiation for Capillary Arrays)

Process Involved in 310/3100 Analysis

- Separation
  - Capillary – 50um fused silica, 43 cm length (36 cm to detector)
  - POP-4 polymer – Polydimethyl acrylamide
  - Buffer - TAPS pH 8.0
  - Denaturants – urea, pyrolidinone
- Injection
  - electrokinetic injection process (formamide, water)
  - importance of sample stacking
- Detection
  - fluorescent dyes with excitation and emission traits
  - CCD with defined virtual filters produced by assigning certain pixels

Ohm’s Law

- \( V = IR \) (where \( V \) is voltage, \( I \) is current, and \( R \) is resistance)
- Current, or the flow of ions, is what matters most in electrophoresis
- CE currents are much lower than gels because of a higher resistance in the narrow capillary
- CE can run a higher voltage because the capillary offers a higher surface area-to-volume ratio and can thus dissipate heat better from the ion flow (current)

Separation Issues

- Electrophoresis buffer –
  - Urea for denaturing and viscosity
  - Buffer for consistent pH
  - Pyrolidinone for denaturing DNA
  - EDTA for stability and chelating metals
- Polymer solution -- POP-4 (but others work also)
- Capillary wall coating – dynamic coating with polymer
  - Wall charges are masked by methyl acrylamide
- Run temperature -- 60 °C helps reduce secondary structure on DNA and improves precision. (Temperature control affects DNA sizing)
Why TAPS instead of Tris-borate (TBE) buffer?

- TBE is temperature/pH sensitive
  - as temperature increases, the pH decreases (0.02 pH units with every 1 °C); this is the principle by which TaqGold activation works
- At lower pH, fluorescence emission of dyes decreases
- Thus when running at 60 °C on the ABI 310, if Tris-borate was used, fluorescent intensity of PCR products would be lower

How to Improve Resolution?

1. Lower Field Strength
2. Increase Capillary Length
3. Increase Polymer Concentration
4. Increase Polymer Length

All of these come at a cost of longer separation run times

Sample Conductivity Impacts Amount Injected

\[
\left[\text{DNA}_{\text{inj}}\right] = \frac{E(t \pi r^2) (\mu_{\text{ep}} + \mu_{\text{eof}})[\text{DNA}_{\text{sample}}] (\lambda_{\text{buffer}})}{\lambda_{\text{sample}}}
\]

- \([\text{DNA}_{\text{inj}}]\) is the amount of sample injected
- \(E\) is the electric field applied
- \(t\) is the injection time
- \(r\) is the radius of the capillary
- \(\mu_{\text{ep}}\) is the mobility of the sample molecules
- \(\mu_{\text{eof}}\) is the electroosmotic mobility
- \([\text{DNA}_{\text{sample}}]\) is the concentration of DNA in the sample
- \(\lambda_{\text{buffer}}\) is the buffer conductivity
- \(\lambda_{\text{sample}}\) is the sample conductivity

Salty samples result in poor injections
Two Major Effects of Sample Stacking
1. Sample is preconcentrated. Effect is inversely proportional to ionic strength
2. Sample is focused. Ions stop moving in low electric field
3. Mobility of sample = \( \mu_{ep} = \frac{v}{E} \) with electric field

Steps Performed in Standard Module

- **Capillary fill** – polymer solution is forced into the capillary by applying a force to the syringe
- **Pre-electrophoresis** – the separation voltage is raised to 10,000 volts and run for 5 minutes.
- **Water wash of capillary** – capillary is dipped several times in deionized water to remove buffer salts that would interfere with the injection process.
- **Sample injection** – the autosampler moves to position A1 (or the next sample in the sample set) and is moved up onto the capillary to perform the injection; a voltage is applied to the sample and a few nanoliters of sample are pulled onto the end of the capillary; the default injection is 15 kV (kilovolts) for 5 seconds.
- **Water wash of capillary** – capillary is dipped several times in waste water to remove any contaminating solution adhering to the outside of the capillary.
- **Electrophoresis** – autosampler moves to inlet buffer vial (position 1) and separation voltage is applied across the capillary; the injected DNA molecules begin separating through the POP-4 polymer solution.
- **Detection** – data collection begins; raw data is collected with no spectral deconvolution of the different dye colors; the matrix is applied during Genescan analysis.

Comments on Sample Preparation

- **Use high quality formamide (<100 \( \mu \)S/cm)!**
  - ABI sells Hi-Di formamide
  - Regular formamide can be made more pure with ion exchange resin
- **Denaturation with heating and snap cooling**
  - Use a thermal cycler for heating and cold aluminum block for snap cooling
  - Heat/cool denaturation step is necessary only if water is substituted for formamide...

January 6, 2005 Letter from Applied Biosystems to ABI 310 Customers

- "Testing has shown that Hi-Di Formamide denatures DNA **without the need to heat samples**…"
- In other words, no heat denaturation and snap cooling needed!

Detection Issues

- **Fluorescent dyes**
  - Spectral emission overlap
  - Relative levels on primers used to label PCR products
  - Dye “blobs” (free dye)
- **Virtual filters**
  - Hardware (CCD camera)
  - Software (color matrix)

Filters determine which wavelengths of light are collected onto the CCD camera
Fluorescent Labeling of PCR Products

- Dyes are attached to one primer in a pair used to amplify a STR marker.
- Dyes are coupled to oligonucleotides (primers) through NHS-esters and amine linkages on the 5’ end of the primer: Dye-(CH₂)₆-primer
- Dye-labeled oligonucleotides are incorporated during multiplex PCR amplification giving a specific color “tag” to each PCR product.
- PCR products are distinguished using CCD imaging on the 310.

Amine Reactive Dyes used in Labeling DNA

- FAM (Blue)
- JOE (Green)
- TAMRA (Yellow)
- ROX (Red)

The succinimidyl ester reacts rapidly with amine linkers on DNA bases.

Virtual Filters Used in ABI 310

- Wavelength range: 500 nm to 700 nm
- Blue, Green, Yellow, Red, Orange
- Commonly used fluorescent dyes:
  - FAM
  - JOE
  - TAMRA
  - ROX
  - LIZ

Filter sets determine what regions of the CCD camera are activated and therefore what portion of the visible light spectrum is collected.

Fluorescent Emission Spectra for ABI Dyes

- 5-FAM
- JOE
- NED
- ROX
- NED is a brighter dye than TAMRA

Laser excitation (488, 514.5 nm)

Please Note!

- There are no filters in a 310.
- It’s just the choice of pixels in the CCD detector.
- All the light from the grating is collected.
- You just turn some pixels on and some off.

Deciphering Artifacts from the True Alleles

- Biological (PCR) artifacts:
  - Incomplete adenylation
- STR alleles:
  - Dye blob
  - Stutter
  - Pull-up (bleed-through)
  - Spike
  - Blue channel
  - Green channel
  - Yellow channel
  - Red channel

http://www.cstl.nist.gov/biotech/strbase/training.htm
Dye Blobs (“Artifacts”)

- Free dye (not coupled to primer) can be injected into the CE capillary and interfere with detection of true STR alleles
- **Dye blobs are wider and usually of less intensity** than true STR alleles (amount depends on the purity of the primers used)
- Dye blobs usually appear at an apparent size that is unique for each dye (e.g., FAM ~120 bp, PET ~100 bp)

**Poor primer purity**

Dye Blob Problems with Some PCR Primers

<table>
<thead>
<tr>
<th>Individual Y-STR Locus Amplifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR product size (bp)</td>
</tr>
<tr>
<td>90</td>
</tr>
<tr>
<td>DYS392</td>
</tr>
<tr>
<td>HEX</td>
</tr>
</tbody>
</table>

Conclusions

DNA typing by capillary electrophoresis involves:

1) The use of entangled polymer buffers
2) Injection by sample stacking
3) Multichannel laser induced fluorescence
4) Internal and external calibration

Practical Aspects of ABI 310/3100 Use

- ABI 310 x 2 (originally with Mac, then NT)
  - 1st was purchased in 1996
  - 2nd was purchased in June 2002

- ABI 3100 (Data collection v1.0.1)
  - Purchased in June 2002
  - Original data collection software retained

- ABI 3130xl upgrade (Data collection v3.0)
  - Purchased in April 2001 as ABI 3100
  - Upgraded to ABI 3130xl in September 2005
  - Located in a different room

http://www.cstl.nist.gov/biotech/strbase/training.htm
Our Use of the ABI 3100

- Data collection software, version 1.0.1
- **POP-6** with 36 cm capillary array
- STR kits and in-house assays for autosomal STRs, Y-STRs, and miniSTRs
- SNaPshot assays for mtDNA SNPs, Y-SNPs, and autosomal SNPs
- DNA sequencing for mtDNA and STR repeat sequencing

We can routinely get more than 400 runs per capillary array by not changing the polymer between applications.

Comparison of ABI 3100 Data Collection Versions

Same DNA sample run with Identifiler STR kit (identical genotypes obtained)

**ABI 3100** (36 cm array, **POP-6**)
- Data Collection v1.0.1
- 5s@2kV injection

**ABI 3130xl** (50 cm array, **POP-7**)
- Data Collection v3.0
- 5s@2kV injection

Relative peak height differences are due to "variable binning" with newer ABI data collection versions.

Difference in the STR allele relative mobilities (peak positions) are from using **POP-6** vs. **POP-7**.

Consumables for ABI 310/3100

**What we use at NIST**
- A.C.E.™ Sequencing Buffer 10X (Amresco)
  - $155/L = $0.0155/mL 1X buffer (costs 20 times less!)
  - http://www.amresco-inc.com
- 3700 **POP-6 Polymer** (Applied Biosystems)
  - $530 / 200 mL = $2.65/mL (costs 20 times less!)

**What ABI protocols suggest**
- 10X Genetic Analyzer Buffer with EDTA
  - $78/25 mL = $0.312/mL 1X buffer (ABI)
- 3100 POP-4 Polymer
  - $365 / 7 mL = $52/mL (2004 prices)

Maintenance of ABI 310/3100/3130

- Syringe – leaks cause capillary to not fill properly
- Capillary storage & wash – it dries, it dies!
- Pump block – cleaning helps insure good fill
- Change the running buffer regularly

YOU MUST BE CLEAN AROUND A CE!

Overall Thoughts on the ABI 310/3100/3130

- Settling on a common instrument platform has been good for the forensic DNA community in terms of data consistency (this is also true with the use of common STR kits)
- I am concerned that the community is very dependent primarily on one company…
- I really like using the instrument and can usually get nice data from it
- Like any instrument, it has its quirks…

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

- Room temperature
  - Variations in room temperature can cause mobility shifts with band shifts and loss of calibration
  - Temperature is also important due to effects of high humidity on electrical conductance

- Cleanliness
  - Urea left in sample block can crystallize and catalyze further crystal formation causing spikes, clogs and other problems.
  - Best bet is to keep polymer in system and not remove or change block until polymer is used up.

Effect of Temperature on allele size

Slope is 0.14 bases/degree centigrade
Therefore a small change in temperature has a big effect
(A 1-2 degree shift in temperature of the heat plate can produce an OL allele)
Temperature Effects: "OL" Alleles

"OL alleles" - look at the 250 peak

And the 250 peak...

Monitoring Room Temperature Over Time

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

Refrigerator and freezer monitoring

Probes

Room temperature monitoring

Temperature Probes

Frig/Freeze Monitors $240
#DT-23-33-80 – USB Temperature Datalogger
PLUS Software $79.00 (#DT-23-33-80)
Room Monitors, # DT-23018-62 – USB Temperature-Humidity Datalogger $91.00
(Cole Parmer, Vernon Hills IL)

Poor Temperature Control Causes DNA Sizing Imprecision

Ladder Overlay, 6FAM
Comb1, 3130xl

Use of Second Allelic Ladder to Monitor Potential Match Criteria Problems

1st Injection (standard for typing)

15th Injection (treated as a sample)

These alleles have drifted outside of their genotyping bins due to temperature shifting over the course of the sample batch

Cleanliness

- Urea sublimates and breaks down to ionic components - these find a path to ground
- Similarly wet buffer under a vial creates paths to ground
- Capillary windows must be clear or matrix effects will occur
- Laser will often assist in this process
- Vial caps will transfer low levels of DNA to capillary

Carbon Trails

High Humidity or wet buffer vials can create other paths to ground

Keep Your System Clean!

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

- Optical System
  - Sensitivity changes with age, capillary diameter, capillary cleanliness, instrument calibration

- Fluidic System
  - Effects of bubbles, dust, urea crystals, leaks in syringe and capillary ferrule

- Matrix Calculations
  - Changes in buffer, optics, sample dye can alter the software calibrations

- Capillary Problems
  - Chemisorbed materials on capillary surface can produce osmotic flow, DNA band broadening and inconsistent resolution (meltdowns)

The Detection Window

- Make sure that the capillary window is lined up (if it is not, then no peaks will be seen)
- Window may need to be cleaned with ethanol or methanol

Beware of Urea Crystals

- Urea crystals have formed due to a small leak where the capillary comes into the pump block
- Urea sublimates and can evaporate to appear elsewhere
- Use a small balloon to better grip the ferrule and keep it tight

Storage when ABI 310 is not in use

- Keep inlet of capillary in water...if it dries out then urea crystals from the polymer will clog the opening
- The waste vial (normally in position 3) can be moved into position
- A special device can be purchased from Supelco to rinse the capillary off-line
- Store in distilled water
- Note that the laser is on when the instrument is on

Remember that the water in the open tube will evaporate over time...

Buffer Issues

- The buffer and polymer affect the background fluorescence- affecting the matrix
- Urea crystals and dust may produce spikes
- High salt concentrations may produce reannealing of DNA
- High salt concentrations affect current
- Low polymer concentrations affect peak resolution
Capillary Meltdowns

(A) Good resolution
(B) Poor resolution

Meltdowns can be permanent or transitory
as we have seen these may result from sample contamination effects

Does the capillary need to be replaced?

No! The next injection looks fine...

Meltdowns may be the result of

- Bad formamide
- Excess salt in sample/renaturation
- Water in the polymer buffer
- **Syringe leak** or bottom out
- Poisoned capillary
- Conductive polymer buffer due to urea degradation
- Crack/shift in capillary window
- Detergents and metal ions

5. Troubleshooting benchmarks

- Monitor run current
- Observe syringe position and movement during a batch
- Examine ILS (ROX) peak height with no sample
- Observe “250 bp” peak in GS500 size standard
- Monitor resolution of TH01 9.3/10 in allelic ladder and size standard peak shapes
- Keep an eye on the baseline signal/noise
- Measure formamide conductivity
- Reagent blank – are any dye blobs present?
- See if positive control DNA is producing typical peak heights (along with the correct genotype)

Measurement of Current

- \( V/I = R \) where \( R \) is a function of capillary diameter, [buffer], and buffer viscosity

- In a CE system the voltage is fixed, thus changes in resistance in the capillary will be reflected in the current observed

- Air bubbles, syringe leaks, alternate paths to ground, changes in temperature, changes in zeta potential, and contamination, will be reflected in the current

- A typical current for a CE system with POP4 buffer is 8-12 \( \mu A \) (microamps)

Syringe Travel

- The ABI 310 instrument also keeps track of the position of the syringe (in the log file)

- Depending on the resistance to flow, the syringe will travel different lengths

- Syringe leaks may be reflected in a longer distance traveled prior to each injection

- These leaks occur around the barrel of the syringe and at the connection to the capillary block
Use of ABI 310 Log File to Monitor Current and Syringe Travel

Syringe Position

Current

Dye Blobs in the Negative Control Sample

Measuring Formamide Conductivity

(not this way)

The key is to measure the bottle when it comes in or buy the good stuff and immediately pipette it out into small tubes with or without ROX already added. Then freeze the tubes.

Do not ever open a cold bottle of formamide. Water will condense inside and aid in the formation of conductive formic acid.

Conclusion:
Troubleshooting is more than following the protocols

It means keeping watch on all aspects of the operation
1. Monitoring conductivity of sample and formamide
2. Keeping track of current and syringe position in log.
3. Watching the laser current
4. Watching and listening for voltage spikes
5. Monitoring room temperature and humidity

Multiplex_QA Article Published

Multiplex_QA: An exploratory quality assessment tool for multiplexed electrophoretic assays

Multiplex_QA is a data analysis tool for monitoring and long-term changes in the performance of multiplexed electrophoretic assays, particularly the commercial short tandem repeats (STR) kits used by the forensic science community. A suite of tools is included to monitor and automate data analysis and visualization.Multiplex_QA uses Microsoft Excel for data analysis and visualization. It provides numerical reports that are easy to read and understand, and it can be used to examine and improve the performance of existing and new multiplexed STR kits.

Multiplex_QA Overview

- Research tool that provides quality metrics to review instrument performance over time (e.g., examines resolution and sensitivity using internal size standard peaks)
- Runs with Microsoft Excel macros. Requires STR data to be converted with NCBI's BatchExtract program into numerical form.

Available for download from STRBase:
http://www.cstl.nist.gov/biotech/strbase/software.htm

User manual (127 pages) available for download from STRBase
Acknowledgments

Pete Vallone
John Butler (Leader)
Margaret Kline
Amy Decker
Becky Hill
Dave Duewer
Jan Redman

Funding from interagency agreement 2003-IJ-R-029 between the National Institute of Justice and the NIST Office of Law Enforcement Standards

NIST Human Identity Project Team

Many wonderful collaborators from industry, university, and government laboratories.

Bruce McCord (Florida International University) for many of the slides

Thank you for your attention...

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

john.butler@nist.gov

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

See also http://www.dna.gov/research/nist

Questions?
Mixture Interpretation Questions  

Homework for Monday Night

Name (leave blank if you want to be anonymous): ___________________ Email address: ___________________

Interpretation Guidelines

What would you like to see in national guidelines on how to perform DNA mixture interpretation and statistical analysis?

How does your lab handle reference samples during interpretation of evidence? Do you try to solve the mixture entirely without looking at either victim or suspect profiles?

What kind of pre-case assessment do you perform when approaching a case where a possible mixture is involved?

Does your lab attempt statistics on a minor component? If so, what types of statistics are used?

Do you have a decision point whereby you consider a mixture too complicated and do not try to solve it? How do you know when to stop in terms of mixture interpretation?

Are composite profiles acceptable – e.g., high injection for minor component and low injection for major component allele identification?

How do you report mixture statistics in court?

Would a flowchart for mixture interpretation be helpful?
Validation and Training

For your lab validation studies of a new STR kit or instrument, how many mixtures should be evaluated? How do you decide on what combination of alleles to include in such a study?

What kind of training materials would be beneficial to help your laboratory more effectively solve mixtures?

Suggestions for training staff to have more analyst consistency within your lab:

Other Topics

What percentage of time is spent in a case trying to deduce the mixture components?

Have you seen performance differences between various STR typing kits that would impact mixture interpretation?

Is your lab using Y-STRs to help with mixtures?

What kinds of software features would be valuable to aid mixture interpretation?

What are the biggest obstacles you face in your lab in terms of mixture interpretation?
Mixture Interpretation Discussion

Florida Statewide Training Meeting
Indian Rocks Beach, FL
May 12-13, 2008

Dr. John M. Butler
National Institute of Standards and Technology
john.butler@nist.gov

Mixture Interpretation Discussion

NIST and NIJ Disclaimer

Funding: Interagency Agreement 2003-IJ-R-029 between the National Institute of Justice and NIST Office of Law Enforcement Standards

Points of view are mine and do not necessarily represent the official position or policies of the US Department of Justice or the National Institute of Standards and Technology.

Certain commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose.

SWGDAM Disclaimer...

Training Information Available on STRBase
http://www.cstl.nist.gov/biotech/strbase/training.htm

AAFS 2008 Workshop Presenters

Ann Marie Gross  
MN BCA

John M. Butler  
NIST

George Carmody  
Carleton University/Statistical Consultant

Gary Shutler  
Wash State Police Crime Lab

Angie Dolph  
Marshall University (NIST Summer Intern)

Joanne B. Sgueglia  
Mass State Police Crime Lab

Tim Kalafut  
US Army Crime Lab

Purpose for Teaching AAFS Workshop

We hope that participants:

• Gain a better understanding of the current approaches being used throughout the community for mixture interpretation

• See worked examples of mixture component deconvolution and statistical analysis

• Come away with ideas to improve your laboratory’s interpretation guidelines and training regarding mixtures in forensic casework

AAFS Workshop Morning Agenda - Theory

Background and Introductory Information
8:30 a.m. – 9:00 a.m. – John Butler

Survey Results on Numbers and Types of Casework Mixtures
9:00 a.m. – 9:15 a.m. – Ann Gross

Principles in Mixture Interpretation
9:15 a.m. – 10:15 a.m. – John Butler

10:15 a.m. – 10:30 a.m. BREAK

Strategies for Mixture Deconvolution with Worked Examples
10:30 a.m. – 11:30 a.m. – John Butler

Different Approaches to Statistical Analysis of Mixtures
11:30 a.m. – 12:00 p.m. – George Carmody

12:00 p.m. – 1:15 p.m. LUNCH

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


- Mixtures arise when two or more individuals contribute to the sample being tested.
- Mixtures can be challenging to detect and interpret without extensive experience and careful training. Even more challenging with poor quality data when degraded DNA is present.
- Differential extraction can help distinguish male and female components of many sexual assault mixtures. Y-chromosome markers can help here in some cases.

Sources of DNA Mixtures

- Two (or more) individuals contribute to the biological evidence examined in a forensic case (e.g., sexual assault with victim and perpetrator or victim, consensual sexual partner, and perp).

Example Mixture Data (MIX05 Study-Profiler Plus)

<table>
<thead>
<tr>
<th>Loci</th>
<th>Mix05 Case #1, Profiler Plus green loci</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>12,12</td>
</tr>
<tr>
<td>X</td>
<td>28,31.2</td>
</tr>
<tr>
<td>T</td>
<td>15,16</td>
</tr>
<tr>
<td>Amelogenin</td>
<td>D8S1179  D21S11  D18S51</td>
</tr>
<tr>
<td>Victim</td>
<td>major</td>
</tr>
<tr>
<td>Perpetrator</td>
<td>minor</td>
</tr>
</tbody>
</table>

More on Mixtures...

Ann Gross will discuss some recent collected casework summaries.

Recent Mixture Workshops

Conducted by John Butler

- Southern Association of Forensic Scientists (SAFS)
  - September 11, 2007 (Atlanta, GA)
  - Mixture Interpretation (theory)
  - Along with Software discussion (Rhonda Roby) and demonstration (Tom Overson/Tim Kalafut)
  - 33 attendees from 13 different labs

- Northeastern Association of Forensic Scientists (NEAFS)
  - November 2-3, 2007 (Bolton Landing, NY)
  - The Cutting Edge of DNA Testing: Mixture Interpretation, miniSTRs, and Low Level DNA
  - 42 attendees from 13 different labs

NEAFS Workshop materials (75 pages) available on STRBase:

Helpful feedback obtained from workshop participants.
Mixtures: Issues and Challenges

- The probability that a mixture will be detected improves with the use of more loci and generic markers that have a high incidence of heterozygotes.
- The detectability of multiple DNA sources in a single sample relates to the ratio of DNA present from each source, the specific combinations of genotypes, and the total amount of DNA amplified.
- Some mixtures will not be as easily detectable as other mixtures.

Detecting Mixtures
- Review and compile information from the entire profile – don’t just focus on a single locus!
- Tri-allelic patterns exist in single source samples
  - 145 different tri-alleles recorded for the 13 core CODIS loci on STRBase as of Jan 22, 2008
    - CSF1PO (5), FGA (22), TH01 (1), TPOX (15), VWA (18), D3S1358 (6), D5S818 (4), D7S820 (7), D8S1179 (11), D13S317 (8), D16S539 (8), D18S51 (21), D21S11 (19)
  - A mixture often declared when >2 peaks in ≥2 loci

Mixtures: Issues and Challenges
- Artifacts of PCR amplification such as stutter products and heterozygote peak imbalance complicate mixture interpretation
- Thus, only a limited range of mixture component ratios can be solved routinely

Responses to Questions from a Previous Mixture Workshop (Fall 2007)
What are the biggest obstacles you face in your lab in terms of mixture interpretation?
- Trying to be consistent in my interpretation and with coworkers
- Consistency between analysts
- No consistency – based on analysts discretion/experience; due to lack of consistent training
- Vague SOP leading to inconsistency between analysts due to differences in how “conservative” or not each analyst is
- There is a lot of “individual interpretation” in our lab
- Varying opinions between interpreting analysts due to lack of uniform guidelines
- Resistance to change from other analysts/ supervisors
- Getting management to commit to guidelines that will be followed by everyone

DNA Mixture Interpretation: Principles and Practice in Component Deconvolution and Statistical Analysis

Numbers and Types of Casework Mixtures

Handouts available on STRBase at http://www.cstl.nist.gov/biotech/strbase/training/AAFS2008_MixtureWorkshop.htm

AAFS 2008 Workshop #16
Washington, DC
February 19, 2008
Ann Marie Gross
ann.gross@state.mn.us
Mixtures......

- How often are mixtures obtained
- What types of mixtures are we seeing
  - Where should we focus our attention for training
  - What info can we give to the forensic community regarding mixtures
- What types of samples most often yield mixtures

Torres et al. 4 year Spanish study

- Four year study (1/1997 to 12/2000)
- 2412 samples typed
  - 955 samples from sexual assaults
  - 1408 samples from other offenses
  - 49 samples from human remains identifications
- 163/2412 samples (6.7% showed mixed profile)

Spreadsheet Information Requested

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

Labs requested to also provide info on kit, PCR volume used, etc.

- Case#
- Item#
- Type of sample (biological material if ID'd)
- Type of substrate
- Quantity amp'd
- Minimum # of contributors (1, 2, 3, 4, or >4)
- Predominant type (major profile) determined?
- Stats reported
- Comments

We would love to have your lab mixture numbers...
Email information to Ann.Gross@state.mn.us

12 Labs Submitted Data
(prior to AAFS meeting)

- Palm Beach Sheriff's Office Crime Lab, Florida
- Centre for Forensic Science, Toronto
- Connecticut State Police
- Washington State Police
- New Jersey State Police
- Georgia Bureau of Investigation
- Royal Canadian Mounted Police, Ottawa
- USACIL, Georgia
- Michigan State Police
- Kern County Crime Lab, California
- CAL DOJ
- Minnesota Bureau of Criminal Apprehension

We would still like to collect more case summary data...

All Laboratory Data Combined

<table>
<thead>
<tr>
<th># contributors</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>&gt;4</th>
</tr>
</thead>
<tbody>
<tr>
<td>N = 3106</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sexual Assault</td>
<td>N = 1408</td>
<td>51%</td>
<td>40%</td>
<td>8%</td>
<td>--</td>
</tr>
<tr>
<td>Major Crime</td>
<td>N = 1388</td>
<td>66%</td>
<td>24%</td>
<td>8%</td>
<td>2%</td>
</tr>
<tr>
<td>High Volume</td>
<td>N = 310</td>
<td>43%</td>
<td>37%</td>
<td>19%</td>
<td>1%</td>
</tr>
</tbody>
</table>

Overall Summary – 3106 samples

- 57% of samples from all types of cases are single source
- 43% of samples from all types of cases are mixtures
  - 33% of mixtures of at least two contributors
  - 9% of mixtures of at least three contributors
  - 1% of mixtures of at least four contributors

Focus in training materials will be on two-person mixtures as they presently predominate
Principles of Mixture Interpretation

SWGDAM Mixture Interpretation Subcommittee
- John Butler (NIST) - chair
- Gary Sims (CA DOJ) - co-chair
- Mike Adamowicz (CT)
- Jack Ballantyne (UCF/NCFS)
- George Carmody (Carleton U)
- Cecelia Crouse (PBSO)
- Allison Eastman (NYSP)
- Roger Frappier (CFS-Toronto)
- Ann Gross (MN BCA)
- Phil Kinsey (MT)
- Jeff Modler (RCMP)
- Gary Shutler (WSP)

Started in January 2007

Progress and Plans for Mixture Committee
- Guidelines in process of being discussed and written
- Collecting data on number and type of mixture cases observed in various labs
- Plan to create a training workbook with worked examples
- Considering flow charts to aid mixture interpretation
- Have discussed responses to ISFG Recommendations

I invite your input as to what should be included in the guidelines…

Your HOMEWORK…

Elements of DNA Mixture Interpretation

- Principles (theory)
- Protocols (validation)

We discussed and would advocate periodic training to aid accuracy and efficiency within your laboratory.

ISFG Recommendations on Mixture Interpretation

Our discussions have highlighted a significant need for continuing education and research into this area.

DNA commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures
P. Gill, J. B. Becker, J. A. Lucchese, L. M. Kozokar, W. B. May, R. M. Parson, B. S. West

Abstract
- We discussed and would advocate periodic training to aid accuracy and efficiency within your laboratory.

http://www.cstl.nist.gov/biotech/strbase/training.htm
Who is the ISFG and why do their recommendations matter?

International Society of Forensic Genetics
http://www.isfg.org/

- An international organization responsible for the promotion of scientific knowledge in the field of genetic markers analyzed with forensic purposes.
- Founded in 1968 and represents more than 1100 members from over 60 countries.
- A DNA Commission regularly offers recommendations on forensic genetic analysis.

DNA Commission of the ISFG
- DNA polymorphisms (1989)
- PCR based polymorphisms (1992)
- Naming variant alleles (1994)
- Repeat nomenclature (1997)
- Mitochondrial DNA (2000)
- Y-STR use in forensic analysis (2001)
- Mixture Interpretation (2006)
- Disaster Victim Identification (2007)

http://www.isfg.org/Publications/DNA+Commission

ISFG Executive Committee
- President: Niels Morling (Copenhagen, Denmark)
- Vice-President: Peter Schneider (Köl, Germany)
- Working Party Representative: Mecki Prinz (New York City, USA)
- Treasurer: Leonor Gusmão (Porto, Portugal)
- Secretary: Wolfgang Mayr (Vienna, Austria)

Authors of ISFG Mixture Article
- Peter Gill: Pioneer of forensic DNA techniques and applications
- John Buckleton: University of Strathclyde (Apr 2008 – present)
- Michael Krawczak: Christian-Albrechts-University, Kiel, Germany
- Bruce Weir: U. Washington, Seattle, USA

The Statisticians
- Charles Bremer
- John Buckleton
- Michael Krawczak
- Bruce Weir

My perspective…

Hierarchy of Rules for Forensic DNA Labs

- United States
  - FBI (DAB) Quality Assurance Standards
  - NDIS Procedures
  - SWGDAM Guidelines
- Europe
  - ENFSI Policies
  - ISFG Recommendations (DNA Commission)
  - National Recommendations

- Audits
  - Laboratory Protocols (SOPs)
  - Individual Analyst Practice
  - Each Case Report
- Training & Experience
  - Laboratory Protocols (SOPs)
  - Individual Analyst Practice
  - Each Case Report

Hopefully each conforms to the levels above it…
UK Response to ISFG Mixture Recommendations

Gil, P., et al. (2008) National recommendations of the technical UK DNA working group on mixture interpretation for the NDNAD and for court going purposes. FSI Genetics 2(1): 76–82

Using the published UK response as a model, let us review the nine ISFG Recommendations on mixture interpretation...

From Report to the Virginia Scientific Advisory Committee by the DNA Subcommittee – Addendum January 8, 2008 (authored by Dr. Norah Rudin and Dr. Artie Eisenberg)

- "Among the many reasons that Forensic DNA analysis has become the gold standard for forensic science is the relatively discrete nature of the data. For strong, single source samples, a profile can readily be determined, and is subject to little or no analyst judgment. However, ambiguity may arise when interpreting more complex samples, such as those containing multiple contributors, of poor quality (e.g. degraded or inhibited DNA), of low quantity (e.g. contact samples), or various combinations of these challenging situations..."

http://www.dfs.virginia.gov/about/minutes/saCommittee/20080108.pdf

From Report to the Virginia Scientific Advisory Committee by the DNA Subcommittee – Addendum January 8, 2008 (authored by Dr. Norah Rudin and Dr. Artie Eisenberg)

- "...These kinds of samples are encountered with increasing frequency, as the sensitivity of the technology has increased, and as law enforcement has become more sophisticated about the kinds of samples they submit for analysis. Difficult samples are also frequently encountered when reanalyzing historical cases, in which samples were not collected and preserved using the precautions necessary for DNA analysis..."

http://www.dfs.virginia.gov/about/minutes/saCommittee/20080108.pdf

"Cold cases" or Innocence Project samples...

http://www.dfs.virginia.gov/about/minutes/saCommittee/20080108.pdf

From Report to the Virginia Scientific Advisory Committee by the DNA Subcommittee – Addendum January 8, 2008 (authored by Dr. Norah Rudin and Dr. Artie Eisenberg)

- "It is for these types of challenging samples, where the evidence profile may not exactly "match" a reference profile, that confirmation bias becomes a concern. The interpretation of an evidentiary DNA profile should not be influenced by information about a subject's DNA profile. Each item of evidence must be interpreted independently of other items of evidence or reference samples. Yet forensic analysts are commonly aware of submitted reference profiles when interpreting DNA test results, creating the opportunity for confirmatory bias, despite the best intentions of the analyst..."

http://www.dfs.virginia.gov/about/minutes/saCommittee/20080108.pdf

Two Parts to Mixture Interpretation

- Determination of alleles present in the evidence and deconvolution of mixture components where possible
  - Many times through comparison to victim and suspect profiles

- Providing some kind of statistical answer regarding the weight of the evidence
  - There are multiple approaches and philosophies

Software tools can help with one or both of these...
Status of Software for Mixture Interpretation

- **NIJ Expert System Testbed (NEST) Project**
  - Evaluating software programs for DNA analysis of single-source (Phase I) and mixtures (Phase II)
  - [http://forensics.marshall.edu/NEST/NEST-Intro.html](http://forensics.marshall.edu/NEST/NEST-Intro.html)

- **US Army Crime Laboratory (USACIL)**
  - Commonly deal with complex sexual assaults
  - Developed software for aiding mixture interpretation and statistical analysis

---

**Steps in the Interpretation of Mixtures**

1. **Identify the Presence of a Mixture**
2. **Designate Allele Peaks**
3. **Identify the Number of Potential Contributors**
4. **Estimate the Relative Ratio of the Individuals Contributing to the Mixture**
5. **Consider All Possible Genotype Combinations**
6. **Compare Reference Samples**

**Mixture Classification Scheme**

(German Stain Commission, 2006):

- **Type A**: no obvious major contributor, no evidence of stochastic effects
- **Type B**: clearly distinguishable major and minor contributors; consistent peak height ratios of approximately 4:1 (major to minor component) for all heterozygous systems, no stochastic effects
- **Type C**: mixtures without major contributor(s), evidence for stochastic effects

---

**Biostatistical approaches**

- Calculation of the **probability of exclusion** for a randomly selected stain donor* [P(E)]
  (*RMNE - "random man not excluded")
- Calculation of the **likelihood ratio** [LR] based on defined hypotheses for the origin of the mixed stain

---

**Type of mixture and interpretation**

- **Type A**: Mixed profile without stochastic effects, a biostatistical analysis has to be performed
- **Type B**: Profile of a major contributor can be unambiguously described and interpreted as a profile from an unmixed stain
- **Type C**: due to the complexity of the mixture, the occurrence of stochastic effects such as allele and locus drop-outs have to be expected:
  - a clear decision to include or exclude a suspect may be difficult to reach, thus a biostatistical interpretation is not appropriate.

---

**Which approach should be used?**

- If the basis for clearly defined and mutually exclusive hypotheses is given, i.e.:
  - the number of contributors to the stain can be determined,
  - unambiguous DNA profiles across all loci are observed (type A mixtures, or type B, if the person considered as "unknown" contributor is part of the minor component of the mixture), then the calculation of a likelihood ratio is appropriate.
Which approach should be used?

- If major/minor contributors cannot be identified based on unambiguous DNA profiles, or if the number of contributors cannot be determined, then the calculation of the probability of exclusion is appropriate.
- The calculation of P(E) is always possible for type A and type B mixtures.

Not acceptable …

- … is the inclusion of a genotype frequency of a non-excluded suspect into the report, if the given mixed stain does not allow a meaningful biostatistical interpretation.
  - this would lead to the wrongful impression that this genotype frequency has any evidentiary value regarding the role of the suspect as a contributor to the mixed stain in question.

Conclusions

- The likelihood ratio has a significant weight of evidence, as it relates directly to the role of the suspect in the context of the origin of the stain.
- The exclusion probability makes a general statement without relevance to the role of the suspect.
- However, this does not imply that P(E) is always more "conservative" in the sense that the weight of evidence is not as strong compared to the LR.

GEDNAP 32

Mixture interpretation exercise:
- 3 person mixture without major contributor
- Person A from group of reference samples was not excluded
- Allele frequencies for eight German database systems provided for exercise
- German-speaking GEDNAP participants invited to participate based on published recommendations

GEDNAP 32

Results:
- 22 labs submitted results (from approx. 80 German-speaking GEDNAP participants)
- Calculations submitted were all correct and consistent:
  - 15x LR approach:
    - Person A + 2 unknown vs. 3 unknown contributors
    - 11x RMNE calculation
  - Will be offered again next time

Training and Specific Guidelines-Classification Schemes yielded consistent results among laboratories

http://www.cstl.nist.gov/biotech/strbase/training.htm
German Type A, B, and C mixture classifications

- **Type A**, where major/minor contributors cannot be deduced, require stats
  - LR
  - RMNE

- **Type B** enables major contributor to be deduced
  - RMP (which is 1/LR)

- **Type C** no stats should be attempted because of the possibility of failure to account for allele dropout due to stochastic effects with low level DNA samples

---

**Mixture Example**
Comparing Alleles Only

<table>
<thead>
<tr>
<th>Locus 1</th>
<th>Locus 2</th>
<th>Locus 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed stain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>16</td>
<td>12</td>
</tr>
</tbody>
</table>

**Mixture Example**
Showing Importance of Using Peak Height Information

<table>
<thead>
<tr>
<th>Locus 1</th>
<th>Locus 2</th>
<th>Locus 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed stain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>16</td>
<td>12</td>
</tr>
</tbody>
</table>

Yes, the reference alleles are present in the evidence mixed stain
BUT the peak height patterns do not fit...

**Mixture Example**
Solving Components Prior to Comparison to Suspect Reference

<table>
<thead>
<tr>
<th>Locus 1</th>
<th>Locus 2</th>
<th>Locus 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed stain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Component 1: 15</td>
<td>17</td>
<td>12</td>
</tr>
<tr>
<td>Component 2: 16</td>
<td>18</td>
<td>14,14</td>
</tr>
<tr>
<td>Reference (suspect) does not match either component of the mixed stain and therefore could not have contributed to the evidence sample</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Mixture Example**
Different Evidence Sample...

<table>
<thead>
<tr>
<th>Locus 1</th>
<th>Locus 2</th>
<th>Locus 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed stain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Component 1: 15</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Component 2: 17</td>
<td>18</td>
<td>13,13</td>
</tr>
<tr>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>16</td>
<td>12</td>
</tr>
</tbody>
</table>

Possibilities include
- 10,10 with 11,12
- 11,11 with 10,12
- 12,12 with 10,11

Evidence (mixture) was expanded

**Conclusions from the evidence:**
1. Major contributor = 13,15 (victim) – to be expected with an intimate sample like a fingernail or vaginal swab
2. Alleles 12 and 14 are likely stutter products of the major contributor’s 13 and 15 alleles but could also be masking minor contributor alleles
3. A number of minor contributor combinations are possible (e.g., 10,11 or 10,12 or 10,13 or 11,13, etc.)
4. Could have more than two contributors present in this mixture

**Another Mixture Example**

<table>
<thead>
<tr>
<th>Locus 1</th>
<th>Locus 2</th>
<th>Locus 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evidence (mixture)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

Conclusion: "Suspect cannot be excluded" BUT statement needs to be qualified by statistics because a large percentage of the population might also not be able to be excluded...
The fact that in this case a suspect is included is not very informative because ~9 out of 10 people examined from any population could potentially be included in the evidence mixture.

The case may grow stronger against a suspect with information from additional STR loci.

The reference sample is still a "match" – just not as much information is available from the evidence for comparison.

The numerator, \( H_p \) (the suspect is the perpetrator) and the defense hypothesis, \( H_d \) (an unknown individual with a matching profile is the perpetrator)

Likelihood Ratio (LR)

- Provides ability to express and evaluate both the prosecution hypothesis, \( H_p \) (the suspect is the perpetrator) and the defense hypothesis, \( H_d \) (an unknown individual with a matching profile is the perpetrator)

\[
LR = \frac{H_p}{H_d}
\]

- The numerator, \( H_p \) is usually 1 – since in theory the prosecution would only prosecute the suspect if they are 100% certain he/she is the perpetrator

- The denominator, \( H_d \) is typically the profile frequency in a particular population (based on individual allele frequencies and assuming HWE) – i.e., the random match probability

LR is not a probability but a ratio of probabilities
DAB Recommendations on Statistics
February 23, 2000
Forensic Sci. Comm. 2(3); available on-line at

“The DAB finds either one or both PE or LR
calculations acceptable and strongly
recommends that one or both calculations be
carried out whenever feasible and a mixture
is indicated”

- Probability of exclusion (PE)
    Statistical Methods in Medical Research, 2, 241–262.
- Likelihood ratios (LR)
    Sinauer, Sunderland, Massachusetts.

Available for download from the ISFG Website:
http://www.isfg.org/Publication;Gill2006

DNA commission of the International Society of Forensic Genetics:
Recommendations on the interpretation of mixtures.
Gill et al. (2006) DNA Commission of the
International Society of Forensic Genetics:
Recommendations on the interpretation of mixtures.
Forensic Sci. Int. 160: 90-101

Summary of ISFG Recommendations
on Mixture Interpretation

1. The likelihood ratio (LR) is the
   preferred statistical method for
   mixtures over RMNE
2. Scientists should be trained in
   and use LRs
3. Methods to calculate LRs of
   mixtures are cited
4. Follow Clayton et al. (1998)
guidelines when deducing
   component genotypes
5. Prosecution determines H_p and
   defense determines H_d and
   multiple propositions may be
   evaluated
6. When minor alleles are the same
   size as stutters of major alleles,
   then they are indistinguishable
7. Allele dropout to explain evidence
   can only be used with low signal
   data
8. No statistical interpretation should
   be performed on alleles below
   threshold
9. Stochastic effects limit usefulness
   of heterozygote balance and
   mixture proportion estimates with
   low level DNA

Thoughts by Peter Gill on Recommendation #5
(ENFSI meeting, Krakow, Poland, April 19, 2007)

- Prosecution and defense each want to maximize their respective probabilities
- Recommendation 5 places ownership for each hypothesis.
- In order to perform the LR calculation(s), the forensic scientist decides on both
  the prosecution and defense hypotheses.
- Since the forensic scientists usually cannot discover the defense hypothesis
  before the trial (as they are typically working with the prosecution if the DNA
  matches...), assumptions must be clearly stated with the important caveat that
  you cannot perform calculations on the stand! (For example, you need three
  weeks warning to make and check calculations.)
- By anchoring the respective hypotheses to each side, the defense can change
  their hypothesis but the prosecution does not need to change theirs...
- It is worth noting that the likelihood ratio always goes up if the defense lowers
  their hypothesis (H_d gets lower with more possible combinations)

ISFG (2006) Recommendations

- Recommendation 6: If the crime profile is a
  major/minor mixture, where minor alleles are
  the same size (height or area) as stutters of
  major alleles, then stutters and minor alleles
  are indistinguishable. Under these
  circumstances alleles in stutter positions that do
  not support H_p should be included in the
  assessment.
- In general, stutter percentage is <15%

Gill et al. (2006) DNA Commission of the International Society of Forensic Genetics:
Consideration of Peak in Stutter Position

Fig. 4: c and d are unambiguous alleles, b is a minor allele in a stutter position and a is an unambiguous minor allele.


Measured Stutter Percentages
Variable by Allele Length and Composition

THO1 9.3 allele: [TCAT]4 -CAT [TCAT]5

Gill et al. (2008) FSI Genetics 2(1): 76–82

UK Response

Recommendation 6:
• Stutters are locus-dependent…
• It is recommended that laboratories make their own maximum experimentally observed stutter sizes per locus determinations since the effects may be technique dependent.
• It is recommended that [maximum stutter percentages be] evaluated per locus.

ISFG (2006) Recommendations

• Recommendation 7: If drop-out of an allele is required to explain the evidence under H_s (S = ab; E = a), then the allele should be small enough (height/area) to justify this. Conversely, if a full crime stain profile is obtained where alleles are well above the background level, and the probability of drop-out approaches Pr(D) = 0, then H_s is not supported.


UK Response

Recommendation 7:
• We recommend slight rewording…[with mention of companion allele]
• If a full crime-stain profile is obtained where alleles are well above the background level, and the probability of dropout Pr(D) approaches zero, then H_s is not supported (Figure 6).
Hypothetical Examples

**If Below Dropout Threshold…**

Gill et al. (2008) FSI Genetics 2(1): 76–82

![Diagram](image1)

If below dropout threshold, we cannot be confident that it is from a heterozygote AA individual. It could also be from an individual who is homozygous, where the missing allele is the other allele. The probability Pr(D) approaches zero (Fig. 4).

**If Above Dropout Threshold…**

Gill et al. (2008) FSI Genetics 2(1): 76–82

![Diagram](image2)

If above dropout threshold, we can be confident that it is from a heterozygote AA individual. The probability Pr(D) is Pr(AB).

**Setting Thresholds**

- **Detection (analytical) threshold**
  - Dependent on instrument sensitivity
  - ~50 RFU
  - Impacted by instrument baseline noise

- **Dropout (stochastic) threshold**
  - Dependent on biological sensitivity
  - ~150-200 RFU
  - Impacted by assay and injection parameters

**Determining the Dropout (Stochastic) Threshold**

Gill et al. (2008) FSI Genetics 2(1): 76–82

- The dropout threshold can be determined experimentally for a given analytical technique from a series of pre-PCR dilutions of extracts of known genotype technique (it will probably vary between analytical methods). These samples can be used to determine the point where allelic dropout of a heterozygote is observed relative to the size of the survivor companion allele. The threshold is the maximum size of the companion allele observed. This is also the point where Pr(D) approaches zero (Fig. 4).

**ISFG (2006) Recommendations**

- **Recommendation 8**: If the alleles of certain loci in the DNA profile are at a level that is dominated by background noise, then a biostatistical interpretation for these alleles should not be attempted.


---

http://www.cstl.nist.gov/biotech/strbase/training.htm
UK Response
Gill et al. (2008) FSI Genetics 2(1): 76–82

Recommendation 8:
• If there is a band below the experimental threshold where background noise might be prevalent, and it is distinct and clear from the background, then it should be recorded and available on the case file.

ISFG (2006) Recommendations

• Recommendation 9: In relation to low copy number, stochastic effects limit the usefulness of heterozygous balance and mixture proportion estimates. In addition, allelic drop-out and allelic drop-in (contamination) should be taken into consideration of any assessment.


UK Response
Gill et al. (2008) FSI Genetics 2(1): 76–82

Recommendation 9:
• Case pre-assessment is necessary in order to determine the best scientific method to process a sample. To facilitate this, it is recommended that wherever possible, this should include quantification. Quantification is used to determine the optimum method to process—if low-level DNA, a sample would benefit from procedures to enhance sensitivity of detection. There may be reasons where quantification is not practicable, especially if low levels of DNA are expected, since the result itself may be compromised if a portion of the sample is sacrificed. At low DNA levels, the accuracy of the quantification test itself may be inefficient.

Recommendation 9 (cont):
• It is possible that a given DNA profile may simultaneously comprise both ‘conventional’ and ‘low-level’ loci: for example, if degradation has occurred then low molecular weight loci may be above the dropout threshold, whereas high molecular weight loci may be below the dropout threshold.
• Similarly, if the sample is a mixture, then at a given locus there may be some alleles that are above the dropout threshold (from a major contributor) and others that are below the dropout threshold (from a minor contributor), i.e. different interpretation rationale may be simultaneously applied to different contributors within a locus.

Thank you for your attention…

Questions or Comments?

http://www.cstl.nist.gov/biotech/strbase/john.butler@nist.gov
301-975-4049

Our team publications and presentations are available at:
http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm
Summary of ISFG Recommendations on Mixture Interpretation

1. The likelihood ratio (LR) is the preferred statistical method for mixtures over RMNE

2. Scientists should be trained in and use LRs

3. Methods to calculate LRs of mixtures are cited

4. Follow Clayton et al. (1998) guidelines when deducing component genotypes

5. Prosecution determines $H_p$ and defense determines $H_d$ and multiple propositions may be evaluated

6. When minor alleles are the same size as stutters of major alleles, then they are indistinguishable

7. Allele dropout to explain evidence can only be used with low signal data

8. No statistical interpretation should be performed on alleles below threshold

9. Stochastic effects limit usefulness of heterozygote balance and mixture proportion estimates with low level DNA

Define what is a mixture (>2 alleles at ≥2 loci)

>2 alleles at a locus, except triallelics?

NO

Single Source DNA Sample

Determine STR profile and compute RMP

YES

Mixed DNA Sample

Differentiate a Major/Minor Component?

NO

Probability of Exclusion \([P_E]\) "RMNE"

NO

Stochastic Effects? Possible Low Level DNA?

NO

Define reliable ratio ranges (4:1 to 10:1)

YES

TYPE B

Determine component profile(s) and compute RMP for major

NO

Define LCN limits (<200 pg)

YES

TYPE C

A biostatistical analysis should not be performed

NO

Assume # Contributors?

YES

Likelihood Ratio [LR]

A biostatistical analysis must be performed

TYPE A

Developed by John Butler based on German classifications Schneider et al. (2006) Rechtsmedizin 16:401-404
Mixture Deconvolution

Ohio Statewide Training Meeting
Indian Rocks Beach, FL
May 12-13, 2008

Dr. John M. Butler
National Institute of Standards and Technology
john.butler@nist.gov

Points for Consideration

• Peak height vs peak area
• Thresholds – analytical vs stochastic levels
• Other lab-specific values:
  – Heterozygote peak height balance
  – Locus-specific stutter percentage
• DNA quantity and quality
  – problems with low-level or degraded DNA

What is a true peak (allele)?

Validation Studies

• Information from validation studies should be used to set laboratory-specific
  • Stutter %
  • Peak Height Ratios
  • Minimum Peak Heights (detection thresholds)
  • Relative balance across loci
• These values are all dependent on amount of input DNA
  • If low-level DNA is amplified, stutter % may be higher and peak height ratios may be lower

Thresholds

• Validation studies should be performed in each laboratory
• Some labs have set two thresholds:
  – Analytical thresholds – what is a peak? (50 RFU)
  – Stochastic thresholds – what is reliable PCR data? (150 RFU)

http://www.cstl.nist.gov/biotech/strbase/training.htm
Different Thresholds of Detection Influence Allele Calls

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.

Identifiler Results: NEST I1, I2, I3, I4 (varying input DNA)

<table>
<thead>
<tr>
<th>Minor component amount</th>
<th>100 pg</th>
<th>50 pg</th>
<th>25 pg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identify the Presence of a Mixture</td>
<td>Is a DNA Profile Consistent with Being a Mixture?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>If the answer to any one of the following three questions is yes, then the DNA profile may very well have resulted from a mixed sample:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Step #1: Is a Mixture Present in an Evidentiary Sample?</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Examine the number of peaks present in a locus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>More than 2 peaks at a locus (except for tri-allelic patterns at perhaps one of the loci examined)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Examine relative peak heights</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterozygote peak imbalance &lt;60%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak at stutter position &gt;15%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Consider all loci tested</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Is a DNA Profile Consistent with Being a Mixture?</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Step #6: Compare Reference Samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>If the answer to any one of the following three questions is yes, then the DNA profile may very well have resulted from a mixed sample:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do any of the loci show more than two peaks in the expected allele size range?</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Is there a severe peak height imbalance between heterozygous alleles at a locus?</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Does the stutter product appear abnormally high (e.g., >15-20%)?
Step #2: Designate Allele Peaks

- Use regular data interpretation rules to decipher between true alleles and artifacts.
- Use stutter filters to eliminate stutter products from consideration (although stutter may hide some of minor component alleles at some loci).
- Consider heterozygote peak heights that are highly imbalanced (<60%) as possibly coming from two different contributors.

Step #3: Identifying the Potential Number of Contributors

- Important for some statistical calculations.
- Typically if 2, 3, or 4 alleles then 2 contributors.
- If 5 or 6 alleles per locus then 3 contributors.
- If >6 alleles in a single locus, then >4 contributors.

- JFS Nov 2005 paper by Forensic Bioinformatics on number of possible contributors.
  - Relies on maximum allele count alone.
  - Does not take into account peak height information.

Recent Article by Buckleton et al.

Buckleton et al. (2007) Towards understanding the effect of uncertainty in the number of contributors to DNA stains.

<table>
<thead>
<tr>
<th>Locus</th>
<th>No. of alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>D3</td>
<td>0.011 ± 0.240</td>
</tr>
<tr>
<td>D16</td>
<td>0.016 ± 0.287</td>
</tr>
<tr>
<td>D32</td>
<td>0.003 ± 0.394</td>
</tr>
<tr>
<td>D21</td>
<td>0.007 ± 0.417</td>
</tr>
<tr>
<td>D8</td>
<td>0.003 ± 0.264</td>
</tr>
<tr>
<td>TRHO</td>
<td>0.016 ± 0.271</td>
</tr>
<tr>
<td>PFA</td>
<td>0.003 ± 0.116</td>
</tr>
</tbody>
</table>

Use of 959 complete 13-locus STR profiles from FBI dataset.

3.39 % (4,967,034 combinations) would only show a maximum of four alleles (i.e., appear based on maximum allele count alone to be a 2-person mixture).
Levels of Locus Heterozygosity Impact Number of Alleles Observed in Mixtures

<table>
<thead>
<tr>
<th>Loci</th>
<th>No. of alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>D3</td>
<td>0.011 0.240 0.355 0.119</td>
</tr>
<tr>
<td>vWA</td>
<td>0.006 0.194 0.548 0.250</td>
</tr>
<tr>
<td>D16</td>
<td>0.016 0.287 0.433 0.166</td>
</tr>
<tr>
<td>D2</td>
<td>0.003 0.094 0.203 0.600</td>
</tr>
</tbody>
</table>

Three-Person Mixtures for Simulated Profiles: Probability by Locus of A Particular Number of Alleles Being Observed

<table>
<thead>
<tr>
<th>Loci</th>
<th>No. of alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>D3</td>
<td>0.000 0.055 0.266 0.463 0.115 0.032</td>
</tr>
<tr>
<td>vWA</td>
<td>0.000 0.033 0.283 0.401 0.178 0.016</td>
</tr>
<tr>
<td>D16</td>
<td>0.001 0.086 0.397 0.411 0.100 0.008</td>
</tr>
<tr>
<td>D2</td>
<td>0.000 0.048 0.104 0.358 0.965 0.119</td>
</tr>
<tr>
<td>D21</td>
<td>0.000 0.023 0.192 0.428 0.302 0.055</td>
</tr>
<tr>
<td>D31</td>
<td>0.000 0.037 0.109 0.399 0.966 0.006</td>
</tr>
<tr>
<td>D39</td>
<td>0.003 0.078 0.352 0.404 0.152 0.015</td>
</tr>
<tr>
<td>TH0</td>
<td>0.001 0.074 0.295 0.439 0.648 0.002</td>
</tr>
<tr>
<td>PGA</td>
<td>0.000 0.012 0.144 0.424 0.346 0.074</td>
</tr>
</tbody>
</table>

Number of Alleles Observed with Simulated Four-Person Mixtures

- The simulation of four person mixtures suggests that 0.014% of four person mixtures would show four or fewer alleles and that 60% would show six or fewer alleles for the SGM Plus loci.
- The results for the Profiler Plus loci were 0.6% and 75%.
- The equivalent values for the CODIS set from Paolletti et al. were 0.02% showing four or fewer and 76.35% showing six or fewer.

Step #4: Estimation of Relative Ratios for Major and Minor Components to a Mixture

- Mixture studies with known samples have shown that the mixture ratio between loci is fairly well preserved during PCR amplification.
- Thus it is generally thought that the peak heights (areas) of alleles present in an electropherogram can be related back to the initial component concentrations.
- Start with loci possessing 4 alleles...

Step #5: Consider All Possible Genotype Combinations

Clayton et al., Forensic Sci. Int. 1998; 91:55-70
Considering Genotype Combinations

Peptide Height Ratios (PHR)
Minimum Peak Height (mPH)
Proportion (p) or mixture proportion (Mx)

Step #6: Compare Reference Samples

- If there is a suspect, a laboratory must ultimately decide to include or exclude him...
- If no suspect is available for comparison, does your laboratory still work the case? (Isn’t this a primary purpose of the national DNA database?)
- Victim samples can be helpful to eliminate their allele contributions to intimate evidentiary samples and thus help deduce the perpetrator

Worked Example

NEST Project Mixture Sample Set

- NIJ Expert Systems Testbed (NEST) Project
  - Marshall University with Rhonda Roby (NIJ consultant)
- Phase II Mixture Sample Analysis
  - Amy Christen (Marshall University) produced a dataset while interning at Forensic Science Service in Summer 2006
  - Data to be used for evaluating “expert systems”
- Mixtures tested (280 total samples)
  - 2 different female/male sample combinations: A:X and B:Y
  - 4 input DNA amounts: 1.5 ng, 1.0 ng, 0.5 ng, 0.25 ng
  - 5 kits: Identifiler, ProfilerPlus, COFiler, PowerPlex 16, SGM Plus
  - 7 mixture ratios: 30:1, 10:1, 3:1, 1:1, 1:3, 1:10, 1:30

I will focus on a subset of this data... e.g., B:Y, 1.0 ng, Identifiler, 3:1

http://www.cstl.nist.gov/biotech/strbase/training.htm
Identifiler Results: NEST H2 – N2 (1.0 ng input DNA)

Data courtesy of Amy Christen, Marshall University NEST Project Team

Calculate ratios based on peak heights

Identifiler Results: NEST H2 – N2 (1.0 ng input DNA)

Data courtesy of Amy Christen, Marshall University NEST Project Team

Identifiler Mixture Example

Profile Overview

Evaluation Notes:
1. Look at ratios 1.2:3.4 (alleles in mixture with at least 2 contributors)
2. Imbalance at amelogenin (female & male mixture with female as major)
3. Decent overall signal with D8 in 1500-1600 RFU range (degradation unlikely)
4. Ratio of major to minor around 3:1

Anomalous Amelogenin Alleles

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

Males possessing only a single X amelogenin amplicon (Y null):
- a male DNA sample will falsely look like a female DNA sample:
  - Santos et al. (1998) reported a rare deletion of the amelogenin gene on the Y-chromosome
  - Y-STR typing can be performed to verify that other portions of the Y-chromosome are present

Males possessing only a single Y amelogenin amplicon (X null):
- Shewale et al. (2000) observed loss of the X chromosome amplicon in three out of almost 7,000 males examined
  - while this phenomenon should not result in a gender misclassification (as the Y null situation might), its occurrence can impact the expected X and Y amplicon ratios in a mixture (see NIST MIX05 interlab study, case #3)

Running reference samples from suspect and/or victim may help discover potential amelogenin anomalies

http://www.cstl.nist.gov/biotech/strbase/training.htm
Locus-by-Locus Breakdown…

• Start with 4 allele loci…
  – Assume two person mixture
  – With non-overlapping heterozygotes
  – Pair peaks with similar peak heights
  Possible but not as likely depending on ratios

Possible Genotype Combinations

Four Peaks (4 allele loci)
- heterozygote + heterozygote, no overlapping alleles (genotypes are unique)
- heterozygote + heterozygote, one overlapping allele
- heterozygote + homozygote, no overlapping alleles (genotypes are unique)

Three Peaks (3 allele loci)
- heterozygote + heterozygote, one overlapping allele
- heterozygote + homozygote, no overlapping alleles (genotypes are unique)

Two Peaks (2 allele loci)
- heterozygote + heterozygote, two overlapping alleles (genotypes are identical)
- heterozygote + homozygote, one overlapping allele
- homozygote + homozygote, no overlapping alleles (genotypes are unique)

Single Peak (1 allele loci)
- homozygote + homozygote, overlapping allele (genotypes are identical)

MUST ALSO CONSIDER STUTTER POSITION

Population Database Used for STR Allele Frequencies

• U.S. population data contained in J.M. Butler (2005) Forensic DNA Typing, 2nd Edition, Appendix II (pp. 577-583)
• Published in Butler et al. (2003) J. Forensic Sci. 48(4): 908-911
• Available at http://www.cstl.nist.gov/biotech/strbase/NISTpop.htm
• Will focus on Caucasians for simplicity

Remember that different population databases will have different allele frequencies because they are based on different samples

4 Allele Locus: TH01

<table>
<thead>
<tr>
<th>Allele</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>0.190</td>
</tr>
<tr>
<td>8</td>
<td>0.084</td>
</tr>
<tr>
<td>9</td>
<td>0.114</td>
</tr>
<tr>
<td>9.3</td>
<td>0.368</td>
</tr>
</tbody>
</table>

\[
PI = (P_A + P_B + P_C + P_D)^2 \\
= (0.190 + 0.084 + 0.114 + 0.368)^2 \\
= (0.756)^2 \\
= 0.572
\]

\[
PE = 1 - PI = 1 - 0.572 = 0.428
\]

Thus ~43% of Caucasian population can be excluded from contributing to this mixture (primarily because allele 6 is missing)

4 Allele Locus: TH01

<table>
<thead>
<tr>
<th>Allele</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>0.190</td>
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<td>8</td>
<td>0.084</td>
</tr>
<tr>
<td>9</td>
<td>0.114</td>
</tr>
<tr>
<td>9.3</td>
<td>0.368</td>
</tr>
</tbody>
</table>

Mix Ratio

Total of all peak heights
= 1370 + 638 + 1121 + 494 = 3623 RFUs

Minor component: (B+D)/Total = (638+494)/3623 = 0.312

Major component: (A+C)/Total = (1370+1121)/3623 = 0.688

Close to the ~3:1 predicted by amelogenin X/Y allele ratio – thus major component = female

http://www.cstl.nist.gov/biotech/strbase/strbase.htm
4 Allele Locus: D2S1338

Mix Ratio

Total of all peak heights
= 438 + 1110 + 1326 + 523 = 3397 RFUs

STR allele call
RFU peak height
Major: 23,24
Minor: 19,25

Minor component:
(A+D)/total = (438+523)/3397 = 0.283

Major component:
(B+C)/total = (1110+1326)/3397 = 0.717

Three Peaks (3 allele loci)
- heterozygote + heterozygote, one overlapping allele
- heterozygote + homozygote, no overlapping alleles (genotypes are unique)

4 Allele Locus: vWA

Mix Ratio

Total of all peak heights
= 880 + 244 + 468 + 736 = 2330 RFUs

STR allele call
RFU peak height
Major: 14,18
Minor: 15,17

Minor component:
(B+C)/total = (244+468)/2330 = 0.306

Major component:
(A+D)/total = (880+736)/2330 = 0.694

Three Peaks (3 allele loci)
- heterozygote + heterozygote, one overlapping allele
- heterozygote + homozygote, no overlapping alleles (genotypes are unique)

3 Allele Locus: D8S1179

Three Peaks (3 allele loci)
- heterozygote + heterozygote, one overlapping allele
- heterozygote + homozygote, no overlapping alleles (genotypes are unique)

3 Allele Locus: D21S11

Three Peaks (3 allele loci)
- heterozygote + heterozygote, one overlapping allele
- heterozygote + homozygote, no overlapping alleles (genotypes are unique)

3 Allele Locus: D7S820

Three Peaks (3 allele loci)
- heterozygote + heterozygote, one overlapping allele
- heterozygote + homozygote, no overlapping alleles (genotypes are unique)

3 Allele Locus: CSF1PO

Three Peaks (3 allele loci)
- heterozygote + heterozygote, one overlapping allele
- heterozygote + homozygote, no overlapping alleles (genotypes are unique)

http://www.cstl.nist.gov/biotech/strbase/training.htm
3 Allele Locus: D3S1358

- Heterozygote + heterozygote, one overlapping allele
- Heterozygote + homozygote, no overlapping alleles (genotypes are unique)

<table>
<thead>
<tr>
<th>Allele</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0.262</td>
</tr>
<tr>
<td>16</td>
<td>0.253</td>
</tr>
<tr>
<td>18</td>
<td>0.152</td>
</tr>
</tbody>
</table>

3 Allele Locus: D18S51

- Heterozygote + heterozygote, one overlapping allele
- Heterozygote + homozygote, no overlapping alleles (genotypes are unique)

<table>
<thead>
<tr>
<th>Allele</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0.169</td>
</tr>
<tr>
<td>16</td>
<td>0.139</td>
</tr>
<tr>
<td>17</td>
<td>0.126</td>
</tr>
</tbody>
</table>

3 Allele Locus: FGA

- Heterozygote + heterozygote, one overlapping allele
- Heterozygote + homozygote, no overlapping alleles (genotypes are unique)

<table>
<thead>
<tr>
<th>Allele</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.127</td>
</tr>
<tr>
<td>23</td>
<td>0.134</td>
</tr>
<tr>
<td>25</td>
<td>0.071</td>
</tr>
</tbody>
</table>

2 Allele Locus: D19S433

- Heterozygote + heterozygote, two overlapping alleles (genotypes are identical)
- Heterozygote + homozygote, one overlapping allele
- Homozygote + homozygote, no overlapping alleles (genotypes are unique)

<table>
<thead>
<tr>
<th>Allele</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>0.369</td>
</tr>
<tr>
<td>15</td>
<td>0.152</td>
</tr>
</tbody>
</table>

2 Allele Locus: D5S818

- Heterozygote + heterozygote, two overlapping alleles (genotypes are identical)
- Heterozygote + homozygote, one overlapping allele
- Homozygote + homozygote, no overlapping alleles (genotypes are unique)

<table>
<thead>
<tr>
<th>Allele</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>0.361</td>
</tr>
<tr>
<td>12</td>
<td>0.384</td>
</tr>
</tbody>
</table>

2 Allele Locus: D13S317

- Heterozygote + heterozygote, two overlapping alleles (genotypes are identical)
- Heterozygote + homozygote, one overlapping allele
- Homozygote + homozygote, no overlapping alleles (genotypes are unique)

<table>
<thead>
<tr>
<th>Allele</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>0.339</td>
</tr>
<tr>
<td>12</td>
<td>0.124</td>
</tr>
</tbody>
</table>
### 2 Allele Locus: D16S539

<table>
<thead>
<tr>
<th>Allele</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>0.113</td>
</tr>
<tr>
<td>12</td>
<td>0.326</td>
</tr>
</tbody>
</table>

Two Peaks (2 allele loci):
- Heterozygote + heterozygote, two overlapping alleles (genotypes are identical)
- Heterozygote + homozygote, one overlapping allele
- Homozygote + homozygote, no overlapping alleles (genotypes are unique)

### 1 Allele Locus: TPOX

<table>
<thead>
<tr>
<th>Allele</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>0.535</td>
</tr>
</tbody>
</table>

Single Peak (1 allele loci):
- Homozygote + homozygote, overlapping allele (genotypes are identical)

### Profiles Used In Mixture Samples

<table>
<thead>
<tr>
<th>Victim</th>
<th>Suspect</th>
</tr>
</thead>
<tbody>
<tr>
<td>D8S1179</td>
<td>13.15</td>
</tr>
<tr>
<td>D21S11</td>
<td>29.30</td>
</tr>
<tr>
<td>D3S138</td>
<td>10.12</td>
</tr>
<tr>
<td>CSF1PO</td>
<td>11.12</td>
</tr>
<tr>
<td>D31S108</td>
<td>18.18</td>
</tr>
<tr>
<td>TH01</td>
<td>7.8</td>
</tr>
<tr>
<td>D16S539</td>
<td>9.12</td>
</tr>
<tr>
<td>D3S1331</td>
<td>23.19</td>
</tr>
<tr>
<td>D19S433</td>
<td>14.15</td>
</tr>
<tr>
<td>vWA</td>
<td>14.18</td>
</tr>
<tr>
<td>TPOX</td>
<td>6.6</td>
</tr>
<tr>
<td>D16S511</td>
<td>15.12</td>
</tr>
<tr>
<td>AMEL</td>
<td>X.X</td>
</tr>
<tr>
<td>D2S1358</td>
<td>12.12</td>
</tr>
<tr>
<td>PGA</td>
<td>20.25</td>
</tr>
</tbody>
</table>

### Software Programs (Expert Systems) for Mixture Deconvolution

These programs do not supply stats (only attempt to deduce mixture components)

- **Linear Mixture Analysis (LMA)** - U.S. Patent 6,807,490
  - Part of TrueAllele system developed by Mark Perlin (Cybergenetics)

- **Least Squares Deconvolution (LSD)**
  - Available for use at [https://lsd.lit.net/](https://lsd.lit.net/)

- **PENDULUM**
  - Part of FSS i-3 software suite (i-STReam)

**USACIL program developed by Tom Overson called DNA_DataAnalysis**

### Acknowledgments

- Amy Christen (Marshall University NEST Project Team)
- Angie Dolph (NIST intern/ Marshall University)
- Tim Kalafut (USACIL)

**Mixture Statistics**

**German Type A, B, and C mixture classifications**

- **Type A**, where major/minor contributors cannot be deduced, require stats
  - LR
  - RMNE

- **Type B** enables major contributor to be deduced
  - RMP (which is 1/LR)

- **Type C** no stats should be attempted because of the possibility of failure to account for allele dropout due to stochastic effects with low level DNA samples

---

**Statistical Interpretation of DNA Mixtures**

Ladd et al. 2001. Croatian Medical Journal 43(3): 244-246

1. Qualitative statement (‘.cannot exclude.’)
2. Interpret as single source from peak height differences, differential extraction, etc. and calculate random match probability (RMP)
3. Calculate probability of exclusion (CPE)
4. Calculate likelihood ratio (LR)

**Random Man Not Excluded (RMNE)**

- = Probability of Exclusion (PE)

- John Buckleton (Forensic DNA Evidence Interpretation, p. 222) quotes Laszlo Szabo of Tasmania Forensic Science Laboratory: "Intuitively, RMNE is easier to explain to a jury and express in reports than the likelihood ratio, and is probably closer to what the court wants—e.g., the suspect matches the mixture, but what if this is the wrong person—then what is the probability that someone else in the population would also match the mixture (i.e., not be excluded as a contributor)."

- Buckleton (Forensic DNA Evidence Interpretation, p. 222) also quotes Bruce Weir: that exclusion probabilities “often rob the items of probative value”

**Probability of Exclusion (RMNE)**

- **Advantages**
  - Does not require an assumption of the number of contributors to a mixture
  - Easier to explain in court

- **Disadvantages**
  - Weaker use of the available information (robs the evidence of its true probative power because this approach does not consider the suspect’s genotype)
  - Likelihood ratio approaches are developed within a consistent logical framework

John Buckleton, Forensic DNA Evidence Interpretation, p. 223

**RMNE (CPE)**

- Statements from DAB Recommendations on Statistics (FDT2e, p. 617)

- CPE provides a calculation of the estimated proportion of individuals from a defined population group that can be excluded as a contributor to an observed DNA mixture
Probability of Exclusion

The probability that a random person (unrelated individual) would be excluded as a contributor to the observed DNA mixture

For each locus, 1 minus the square of the sum of frequencies for the observed alleles

\[ PE_i = 1 - \left( \sum_{i=1}^{n} A_i \right)^2 \]

Buckleton (2005) Forensic DNA Evidence Interpretation, p. 219

Across multiple loci (i.e., combined probability of exclusion, CPE):

\[ PE = 1 - \prod_{i} \left(1 - PE_i\right) \]

Buckleton (2005) Forensic DNA Evidence Interpretation, p. 221

Combined Probability of Exclusion (CPE)

Each locus is calculated separately and then combined for CPE

\[ CPE = 1 - (1 - PE_1)(1 - PE_2)(1 - PE_3)...(1 - PE_n) \]

Probability of exclusion at a single locus:

- The combined frequency of alleles detected (P)
  \[ P = frequency\ of\ allele\ 1 + frequency\ of\ allele\ 2 + \ldots + frequency\ of\ allele\ N \]

- The combined frequency of alleles not detected (Q)
  \[ Q = 1 - P \]

\[ PE = Q^2 + 2Q(1-Q) \]

Across multiple loci (i.e., combined probability of exclusion, CPE):

\[ CPE = 1 - (1 - PE_1)(1 - PE_2)(1 - PE_3)...(1 - PE_n) \]

Calculation from CPI Perspective

Each locus is calculated separately and then combined for CPI

CPI or P_{profile} = (P_{locus1}) (P_{locus2}) (P_{locus3}) \ldots (P_{locusN})

Probability of inclusion at a single locus:

- Individual frequencies are summed and then squared
  \[ P_{locus} = (p_1^2 + p_2^2 + \ldots + p_N^2) \]

\[ PE = 1 - P_{locus} = 1 - PI \]
\[ PE = Q^2 + 2Q(1-Q) \]

Provides probability of an unrelated individual in the population is a contributor to the mixture at the loci examined

Basic Math Terms

- When ‘+’ is used, this means ‘OR’
- When ‘x’ is used, this means ‘AND’
- Pr. is shorthand for probability
- Therefore...
  - the probability of an ‘AND’ b happening together is \( Pr(a \ and \ b) = a \times b \)
  - the probability of an ‘OR’ b happening together is \( Pr(a \ or \ b) = a + b \)

Likelihood Ratios

The combined frequency of alleles detected (P)

\[ P = 0.151 + 0.243 + 0.166 = 0.56 \]

\[ Q = 1 - 0.56 = 0.44 \]

\[ PE = (0.44)^2 + 2(0.44)(1-0.44) = 0.1936 + 0.4928 = 0.686 \]

US Caucasian Data

<table>
<thead>
<tr>
<th>Allele</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>0.151</td>
</tr>
<tr>
<td>10</td>
<td>0.243</td>
</tr>
<tr>
<td>12</td>
<td>0.166</td>
</tr>
</tbody>
</table>

Provides probability of an unrelated individual in the population is a contributor to the mixture at the loci examined

Conditioning

- Probabilities are conditional, which means that the probability of something is based on a hypothesis
- In math terms, conditioning is denoted by a vertical bar
  - Hence, \( Pr(a|b) \) means ‘the probability of a given that b is true’
- The probability of an event a is dependent upon various assumptions—and these assumptions or hypotheses can change...

Slide information from Peter Gill (ISFG 2007 workshop, Copenhagen, August 20-21, 2007)

http://www.cstl.nist.gov/biotech/strbase/training.htm
Probability Example – Will It Rain? (1)

Defining the Event and Assumptions/Hypotheses
• Let's suppose that $a$ is the probability of an event (e.g., will it rain?)
• What is the probability that it will rain in the afternoon – $Pr(a)$?
• This probability is dependent upon assumptions
  – We can look at the window in the morning and observe if it is sunny (s) or cloudy (c)
  – $Pr(a)$ if it is sunny (s) is less than $Pr(a)$ if it is cloudy (c)
• We can write this as $Pr(a|s)$ and $Pr(a|c)$
  – Since sunny or cloudy are the only possibilities, $Pr(s) + Pr(c) = 1$
  – or $Pr(s) = 1 – Pr(c)$

Examining Available Data
• $Pr(a|s)$ and $Pr(a|c)$ can be calculated from data
  • How often does it rain in the afternoon when its sunny in the morning?
    – 20 out of 100 observations so $Pr(a|s) = 0.2$
  • How often does it rain in the afternoon when it is cloudy in the morning?
    – 80 out of 100 observations so $Pr(a|c) = 0.8$

Formation of the Likelihood Ratio (LR)
• The LR compares two probabilities to find out which of the two probabilities is the most likely
  The probability that it will rain in the afternoon when it is cloudy in the morning or $Pr(a|c)$ is divided by the probability that it will rain in the afternoon when it is sunny in the morning or $Pr(a|s)$
  
  $LR = \frac{Pr(a|c)}{Pr(a|s)} = \frac{0.8}{0.2} = 4$

Likelihood Ratios in Forensic DNA Work
• We evaluate the evidence ($E$) relative to alternative pairs of hypotheses
  • Usually these hypotheses are formulated as follows:
    – The probability of the evidence if the crime stain originated with the suspect or $Pr(E|S)$
    – The probability of the evidence if the crime stain originated from an unknown, unrelated individual or $Pr(E|U)$
  
  $LR = \frac{Pr(E|S)}{Pr(E|U)}$ The numerator

The Likelihood Ratio Must Be Stated Carefully
• The probability of the evidence is $x$ times more likely if the stain came from the suspect Mr. Smith than if it came from an unknown, unrelated individual.
  • It is not appropriate to say: “The probability that the stain came from Mr. Smith.” because we must always include the conditioning statement – i.e., always make the hypothesis clear in the statement.
  • Always use the word ‘if’ when using a likelihood ratio to avoid this trap
Likelihood Ratio (LR)

- Provides ability to express and evaluate both the prosecution hypothesis, \(H_p\) (the suspect is the perpetrator) and the defense hypothesis, \(H_d\) (an unknown individual with a matching profile is the perpetrator)

\[
LR = \frac{H_p}{H_d}
\]

- The numerator, \(H_p\), is usually 1 – since in theory the prosecution would only prosecute the suspect if they are 100% certain he/she is the perpetrator

- The denominator, \(H_d\), is typically the profile frequency in a particular population (based on individual allele frequencies and assuming HWE) – i.e., the random match probability

Relationship between Likelihood Ratio (LR) and Random Match Probability (RMP)

- For single source samples or deduced major component profiles in a mixture...

\[
LR = \frac{1}{RMP} \quad \text{or} \quad RMP = \frac{1}{LR}
\]

Example #1

A Single Locus from a 2-Person Mixture

- Consider a simple two person mixture with one locus consisting of two heterozygotes with non-overlapping alleles

- If the suspect is \(ab\), then there must be another (unknown person) who is \(cd\)

Example #1

The Two Hypotheses Are Formed...

- Prosecution (\(H_p\)): The DNA result has come from the suspect and one unknown person, or \(Pr(E|S,U)\)

- Defense (\(H_d\)): The DNA result has come from two unknown people, or \(Pr(E|U_1,U_2)\)

\[
LR = \frac{Pr(E|S,U)}{Pr(E|U_1,U_2)}
\]

Example #1

Formulating the Numerator (Prosecution Hypothesis)

- If the prosecution hypothesis is true, then we would expect genotype \(ab\) to be present with 100% probability or \(Pr=1\).

- The chance of seeing an unknown person of type \(cd\) is the frequency of that type in the population or \(2p_c p_d\), where \(p_c\) is the allele frequency for allele \(c\).

- \(Pr(E|S,U) = 1 \times 2p_c p_d = 2p_c p_d\)

Example #1

Formulating the Denominator (Defense Hypothesis)

- The defense claims that the evidence could come from any two random individuals

- We must work out all possible pairwise combinations from alleles \(abcd\) and their probabilities (genotype frequencies)

\[
\begin{array}{cccc}
\text{Products} & \text{Individual #1} & \text{Individual #2} \\
ab & 2p_a p_b & 2p_b p_d \\
ac & 2p_a p_c & 2p_c p_d \\
ad & 2p_a p_d & 2p_d p_c \\
bd & 2p_b p_d & 2p_d p_c \\
bc & 2p_b p_c & 2p_c p_a \\
bc & 2p_c p_a & 2p_a p_b \\
\end{array}
\]

\[
Pr(E|U_1,U_2) = 24p_a p_b p_c p_d
\]
Formulating the Likelihood Ratio

- The numerator and denominator are combined to form the LR.
- And common elements in both numerator and denominator are eliminated to simplify the algebraic equation...

\[ LR = \frac{Pr(E \mid S, U)}{Pr(E \mid U, U')} = \frac{2p_ap_b}{12} = \frac{1}{12p_a p_b} \]

Adapted from Peter Gill (ISFG 2007 workshop, Copenhagen, August 20-21, 2007)

All LR Calculations Follow the Same Basic Rules Just Shown

- Form hypotheses – Keep in mind what you are conditioning on.
- The LR numerator belongs to the prosecution.
- The LR denominator belongs to the defense.
- Numerator and denominator are combined and equation is simplified.
- Allele frequency values are placed into the equation for each locus.
- The LR from each locus is combined through multiplication if the loci are independently inherited (i.e., the product rule) to form a LR for the entire profile.

Example #1
Formulating the Likelihood Ratio

Another Example...

- The evidence profile is from a semen stained vaginal swab and possesses alleles a, b, c, and d.
- The suspect is a, b, and the victim is c, d.
- Because it is reasonable to assume that the victim’s alleles would be present on the swab (i.e., an intimate sample), we can condition on this...

With an Intimate Sample, the Hypothesis Changes...

- Prosecution (H_s): The DNA result has come from the suspect and the victim, or \( Pr(E \mid S, V) \)
- Defense (H_d): The DNA result has come from the victim and an unknown person, or \( Pr(E \mid U, V) \)

\[ LR = \frac{Pr(E \mid S, V)}{Pr(E \mid U, V)} \]

Example #2
Formulating the Numerator (Prosecution Hypothesis)

- The prosecution hypothesis \( (S+V) \) is completely explains the evidence. Hence, the probability is \( Pr=1 \)

\[ Pr(E \mid S, V) = 1 \times 1 = 1 \]

Example #2
Formulating the Denominator (Defense Hypothesis)

- The defense hypothesis is that the presence of alleles a and b are the result of an unknown person – and they concede that alleles c and d come from the victim.
- Since the frequency of an unknown, unrelated individual possessing alleles a and b in the population is \( 2p_a p_b \), where \( p_a \) is the allele frequency for allele a and \( p_b \) is the allele frequency for allele b, then

\[ Pr(E \mid U, V) = 2p_a p_b \times 1 = 2p_a p_b \]
Formulating the Likelihood Ratio

- The numerator and denominator are combined to form the LR

\[ LR = \frac{Pr(E \mid S,V)}{Pr(E \mid U,V)} = \frac{1}{2p_a p_b} \]

- Note that this LR is the same as for a non-mixed sample comprising the suspect alone.

- This example then is an illustration of simplification by "subtraction" (victim’s alleles are being removed from mathematical consideration...).

Example #2

Forming the Denominator (H_d) for the LR...

<table>
<thead>
<tr>
<th>Evidence (Mixture)</th>
<th>Victim</th>
<th>Suspect</th>
<th>LR</th>
</tr>
</thead>
<tbody>
<tr>
<td>A_1, A_2, A_3</td>
<td>A_2, A_3</td>
<td>A_1, A_2</td>
<td>8,10,12</td>
</tr>
<tr>
<td>8,10,12</td>
<td>10,12</td>
<td>8,10</td>
<td></td>
</tr>
</tbody>
</table>

\[ LR = \frac{1}{(0.151)((2)(0.243) + 2(0.166) + (0.151))} \]

\[ LR = 6.83 \]

Does not consider peak height information

The prosecution hypothesis (that the suspect is the perpetrator) is 6.83 times more likely than the defense hypothesis (that an unknown, unrelated individual is the perpetrator).

Likelihood Ratio (LR) Calculations

DAB Recommendations on Statistics

February 23, 2000

Forensic Sci. Comm. 2(3); available on-line at

"The DAB finds either one or both PE or LR calculations acceptable and strongly recommends that one or both calculations be carried out whenever feasible and a mixture is indicated"

- Probability of exclusion (PE)
- Likelihood ratios (LR)
Interlaboratory Mixture Studies

Dr. John M. Butler
National Institute of Standards and Technology
john.butler@nist.gov

Outline

- Purpose of Interlaboratory Studies
- Overview of Mixture Studies and Lessons Learned
- NIST MIX05 Study Results

Interlaboratory Studies

- Purpose…
  - Not a proficiency test
  - Most labs see them as opportunity to anonymously directly compare themselves to others
- STRBase section on interlab studies

A High Degree of Variability Currently Exists with Mixture Interpretation

- “If you show 10 colleagues a mixture, you will probably end up with 10 different answers”
  - Peter Gill, Human Identification E-Symposium, April 14, 2005
- Interlaboratory studies help to better understand why variability may exist between laboratories
  - Most analysts are only concerned about their own lab protocols and do not get an opportunity to see the big picture from the entire community that can be provided by a well-run interlaboratory study

Individual Performance in an Interlaboratory Study

DNA Concentration, ng / μL

<table>
<thead>
<tr>
<th>DNA Concentration</th>
<th>Others</th>
<th>You</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Donor Alleles (Major + Minor) Identified, %

<table>
<thead>
<tr>
<th>Yield gel</th>
<th>Quantiblot</th>
</tr>
</thead>
<tbody>
<tr>
<td>99.5</td>
<td>99</td>
</tr>
<tr>
<td>99</td>
<td>98</td>
</tr>
<tr>
<td>98</td>
<td>96</td>
</tr>
<tr>
<td>96</td>
<td>90</td>
</tr>
<tr>
<td>90</td>
<td>80</td>
</tr>
<tr>
<td>80</td>
<td>70</td>
</tr>
</tbody>
</table>

Non-Donor Alleles Reported, %

<table>
<thead>
<tr>
<th>Yield gel</th>
<th>Quantiblot</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5%</td>
<td>25%</td>
</tr>
<tr>
<td>25%</td>
<td>75%</td>
</tr>
<tr>
<td>75%</td>
<td>97.5%</td>
</tr>
</tbody>
</table>

2 different quant methods gave different results; this lab followed the Quantiblot results

Process for Interlaboratory Study

NIST Initiated Interlaboratory Studies

<table>
<thead>
<tr>
<th>Studies Involving STRs</th>
<th>Labs</th>
<th>Publications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixture Interpretation Study (Jan–Aug 2005)</td>
<td>69</td>
<td>Several presentations made ... Poster at 2005 Promega meeting (Sept 2005); available on STRBase</td>
</tr>
</tbody>
</table>

Overall Lessons Learned from NIST MSS 1,2,&3

- Laboratories have instruments with different sensitivities
- Different levels of experience and training plays a part in effective mixture interpretation
- Amount of input DNA makes a difference in the ability to detect the minor component (labs that put in "too much" DNA actually detected minor components more frequently)

NIST MIX05 Summary

Purpose of MIX05 Study

- Goal is to understand the "lay of the land" regarding mixture analysis across the DNA typing community
- One of the primary benefits we hope to gain from this study is recommendations for a more uniform approach to mixture interpretation and training tools to help educate the community
MIX05 Study Design and Purpose

- Permit a large number of forensic practitioners to evaluate the same mixture data
- Provide multiple cases representing a range of mixture scenarios
- Generate data from multiple STR kits on the same mixture samples to compare performance for detecting minor components
- The primary variable should be the laboratory’s interpretation guidelines rather than the DNA extraction, PCR amplification, and STR typing instrument sensitivity
- Are there best practices in the field that can be advocated to others?

Requests for Participants in MIX05

Mixtures representing four different case scenarios have been generated at NIST with multiple STR kits and provided to laboratories as electropherograms.

We would like to receive the following information:

1) Report the results as though they were from a real case including whether a statistical value would be attached to the results. Please summarize the perpetrator(s) alleles in each “case” as they might be presented in court—along with an appropriate statistic (if warranted by your laboratory standard operating procedure) and the source of the allele frequencies used to make the calculation. Please indicate which kit(s) were used to solve each case.

2) Estimate the ratio for samples present in the evidence mixture and how this estimate was determined.

3) Provide a copy of your laboratory mixture interpretation guidelines and a brief explanation as to why conclusions were reached in each scenario.

A MIX05 Participant Noted…

“Things we do not do:

- Calculate mixture ratios for casework
  - Calculation used for this study: Find loci with 4 alleles (2 sets of sister alleles). Make sure sister alleles fall within 70%, then take the ratio of one allele from one sister set to one allele of the second sister set, figure ratios for all combinations and average. Use peak heights to calculate ratios.
- Provide allele calls in reports
- Provide perpetrator(s) alleles or statistics in court without a reference sample to compare to the DNA profile obtained from the evidence. We will try to determine the perpetrator(s) profile for entry into CODIS."

We recognize that some of the information requested in this interlab study may not be part of a lab’s standard operating procedure.

MIX05 Case Scenarios

<table>
<thead>
<tr>
<th>Case</th>
<th>Victim Contribution</th>
<th>Perpetrator Contribution</th>
<th>Evidence Mixture</th>
<th>Perpetrator</th>
<th>Victim</th>
<th>Evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>3F:1M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#2</td>
<td>1F:3M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#3</td>
<td>Balanced</td>
<td>Male lacked amelogenin X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#4</td>
<td>More extreme</td>
<td>Male contained tri-allelic pattern at TPOX</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Female victim DNA profile was supplied for each case.

Labs asked to deduce the perpetrator DNA profile - suspect(s) not provided.

Mixture Interpretation Interlab Study (MIX05)

- Only involves interpretation of data - to remove instrument detection variability and quantitation accuracy issues
- 94 labs enrolled for participation
- 69 labs have returned results (17 from outside U.S.)
- Four mock cases supplied with “victim” and “evidence” electropherograms (GeneScan .fsa files – that can be converted for Mac or GeneMapper, gel files made available to FMBIO labs)
- Data available with Profiler Plus, Coffiler, SGM Plus, PowerPlex 16, Identifier, PowerPlex 16 BIO (FMBIO) kits
- Summary of results will involve training materials to illustrate various approaches to solving mixtures

http://www.cstl.nist.gov/biotech/strbase/training.htm
MIX05 Results on Multiple Kits
http://www.cstl.nist.gov/biotech/strbase/interlab/MIX05.htm

Case 1 evidence (mixture)

- ABI 3100 Generated Data was supplied on CD-ROM to labs as either .fsa files (for Genotyper NT or GeneMapperID) or Mac-converted files for Genotyper Mac

- FMBIO data was also made available upon request

What MIX05 Participants Have Received Back from NIST...

- Certificate of participation in the interlab study
- Copy of the poster presented at the Promega Sept 2005 meeting displaying “correct” results for the perpetrator in each case scenario as well as an explanation of study design and preliminary results

http://www.cstl.nist.gov/biotech/strbase/interlab/MIX05/MIX05poster.pdf

Summary of MIX05 Responses

94 labs enrolled for participation
69 labs returned results (17 from outside U.S.)

- 50 labs made allele calls
- 39 labs estimated ratios
- 29 labs provided stats

STR kit results used

- 34 ProfilerPlus/COfiler
- 10 PowerPlex 16
- 7 PP16 BIO
- 5 Identifier
- 2 SGM Plus
- 1 All ABI kit data
- 9 Various combinations

- All participants were supplied with all data and could choose what kits to examine based on their experience and lab protocols

Generally Identifier data was of poorer quality in the electropherograms we provided...which caused some labs to not return results (they indicated a desire for higher quality data through sample re-injection to reduce pull-up prior to data interpretation)

When is a Sample a Potential Mixture?

According to several MIX05 participant interpretation guidelines

- Number of Observed Peaks
  - Greater than two peaks at a locus
  - More than two alleles are present at two or more loci, although three banded patterns can occur
  - Presence of 3 alleles at a single locus
  - 4 peaked patterns (if observed at any locus), 3 peaked patterns (if observed at two or more loci), significant imbalances (peak height ratios <60%) of alleles for a heterozygous genotype at two or more loci with the exception of low template amplifications, which should be interpreted with caution

- Imbalance of heterozygote alleles
  - thresholds range from 50-70%
- Stutter above expected levels
  - generally 15-20%

These protocol differences can lead to variation in reported alleles and therefore the deduced profile and resulting statistics

Summary of Some MIX05 Reported Results

Case #2 has perpetrator as major component and thus is the easiest to solve...

- Most calls were correct (when they were made)

http://www.cstl.nist.gov/biotech/strbase/interlab/MIX05/MIX05poster.pdf

Some Mixture Ratios Reported in MIX05

Many labs do not routinely report the estimated ratio of mixture components

- LabId: Case #2 (M,F)
- Case #2 (M,F)
- Case #2 (M,F)
- Case #2 (F,M)

- 13 2 6 <2 10
- 34 1.8-3.6 3.6-4.7 1.6-1.8 6.2-7.6
- 70 58.8% 15% 64% 36%
- 73 2.1 6.1 2.1 not determined
- 90 2.1 6.1 2.1 not determined
- 4 10.1 0.1 11 not determined
- 33 not determined
- 12 male 2.1 6.1 2.1 1.6 8.8
- 66 6.6 4.6 1.6 2.1 4.5 1.1
- 79 -3.1 to -2.1 not determined
- 77 2.1 6.1 2.1 10.1
- 61 2.1 6.1 2.1 10.1

http://www.cstl.nist.gov/biotech/strbase/training.htm
Some Reported Stats for MIX05 Case #1

<table>
<thead>
<tr>
<th>LabID</th>
<th>Kits Used</th>
<th>Caucasians</th>
<th>African Americans</th>
<th>Hispanics</th>
</tr>
</thead>
<tbody>
<tr>
<td>77</td>
<td>ProPlus/Coffler</td>
<td>PE calculated</td>
<td>PE calculated</td>
<td>PE calculated</td>
</tr>
<tr>
<td>73</td>
<td>ProPlus/Coffler</td>
<td>none provided</td>
<td>none provided</td>
<td>none provided</td>
</tr>
<tr>
<td>4</td>
<td>ProPlus/Coffler</td>
<td>PE calculated</td>
<td>PE calculated</td>
<td>PE calculated</td>
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<tr>
<td>12</td>
<td>ProPlus/Coffler</td>
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<td>none provided</td>
<td>none provided</td>
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<tr>
<td>20</td>
<td>ProPlus/Coffler</td>
<td>none provided</td>
<td>none provided</td>
<td>none provided</td>
</tr>
<tr>
<td>80</td>
<td>ProPlus/Coffler</td>
<td>1.05E+15</td>
<td>2.13E+14</td>
<td>3.78E+15</td>
</tr>
<tr>
<td>94</td>
<td>ProPlus/Coffler</td>
<td>2.40E+11</td>
<td>7.00E+00</td>
<td>9.80E+10</td>
</tr>
<tr>
<td>46</td>
<td>PPIS</td>
<td>5.05E+00</td>
<td>3.05E+11</td>
<td>none provided</td>
</tr>
<tr>
<td>33</td>
<td>ProPlus/Coffler</td>
<td>2.94E+10</td>
<td>1.12E+08</td>
<td>1.74E+09</td>
</tr>
<tr>
<td>6</td>
<td>ProPlus/Coffler</td>
<td>4.00E+00</td>
<td>3.50E+00</td>
<td>200.00E+00</td>
</tr>
<tr>
<td>9</td>
<td>ProPlus/Coffler</td>
<td>1.94E+02</td>
<td>1.97E+07</td>
<td>1.54E+08</td>
</tr>
<tr>
<td>61</td>
<td>Identifier</td>
<td>1.92E+01</td>
<td>250.00</td>
<td>2.49E+07</td>
</tr>
<tr>
<td>79</td>
<td>ProPlus/Coffler</td>
<td>350.000</td>
<td>47,500</td>
<td>1,350,000</td>
</tr>
<tr>
<td>18</td>
<td>ProPlus/Coffler</td>
<td>434,800</td>
<td>31,710</td>
<td>390,000</td>
</tr>
</tbody>
</table>

Possible Reasons for Variability in Reported Statistics:
- Different types of calculations (CPE vs RMP)
- Different loci included in calculations (due to different thresholds used)
- Different allele frequency population databases (most use PopStats)
- Use of victim (e.g., major component in Case 1) profile stats

Which loci are included in each calculation?

Some Differences in Reporting Statistics

<table>
<thead>
<tr>
<th>LabID</th>
<th>Kits Used</th>
<th>Caucasians</th>
<th>African Americans</th>
<th>Hispanics</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>ProPlus/Coffler</td>
<td>1.16E+15</td>
<td>2.13E+14</td>
<td>3.98E+15</td>
</tr>
<tr>
<td>34</td>
<td>ProPlus/Coffler</td>
<td>2.40E+11</td>
<td>7.00E+00</td>
<td>9.80E+10</td>
</tr>
<tr>
<td>30</td>
<td>ProPlus/Coffler</td>
<td>2.94E+03</td>
<td>1.12E+08</td>
<td>1.74E+05</td>
</tr>
<tr>
<td>6</td>
<td>ProPlus/Coffler</td>
<td>49.00E+00</td>
<td>3.00E+00</td>
<td>200.00E+00</td>
</tr>
<tr>
<td>9</td>
<td>ProPlus/Coffler</td>
<td>4.14E+17</td>
<td>1.92E+07</td>
<td>1.54E+08</td>
</tr>
<tr>
<td>79</td>
<td>ProPlus/Coffler</td>
<td>930.000</td>
<td>47,000</td>
<td>1,350,000</td>
</tr>
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<td>ProPlus/Coffler</td>
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<td>390,000</td>
</tr>
</tbody>
</table>

~10 orders of magnitude difference ($10^5$ to $10^{15}$) based on which alleles were deduced and reported

Remember that these labs are interpreting the same MIX05 electropherograms

Further Examination of These 7 Labs

<table>
<thead>
<tr>
<th>LabID</th>
<th>Kits Used</th>
<th>Case 1</th>
<th>ASCLO-LAB accredited?</th>
<th>Solved loci listed?</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>ProPlus/Coffler</td>
<td>1.16E+15</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>34</td>
<td>ProPlus/Coffler</td>
<td>2.40E+11</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>30</td>
<td>ProPlus/Coffler</td>
<td>2.94E+03</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>ProPlus/Coffler</td>
<td>49.00E+00</td>
<td>Yes</td>
<td>No (CPE)</td>
</tr>
<tr>
<td>9</td>
<td>ProPlus/Coffler</td>
<td>4.14E+17</td>
<td>No (CPE)</td>
<td>Yes</td>
</tr>
<tr>
<td>79</td>
<td>ProPlus/Coffler</td>
<td>930.000</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>18</td>
<td>ProPlus/Coffler</td>
<td>434,800</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

Possible Reasons for Variability in Reported Statistics:
- Different types of calculations (CPE vs RMP)
- Different loci included in calculations (due to different thresholds used)
- Different allele frequency population databases (most use PopStats)
- Use of victim (e.g., major component in Case 1) profile stats

Different Stats Used

- Lab 9 (4.14 x $10^5$) used 1/CPI
- Lab 6 (4.0 x $10^5$) used selected loci and summed all possible genotypes for loci not completely deduced
- Lab 90 (1.18 x $10^{15}$) used theta value of 0.03 and deduced alleles at all 13 loci (correctly deduced all perpetrator alleles)

Different Detection Thresholds Used

<table>
<thead>
<tr>
<th>LabID</th>
<th>Kits Used</th>
<th>Case 1</th>
<th>75RFU</th>
<th>13strs</th>
<th>Results correct</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>ProPlus/Coffler</td>
<td>1.16E+15</td>
<td>Not stated</td>
<td>8</td>
<td>STRs, 2 partial, 3 INC</td>
</tr>
<tr>
<td>34</td>
<td>ProPlus/Coffler</td>
<td>2.40E+11</td>
<td>Not stated</td>
<td>5</td>
<td>STRs, 3 partial, 2 INC</td>
</tr>
<tr>
<td>30</td>
<td>ProPlus/Coffler</td>
<td>2.94E+03</td>
<td>Not stated</td>
<td>3</td>
<td>STRs, 4 partial, 1 INC</td>
</tr>
<tr>
<td>6</td>
<td>ProPlus/Coffler</td>
<td>49.00E+00</td>
<td>Not stated</td>
<td>3</td>
<td>STRs, 2 partial, 1 INC</td>
</tr>
<tr>
<td>9</td>
<td>ProPlus/Coffler</td>
<td>4.14E+17</td>
<td>Not stated</td>
<td>2</td>
<td>STR, 5 partial, 6 INC</td>
</tr>
<tr>
<td>79</td>
<td>ProPlus/Coffler</td>
<td>930.000</td>
<td>Not stated</td>
<td>2</td>
<td>STR, 5 partial, 6 INC</td>
</tr>
<tr>
<td>18</td>
<td>ProPlus/Coffler</td>
<td>434,800</td>
<td>Not stated</td>
<td>2</td>
<td>STR, 5 partial, 6 INC</td>
</tr>
</tbody>
</table>

- Lab 90 has specific, detailed mixture interpretation guidelines with worked examples and a fabulous flowchart
- Lab 16 has vague guidelines that begin with "mixture interpretation is not always straightforward. Analysts must depend on their knowledge and experience...."
Manually Solving Mixture Component Profiles

A Model Report of Analysis...

- "The Profiler Plus and COfiler sample files were evaluated by four different analysts, using both NT and MAC analysis platforms. The analysts checked for concordance, and a single conclusion for each mock case has been issued."

- They detailed all assumptions made outside the course of routine casework:
  - Assumed intimate samples
  - That a comparison of deduced "foreign" alleles had been made with the perpetrator's known standard in order to calculate the significance of the inclusion with the evidentiary profile

- For Case #4: "A Combined Probability of Inclusion was calculated and reported for only those loci where all the alleles were above threshold (75 RFUs). However, a minor profile(s) could not be deduced from this sample. Please note that our laboratory may employ strategies to gain more information from the sample, such as a 10 second injection of the CE and Y-STR analysis."

Quotes from One Lab’s MIX05 Report

- Case 1: STR typing results from the Evidence sample indicate a DNA mixture profile. The victim cannot be excluded as a possible donor of the genetic material in the Evidence sample. No statistics will be generated at this time.

- The Evidence samples would have to be rerun in order to verify any alleles called in the final profiles. This is true for any mixed sample profiles as per our laboratory guidelines.

- Our laboratory does not "pull out" any profile from a mixture for interpretation or statistical purposes. The exception to this is for CODIS profiles where the alleles that can be unambiguously attributed to the victim are removed.

- We currently do not calculate and report statistics on mixture samples.

Examples of MIX05 Report Formats

All examples with Case #1
(~3:1 mixture with female victim as the major component – and victim profile is provided)
Another MIX05 Participant Manually Solving a Mixture

Semi-Automated Locus-by-Locus Interpretation Performed by One MIX05 Participant

Excel spreadsheet used to examine possible component combinations

Different Reporting Formats for MIX05 Data

Table 1: SUMMARY of DNA Typing Results: Alleles Detected

<table>
<thead>
<tr>
<th>Locus</th>
<th>Victim(s)</th>
<th>Deletions Detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>D3S1358</td>
<td>15, 16</td>
<td>15.14 (0.75)</td>
</tr>
<tr>
<td>vWA</td>
<td>17</td>
<td>15.16 (0.97)</td>
</tr>
<tr>
<td>FGA</td>
<td>19.1</td>
<td>15.19 (0.92)</td>
</tr>
<tr>
<td>D21110</td>
<td>18</td>
<td>15.21 (0.92)</td>
</tr>
<tr>
<td>D19515</td>
<td>16</td>
<td>15.16 (0.97)</td>
</tr>
<tr>
<td>D5S811</td>
<td>16</td>
<td>10.16 (0.97)</td>
</tr>
<tr>
<td>D3S1338</td>
<td>10, 14</td>
<td>10.10 (0.97)</td>
</tr>
<tr>
<td>THO1</td>
<td>17</td>
<td>7.6 (0.97)</td>
</tr>
<tr>
<td>TPOX</td>
<td>8</td>
<td>8 (0.97)</td>
</tr>
</tbody>
</table>

No attempt to deduce perpetrator alleles (foreign profile)

Different Reporting Formats for MIX05 Data

Profile that would be put into CODIS

Different Reporting Formats for MIX05 Data

http://www.cstl.nist.gov/biotech/strbase/training.htm
Different Reporting Formats for MIX05 Data

The community would benefit from more uniform reporting formats and mixture solving strategies...

Value of the MIX05 Study

http://www.cstl.nist.gov/biotech/strbase/interlab/MIX05.htm

- Data sets exist with multiple mixture scenarios and a variety of STR kits that can be used for training purposes
- A wide variety of approaches to mixture interpretation have been applied on the same data sets evaluated as part of a single study
- Interpretation guidelines from many laboratories are being compared to one another for the first time in an effort to determine challenges facing future efforts to develop "expert systems" for automated mixture interpretation
- We are exploring the challenges of supplying a common data set to a number of forensic laboratories (e.g., if a standard reference data set was ever desired for evaluating expert systems)

NIST Software Programs to Aid Mixture Work

Excel-based programs developed by David Duewer (NIST)

- **mixSTR** (developed at request of Palm Beach Sheriff's Office)
  - Does not interpret data (relies on user inputted alleles following STR data review)
  - Aids in the organization of STR mixture information
  - Considers only the presence/absence of alleles (no peak heights used)

- **Virtual MixtureMaker** (developed to aid MIX05 sample selection)
  - Creates mixture combinations through pairwise comparisons of input STR profiles
  - Returns information on the number of loci possessing 0,1,2,3,4,5, or 6 alleles in each 2-person mixture (also reports number of loci in each sample with 0,1,2, or 3 alleles)
  - Useful for selection of samples in mixture or validation studies with various degrees of overlapping alleles in combined STR profiles
  - Useful in checking for potentially related individuals in a population database

Programs can be downloaded from NIST STRBase web site: http://www.cstl.nist.gov/div831/strbase/software.htm
Example of suspect to evidence (S/E) comparisons made in this case. Note that the suspect is 21,23 at FGA while the evidence contains 23,24* (* indicates that allele 24 is a minor component). Thus this suspect has allele 23 in common and is missing allele 24 in the evidence.

Virtual MixtureMaker Output

One locus with 5 alleles in this 2-person mixture

When the STR profiles for these two individuals are combined to create a 2-person mixture, the mixture profile will contain 1 locus with a single allele, 7 loci with two alleles, 4 loci with three alleles, and 3 loci with four alleles (and no loci with 5 or 6 alleles, which is only possible if one or both samples possess tri-allelic patterns at the same STR locus).

Some Final Thoughts...

• It is of the highest importance in the art of detection to be able to recognize out of a number of facts, which are incidental and which vital. Otherwise your energy and attention must be dissipated instead of being concentrated (Sherlock Holmes, *The Reigate Puzzle*).

• ”Don’t do mixture interpretation unless you have to” (Peter Gill, Forensic Science Service, 1998).

• Mixture interpretation consumes a large part of DNA analysts’ time – software tools that improve consistency in analysis will speed casework reporting and hopefully cases solved.

Conclusion

"Mixture interpretation theory is well established and used in forensic laboratories. Most mixtures detected in casework are satisfactorily solved. But from this revision we can conclude that the behaviour of each mixed sample can be different and multifactorial and occasionally its interpretation turns out to be complicated—sometimes paralleling the importance of the evidence in the resolution of the case. In some casework mixtures our experience has proved that theoretical assumptions from studies with laboratory samples, albeit very useful, can turn out to be impracticable. We consider that more sharing of day to day forensic laboratory problems is needed to refine our technical procedures in the resolution of specially difficult evidence."

Acknowledgments

Funding from interagency agreement 2003-IJ-R-029 between NIJ and the NIST Office of Law Enforcement Standards

The many forensic scientists and their supervisors who took time out of their busy schedules to examine the MIX05 data provided as part of this interlaboratory study
Relevant Literature on Mixture Interpretation

**General Information**


Schneider, P.M., Fimmers, R., Keil, W., Molsberger, G., Patzelt, D., Pflug, W., Rothamel, T., Schmitter, H., Schneider, H., Brinkman, B. (2006) General recommendations of the (German) stain commission on the interpretation of DNA results from mixed stains. *Rechtsmedizin* 16:401-404. (article in German)


**Mixture Detection and Component Profile Deconvolution**


**Designating True Alleles versus Artifacts**


**Expert System Software Approaches**


**Interlaboratory Studies on Mixture Interpretation**


**Statistical Calculations and Issues**


**Defense Attacks on Mixture Interpretation**


**Y-STRs Can Benefit Some Mixture Samples Compared to Autosomal STRs**


**Y-STR Mixture and Statistical Issues**


