Continuing Education Seminar

Wisconsin DNA Mixture Training

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

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Milwaukee, WI
May 12, 2009

Topics and Techniques for Forensic DNA Analysis
Continuing Education Seminar

Introductions

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Outline for Training Planned

May 12 (morning)

- Introductions
- Background Information and Surveys of Casework
- Principles in Mixture Interpretation

BREAK

- Deconvolution Worked Example

LUNCH

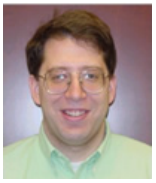
May 12 (afternoon)

- Properly Stating Conclusions
- Brief Review of Literature

BREAK

- Validation & Setting Thresholds
- Low-Level DNA Testing

Please Ask Questions Anytime!



Dr. John M. Butler

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

B.S. Chemistry
1992

Experience

- University of Virginia/FBI Laboratory (1992-1995)
  - Work performed in Bruce McCord's lab
- NIST NRC Postdoc (1995-1997)
- GeneTrace Systems Inc (1997-1999)
- NIST Human Identity Project Leader (1999-present)

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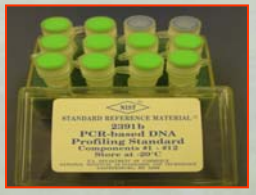
- Ph.D. dissertation (Aug 1995): "Sizing and quantitation of polymerase chain reaction products by capillary electrophoresis for use in DNA typing"
- *Forensic DNA Typing* textbook (now in its 2nd Edition)
- STRBase website: http://www.cstl.nist.gov/biotech/strbase/
- Family: wife Terilynne and 6 children
- Hobbies: reading, writing, and making PowerPoint slides

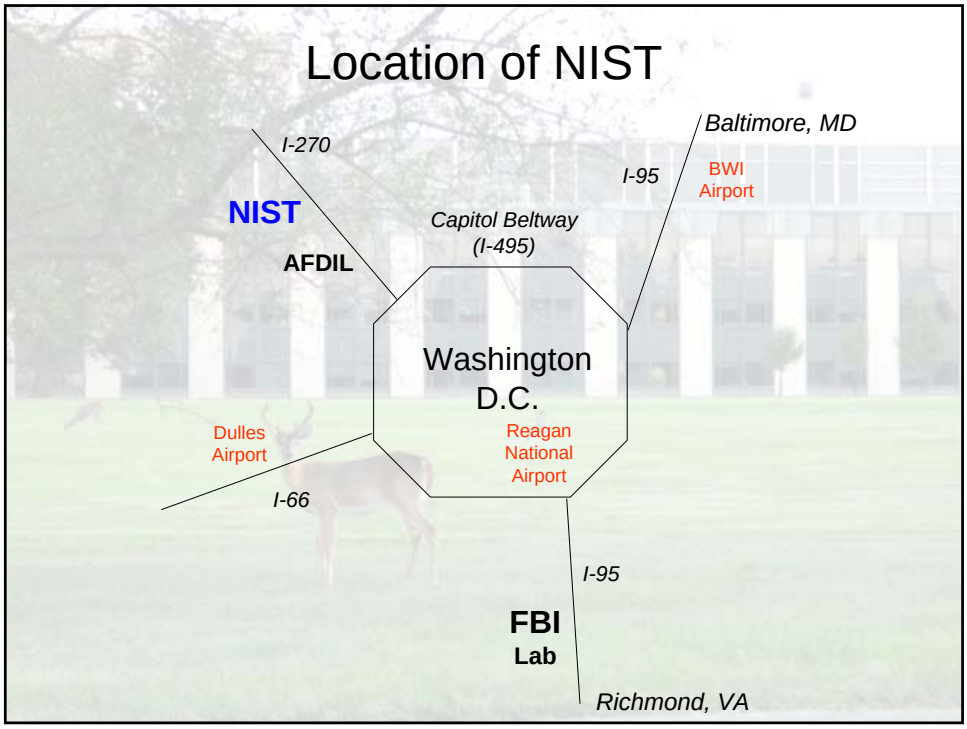
NIST History and Mission

- 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.**

$603 for 3 jars



DNA typing standard



NIST Human Identity Project Team

…Bringing traceability and technology to the scales of justice…


John Butler
Group Leader


Amy Decker


Becky Hill


Margaret Kline


Jan Redman


Pete Vallone


Dave Duewer
(data analysis)


Angie Dolph
(summer 2007)


Michelle Burns
(summer 2008+)

And many wonderful collaborators…

Since 2000:
>100 publications
>250 presentations
>30 training workshops

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

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

National Institute of Justice
The Research, Development, and Evaluation Agency of the U.S. Department of Justice

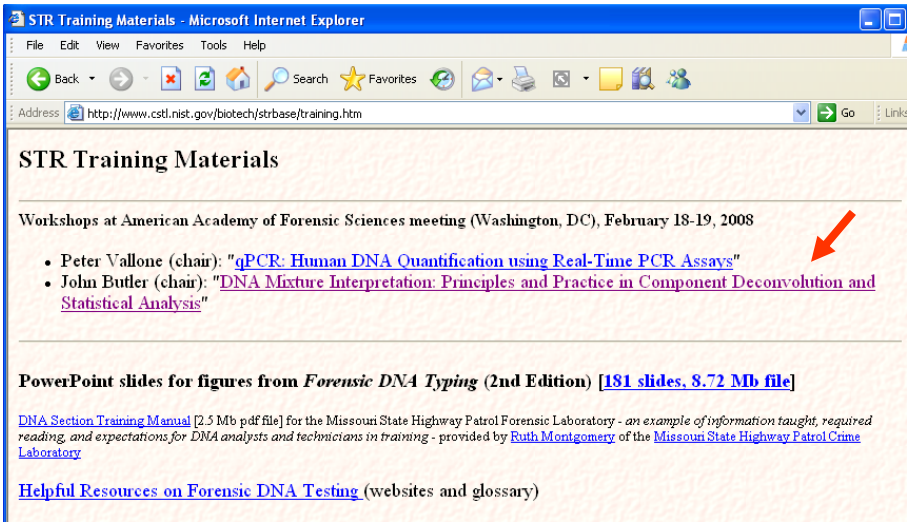
Current Areas of NIST Effort with Forensic DNA

- **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



Contributors to These Workshop Slides

Bruce McCord

Florida International University

CE

Angie Dolph

Marshall U./ NIST

Amy Decker

NIST

mixtures

NIST and NIJ Disclaimer

Funding: Interagency Agreement 2008-DN-R-121 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…

Topics and Techniques for Forensic DNA Analysis
Continuing Education Seminar

Principles of Mixture Interpretation

Wisconsin DNA Mixture Training

Milwaukee, WI
May 12, 2009

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

john.butler@nist.gov

Purpose for Teaching this Workshop

We hope that you:

- 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 for handling DNA mixtures in forensic casework

Mention of Mixtures in the July 2009 Revised Quality Assurance Standards (QAS)

- QAS Standard 5.3.2
  - **A casework CODIS administrator** shall be or have been a current or previously qualified DNA analyst … with **documented mixture interpretation training**.
- QAS Standard 8.3.1
  - **Internal validation studies** conducted after the date of this revision shall include as applicable: known and non-probative evidence samples or mock evidence samples, reproducibility and precision, sensitivity and stochastic studies, **mixture studies**, and contamination assessment. Internal validation studies shall be documented and summarized…
- QAS Standard 8.3.2
  - **Internal validation shall define quality assurance parameters and interpretation guidelines, including as applicable, guidelines for mixture interpretation.**
- QAS Standard 9.6.4
  - **Laboratories analyzing forensic samples shall have and follow a documented procedure for mixture interpretation that addresses major and minor contributors, inclusions and exclusions, and policies for the reporting of results and statistics**.

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SWGDAM Disclaimer…

Did anyone here attend this workshop?

DNA Mixture Interpretation:

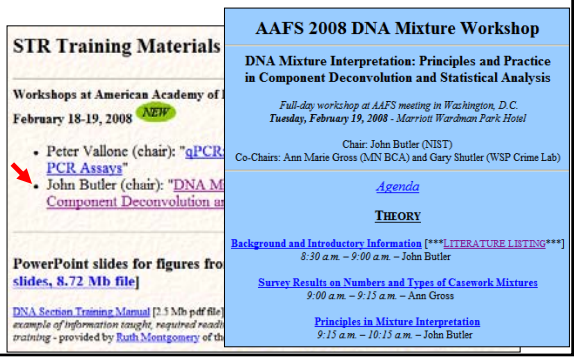
Principles and Practice in Component Deconvolution and Statistical Analysis

AAFS 2008 Workshop #16
Washington, DC
February 19, 2008

John M. Butler
Ann Marie Gross
Gary G. Shutler

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

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**

Afternoon Agenda – **Practical Application**

Real Case Example – Importance of Properly Stating Your Conclusions
1:15 p.m. – 1:30 p.m. – Gary Shutler

Variability between Labs in Approaches & Mixture Interlaboratory Studies
1:30 p.m. – 2:15 p.m. – John Butler

Validation Studies and Preparing Mixture Interpretation Guidelines
2:15 p.m. – 2:45 p.m. – Joanne Sgueglia

***2:45 p.m. – 3:00 p.m.* BREAK**

Testing of Mixture Software Programs
3:00 p.m. – 3:15 p.m. – Angela Dolph

DNA_DataAnalysis Software Demonstration
3:15 p.m. – 4:00 p.m. – Tim Kalafut

Training Your Staff to Consistently Interpret Mixtures
4:00 p.m. – 4:45 p.m. – Panel Discussion with Ann Gross, Gary Shutler, Joanne Sgueglia

***4:45 p.m. – 5:00 p.m.* – Questions and Answers as needed**

Mixture Basics

From J.M. Butler (2005) *Forensic DNA Typing, 2nd Edition*, p. 154

- 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.
- Differential extraction can help distinguish male and female components of many sexual assault mixtures.

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…

More on Mixtures...

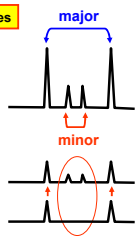
Most mixtures encountered in casework are 2-component mixtures
arising from a combination of victim and perpetrator DNA profiles

Torres *et al.* (2003) *Forensic Sci. Int.* 134:180-186 examined 1,547 cases from 1997-2000 containing **2,424 typed samples** of which **163 (6.7%) contained a mixed profile** with **only 8 (0.3%) coming from more than two contributors**

95.1% (155/163) were 2-component mixtures

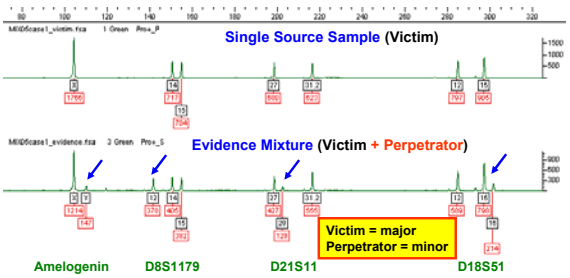
Ratios of the various mixture components stay fairly constant between multiple loci enabling deduction of the profiles for the major and minor components

Some mixture interpretation strategies involve using victim (or other reference) alleles to help isolate obligate alleles coming from the unknown portion of the mixture



http://www.cstl.nist.gov/biotech/strbase/interlab/MIX05.htm
MIX05 Case #1; Profiler Plus green loci

Example Mixture Data (MIX05 Study-Profiler Plus)



Obligate Alleles (not present in the victim reference)

Y	12	28	16

True "Perpetrator" Profile

X,Y	12,12	28,31.2	15,16

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)
  Victim Reference and Spouse or Boyfriend Reference
- **Contamination** of a single source sample from
  - evidence collection staff
  - laboratory staff handling the sample
  - Low-level DNA in reagents or PCR tubes or pipet tips

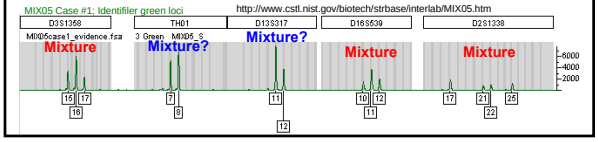
  Examine Staff Profiles (Elimination Database), etc.

Reference elimination samples are useful in deciphering both situations due to possibility of intimate sample profile subtraction

Mixtures: Issues and Challenges

From J.M. Butler (2005) *Forensic DNA Typing, 2nd Edition*, p. 155

- The probability that a mixture will be detected improves with the use of more loci and genetic 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.

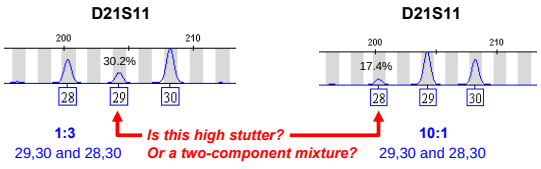


Detecting Mixtures

- 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



Gathered Case Summary Data

During 2007 and early 2008, **Ann Gross** (MN BCA) from the SWGDAM Mixture Interpretation Committee **coordinated the collection of case summary data** from **14 different forensic labs** who collectively reported on **4780 samples**.

A preliminary summary of this information is divided by crime classifications: sexual assault, major crime (homicide), and high volume (burglary). **Over half of the samples examined were single source and ~75% of all reported mixtures were 2-person.**

CFS Toronto Case Summary Data

Case type	N = 276		1	2	3	4	>4
			# contributors				
Sexual Assault	N = 152		42%	52%	7%	1%	--
High Volume	N = 56		69%	16%	16%	--	--
Major Crime	N = 68		59%	34%	7%	--	--
			Single source	Mixtures			

Mixture Case Summaries

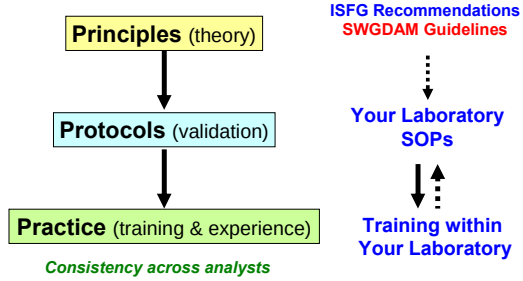
	minimum # of contributors					
Crime Class	1	2	3	4	>4	N
Sexual Assault	884	787	145	11	0	1827
Major Crime	1261	519	182	32	0	1994
High Volume	344	220	140	11	5	720
Total	2489	1526	467	54	5	4541
Single source	54.8%	33.6%	10.3%	1.2%	0.1%	mixtures

http://www.cstl.nist.gov/biotech/strbase/pub_pres/Promega2008poster.pdf

"Final" Data Set from 14 Different Labs

Plan to conduct further data analysis and publish results

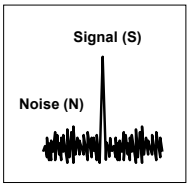
Elements of DNA Mixture Interpretation



We advocate periodic training to aid accuracy and efficiency of mixture interpretation within your laboratory.

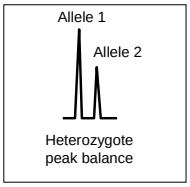
What is a true peak (allele)?

Peak detection threshold



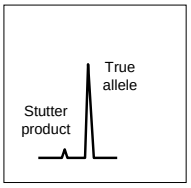
Signal > 3x sd of noise

Peak height ratio (PHR)



PHR consistent with single source
Typically above 60%

Stutter percentage



Stutter location below 15%

Setting Thresholds

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

what is a peak?

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

what is reliable PCR data?

Validation studies should be performed in each laboratory

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

Threshold Values

- Critical for proper interpretation of STR data
- Establish minimum RFU that a PCR product must display for quantitative and/or qualitative evaluation
- Signal-to-noise ratio is really irrelevant as PCR variability is the bigger issue (stochastic effects with low levels of DNA template)

Bruce Budowle, "Guidelines for the Interpretation of Mixtures", Promega 2008 meeting breakout session on mixture interpretation (Hollywood, CA) – Oct 15, 2008

Threshold 1

- A **Peak Amplitude Threshold (PAT)** must be established that operationally defines the minimum peak height in RFUs for confidently ascribing a true PCR amplicon peak
- Defines when confidence is high for peak assignment
- Quantitative threshold based on a signal-to-noise ratio (and may be slightly higher – i.e., 50 RFUs)
- May also be called "Detection Threshold"

Bruce Budowle, "Guidelines for the Interpretation of Mixtures", Promega 2008 meeting breakout session on mixture interpretation (Hollywood, CA) – Oct 15, 2008

Threshold 2

- A **Match Interpretation Threshold (MIT)** must be established based on empirical studies performed in your laboratory
  - FBI's MIT was 200 RFU and has now been lowered to 150 RFUs based on instruments getting better
- The minimum peak height in RFUs that all amplicon peaks at a given locus must display to confidently conclude that no genetic components of the sample failed to be detected due to stochastic affects (such as might occur with low copy number template)
  - Can exclude but not use statistics if alleles fall between PAT and MIT
- Necessary for avoiding standard interpretation where potential stochastic affects may result in allele drop out, peak height ratio variation, or non-reproducible results
  - This threshold does not apply to LCN
- May be called "Interpretation Threshold"

Bruce Budowle, "Guidelines for the Interpretation of Mixtures", Promega 2008 meeting breakout session on mixture interpretation (Hollywood, CA) – Oct 15, 2008

Two Thresholds

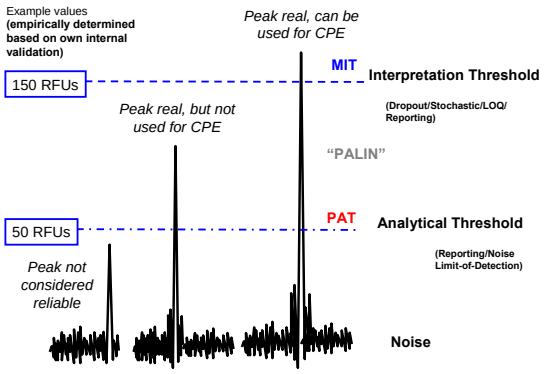
- Peak Amplitude Threshold (**PAT**)
- Match Interpretation Threshold (**MIT**)

Pat Buchanan

If between PAT and MIT, **can exclude** but not use statistics

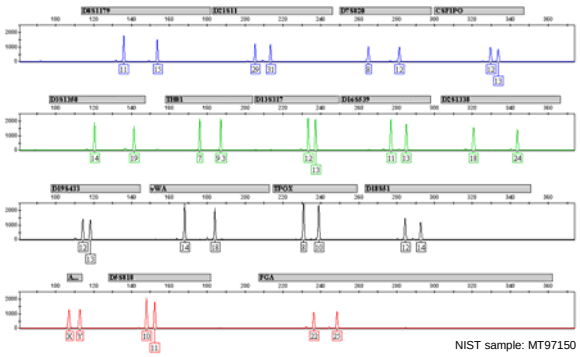
Mitt Romney

Different Thresholds



1 ng template DNA, 28 cycles

Identifiler data (full profile)



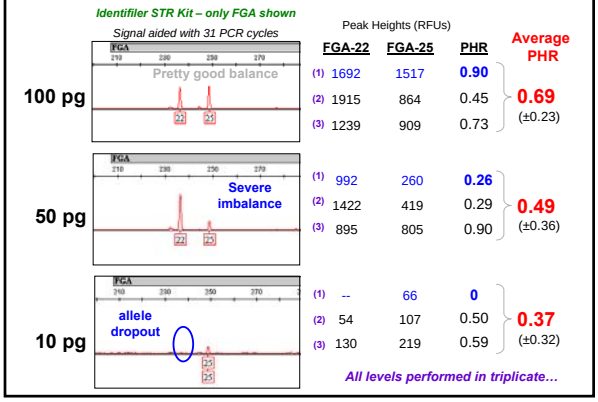
NIST sample: MT97150

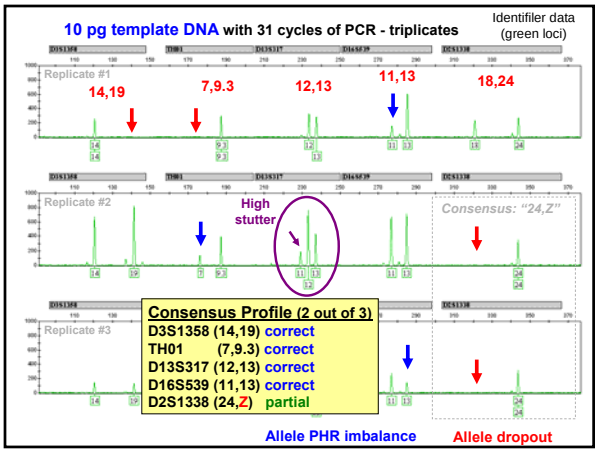
Peak Height Ratios (PHRs) all >0.80

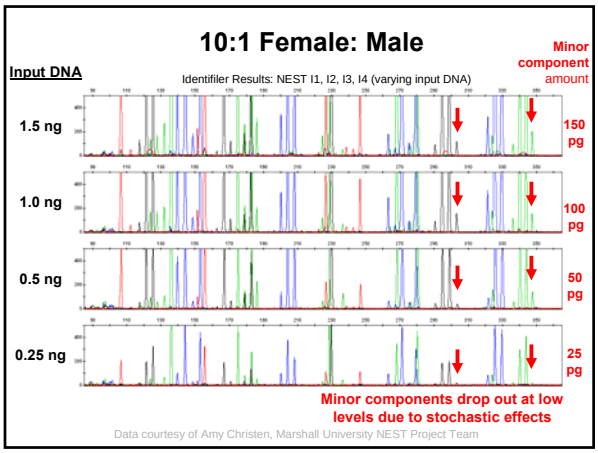
Reliable Mixture Interpretation Cannot Usually Be Performed with Low Level DNA

- Intra-locus peak height ratios vary significantly
- Stutter products can be artificially high
- Allele dropout occurs
- Allele drop-in confuses results
  - can only be caught with replicate amplifications and analyses

Peak Height Ratio Measurements







Statistical Approaches

Statistical Approaches with Mixtures

See Ladd *et al.* (2001) *Croat Med J.* 42:244-246

- **Inferring Genotypes of Contributors** - Separate major and minor components into individual profiles and compute the random match probability estimate as if a component was from a single source
- **Calculation of Exclusion Probabilities** - CPE/CPI (RMNE) – The probability that a random person (unrelated individual) would be excluded as a contributor to the observed DNA mixture
- **Calculation of Likelihood Ratio Estimates** – Comparing the probability of observing the mixture data under two (or more) alternative hypotheses; in its simplest form LR = 1/RMP

RMNE = Random Man Not Excluded (same as CPE)
CPE = Combined Probability of Exclusion (CPE = 1 – CPI)
CPI = Combined Probability of Inclusion (CPI = 1 – CPE)

Advantages and Disadvantages

RMNE (CPE/CPI)

- **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

Likelihood Ratios (LR)

- **Advantages**
  - Enables full use of the data including different suspects
- **Disadvantages**
  - More difficult to calculate

Summarized from John Buckleton, *Forensic DNA Evidence Interpretation*, p. 223

Assumptions for CPE/CPI Approach

- **There is no allele dropout** (i.e., all alleles are above stochastic threshold) – low-level mixtures can not reliably be treated with CPE
- All contributors are from the same racial group (i.e., you use the same allele frequencies for the calculations)
- All contributors are unrelated
- Peak height differences between various components are irrelevant (i.e., **component deconvolution not needed**) – this may not convey all information from the available sample data…

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, $\mathbf{H_d}$, 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
http://www.fbi.gov/hq/lab/fsc/backissu/july2000/dnastat.htm

"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)
  - Devlin, B. (1993) Forensic inference from genetic markers. *Statistical Methods in Medical Research* 2: 241–262.
- Likelihood ratios (LR)
  - Evett, I. W. and Weir, B. S. (1998) *Interpreting DNA Evidence*. Sinauer, Sunderland, Massachusetts.

ISFG DNA Commission on Mixture Interpretation

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

Available for download from the ISFG Website:
http://www.isfg.org/Publication;Gill2006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DNA commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures

P. Gill, C.H. Brenner, J.S. Buckleton, A. Carracedo, M. Krawczak, W.R. Mayr, N. Morling, M. Prinz, P.M. Schneider, B.S. Weir

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

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


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Editorial

Editorial on the recommendations of the DNA commission of the ISFG on the interpretation of mixtures

"…**These recommendations have been written** to serve two purposes: to define a generally acceptable mathematical approach for typical mixture scenarios and to address open questions where practical and generally accepted solutions do not yet exist. This has been done **to stimulate the discussion among scientists in this field**. **The aim is to invite proposals and criticism in the form of comments and letters to the editors of this journal**…**We are hoping to continue the process to allow the DNA Commission to critically revise or extend these recommendations in due time…"**

Responses to ISFG DNA Commission Mixture Recommendations

- UK Response
  - Gill *et al.* (2008) *FSI Genetics* 2(1): 76–82
- **German Stain Commission**
  - Schneider *et al.* (2006) *Rechtsmedizin* 16:401-404 (German version)
  - Schneider *et al.* (2009) *Int. J. Legal Med.* 123: 1-5 (English version)
- ENFSI Policy Statement
  - Morling *et al.* (2007) *FSI Genetics* 1(3):291–292
- New Zealand/Australia Support Statement
  - Stringer *et al.* (2009) *FSI Genetics* (in press)
- **SWGDAM** – nothing yet…
  - a Mixture Interpretation subcommittee was started Jan 2007

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)
- Additional Y-STRs - nomenclature (2006)
- **Mixture Interpretation (2006)**
- Disaster Victim Identification (2007)
- Biostatistics for Parentage Analysis (2007)

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

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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

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

Adapted from Peter Schneider slide (presented at EDNAP meeting in Krakow in April 2007)

Mixture Classification Scheme

Schneider *et al.* (2006) *Rechtsmedizin* 16:401-404

(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

Type A **Type B** **Type C**

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.

Slide from Peter Schneider (presented at EDNAP meeting in Krakow in April 2007)

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

Slide from Peter Schneider (presented at EDNAP meeting in Krakow in April 2007)

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.

Slide from Peter Schneider (presented at EDNAP meeting in Krakow in April 2007)

Which approach should be used?

- If major/minor contributors cannot be identified based on unambiguous DNA profiles, or if the 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.

Slide from Peter Schneider (presented at EDNAP meeting in Krakow in April 2007)

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.

Slide from Peter Schneider (presented at EDNAP meeting in Krakow in April 2007)

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.

Slide from Peter Schneider (presented at EDNAP meeting in Krakow in April 2007)

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

Slide from Peter Schneider (presented at EDNAP meeting in Krakow in April 2007)

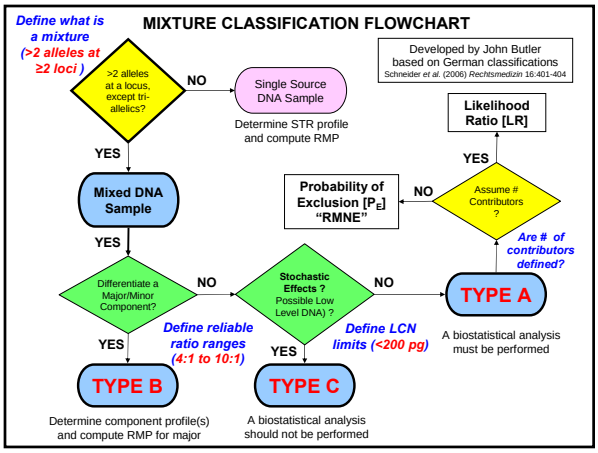
GEDNAP 32

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

Slide from Peter Schneider (presented at EDNAP meeting in Krakow in April 2007)



German Type A,B, and C mixture classifications

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

Summary of ISFG Recommendations on Mixture Interpretation

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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

Steps in Mixture Deconvolution

(Clayton *et al.* 1998)

Step #1 Identify the Presence of a Mixture

↓

Step #2 Designate Allele Peaks

↓

Step #3 Identify the Number of Potential Contributors

↓

Step #4 Estimate the Relative Ratio of the Individuals Contributing to the Mixture

↓

Step #5 Consider All Possible Genotype Combinations

↓

Step #6 Compare Reference Samples


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Analysis and interpretation of mixed forensic stains using DNA STR profiling

T.M. Clayton*, J.P. Whitaker, R. Sparkes, P. Gill

Clayton *et al.* (1998) *Forensic Sci. Int.* 91:55-70

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: Recommendations on the interpretation of mixtures. *Forensic Sci. Int.* 160: 90-101

Consideration of Peak in Stutter Position

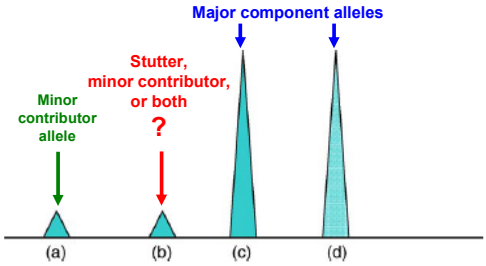
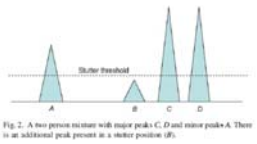


Fig. 4. *c* and *d* are unambiguous alleles, *b* is a minor allele in a stutter position and *a* is an unambiguous minor allele.

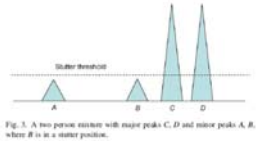
Gill *et al.* (2006) DNA Commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures. *Forensic Sci. Int.* 160: 90-101

ISFG Recommendation #6 Example



Likely a AA

(homozygote)



Possibly AB

(heterozygote)

Could also be AC, AD, AA, or A,? (dropout)

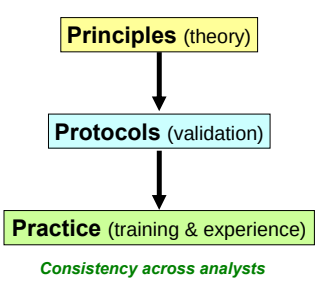
A Few of the Responses…

from the Mixture Workshop Questionnaires (Nov 2007 and May 2008)

Do you have a decision point whereby you consider a mixture too complicated and do not try to solve it?

- 3+ contributors, except determination of a clear major; may give include/exclude, but not completely resolve
- no pre-set guidelines, left to analyst discretion
- 2+ contributors with little variation in peak heights, close to 1:1 ratio
- Our decision point usually comes after 3 hours of discussions with other analysts and a lot of "but what about this… and this…" at which point we decide if we're all so unsure, it would be risky to interpret (and therefore deem it "inconclusive")

Elements of DNA Mixture Interpretation



ISFG Recommendations
SWGDAM Guidelines

Your Laboratory SOPs

Training within Your Laboratory

Consistency across analysts

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

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)
- Terry Coons (OR)
- Roger Frappier (CFS-Toronto)
- Ann Gross (MN BCA)
- Bruce Heidebrecht (MD)
- Phil Kinsey (MT)
- Jeff Modler (RCMP)
- Tamyra Moretti (FBI DNA Unit I)
- Steven Myers (CA DOJ)
- Joanne Sgueglia (MA)
- Gary Shutler (WA)

Everyone not at every meeting…

Have met 5 times:
Jan 2007
July 2007
Jan 2008
July 2008
Nov 2008

Through the Jan 2008 meeting we have also had to deal with Y-STR issues – which has limited our focus on mixtures

Additional Participants (Jan 2008)
Cecelia Crouse (PBSO)
Allison Eastman (NYSP)
Steve Lambert (SC)

Also at Gaithersburg mtg (Nov 2008)
Todd Bille (ATF)
Hiron Poon (RCMP)

From John Butler's CODIS User's Mixture mini-Workshop

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

CE User's Group (December 5, 2008)

- Bruce Heidebrecht organized
- Held at Maryland State Police Forensic Lab
- Presentations & discussion on 4 mixture cases
- ~60 people attended from 16 labs
- Bruce has developed several helpful tools for mixtures…

I will review some of these mixture cases with my worked examples

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

Topics and Techniques for Forensic DNA Analysis

Continuing Education Seminar

Importance of How Conclusions Are Stated

Wisconsin DNA Mixture Training

Milwaukee, WI

May 12, 2009

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

john.butler@nist.gov

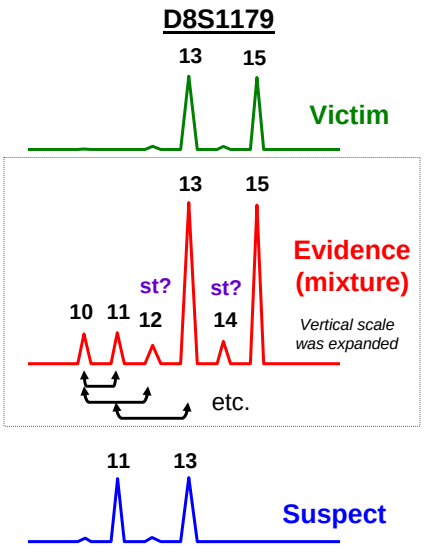
Fox News Atlanta Story

http://tinyurl.com/MixedSampleDNA

- **I-Team: The GBI and DNA**
- **Monday, 27 Apr 2009, 7:08 PM EDT**
- **http://www.myfoxatlanta.com/dpp/news/ITeam_The_GBI_and_DNA_042709**

- **I-Team: The GBI and DNA Part 2**
- Tuesday, 28 Apr 2009, 10:50 PM EDT
- http://www.myfoxatlanta.com/dpp/news/ITeam_The_GBI_and_DNA_Pt_2_042809

Another Mixture Example



Conclusions from the evidence:

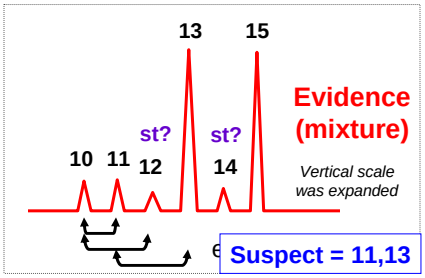
1. **Major contributor = 13,15 (victim) –** *to be expected with an intimate sample like a fingernail or vaginal swab*
2. Alleles 12 and 14 are **likely stutter products** of the major contributor's 13 and 15 alleles but could also be masking minor contributor alleles
3. A number of minor contributor 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

"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…

Probability of Exclusion Calculation

for a Single STR Locus

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



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…

From VA DFS STR Allele Frequencies

http://www.dfs.virginia.gov/manuals/manuals.cfm?id=5

D8S1179 alleles	AA (n=384)	C (n=346)	H (n=366)
10	0.0287	0.1069	0.0820
11	0.0495	0.0925	0.0465
12	0.1094	0.1416	0.1093
13	0.2422	0.3093	0.3224
14	0.2969	0.1965	0.2623
15	0.1849	0.0896	0.1202
SUM	0.9115	0.9364	0.9426
Sq SUM = PI	0.8308	0.8769	0.8886
PE = 1-PI	0.1692	0.1231	0.1114
PE (%)	**16.9%**	**12.3%**	**11.1%**
	African Am.	**Caucasians**	**Hispanics**

"Suspect cannot be excluded" BUT we would expect to see, for example, only 11.1% of Hispanics excluded (or 88.9% cannot be excluded) based on results at this one locus

Relevant Literature on Mixture Interpretation

General Information

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REVIEW ARTICLE

The German Stain Commission: recommendations for the interpretation of mixed stains

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Abstract In the course of forensic DNA analysis, the interpretation of DNA profiles of mixed stains, i.e. cell material from more than a single donor, has become increasingly more important. The German Stain Commission, a joint commission of Institutes of Forensic Science and Legal Medicine, has therefore developed guidelines aiming to harmonize the evaluation of mixed stains in German criminal cases.

Keywords Short tandem repeat typing · Biostatistical analysis · Likelihood ratio · Probability of exclusion · Mixtures

Preface

Since the beginning of forensic stain analysis, mixed stains have been observed [1, 2]. Over the past few years, they have

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gained importance as evidence due to improved analytical methods and the enormous increase in the numbers of investigated stains [3, 4]. While the interpretation of single source stains usually does not cause problems [5], the evaluation and interpretation of mixed DNA stains requires particular attention [6–8]. Our recommendations – first published in German [9] – are intended to build a framework for an adequate means of treating typical cases. However, it is beyond the scope of these basic recommendations to address all possible constellations.

Definitions

A stain exhibiting more than two alleles in a single DNA system[1] shall be considered a mixed stain except in the case of genetic irregularities (e.g., trisomy, somatic mosaicism, or duplication). If more than two alleles are observed in at least two DNA systems, the presence of a mixed stain shall be assumed.

The number of possible contributors to a mixed stain shall be derived, if possible:

- In general, the presence of not more than four alleles in a given system allows the assumption of at least two independent stain donors.
- In general, the presence of not more than six alleles in a given system allows the assumption of at least three independent stain donors.
- In general, if more than six alleles are observed in a given system, the exact number of stain donors cannot be reliably determined.

Classification of mixed stains

Type A has no obvious major contributor with no evidence of stochastic effects.[2] Type B has clearly distinguishable major and minor DNA components; consistent peak height ratios of approximately 4:1 (major to minor component) across all heterozygous systems, and no evidence of stochastic effects. Type C has mixtures with no major component(s) and evidence of stochastic effects.

[1] A DNA system is a genetic locus exhibiting a short tandem repeat polymorphism amplified with a pair of defined primers using the polymerase chain reaction (PCR).

[2] DNA profiles obtained from the amplification of samples with low DNA content and/or poor DNA quality, where the occurrence of allelic drop out and/or locus drop out has to be assumed.

Evaluation criteria

Peak analysis

The morphology of a peak shall be typical and fully consistent with an allele of a given short tandem repeat system. Generally, reproducible peaks with heights >50 relative fluorescence units (RFU) can be considered regular peaks if the noise of the baseline is low and the number of PCR cycles recommended by the manufacturer was used.

The presence of peaks exhibiting a low signal strength (i.e., typically below 100 RFU) and/or peaks exhibiting clearly variable intensities shall be annotated in the table of observed alleles. Tables in the final report shall be accompanied by a legend explaining the designations of peak characteristics.

Stutter peaks

Both $n-1$ and $n+1$ stutter peaks may occur. Their heights depend on the DNA systems and the amplification conditions. A stutter peak may, in certain cases, exhibit up to 15% of the height of the corresponding main peak. Furthermore, the following shall be considered for the evaluation of a stutter peak:

- The relative stutter intensities of the alleles of a locus, as well as those between loci of a multiplex amplification.
- The possibility that a stain allele is in the position of a stutter peak.

In case of reasonable doubt, a peak in the position of a stutter peak shall be considered a true allele and part of the DNA profile and shall be included in the biostatistical calculation.

Inclusion/exclusion criteria

Inclusion

If all alleles of a person in question are uniformly present in a mixed stain, the person shall be considered a possible contributor to the stain.

Exclusion

If alleles of a person in question are not present in a mixed stain, the person shall not be considered as a possible contributor to the stain.

Grey area between inclusion and exclusion

The following effects may occur in type C mixtures due to imbalances between the mixture components and may cause

difficulties in reaching an unambiguous decision about inclusion or exclusion across all analyzed DNA systems:

- Locus drop out and allelic drop out (e.g., caused by the sensitivity of the amplification system, as well as by stochastic effects).
- Allelic drop out is more likely to occur for longer than for shorter alleles, and in particular for DNA systems with long amplicon sizes.

Additional criteria

In every case, the decision about inclusion or exclusion shall be made after careful consideration of the issues described under the "Grey area between inclusion and exclusion" section. The reasons shall be explained in detail. If appropriate, it shall be stated why a clear decision about inclusion or exclusion was not possible.

Biostatistical calculations for mixed stains

Basis

The basis for all calculations is the knowledge of the allele frequencies in the relevant population.

Probability of exclusion (P_E)/probability of inclusion (P_I)

P_I represents the combined probability (relative population frequency) of all combinations of genotypes that cannot be excluded to have contributed to the DNA profile of a stain based on the criteria given in the "Inclusion" section. P_I is equivalent to the match probability in the case of a stain originating from a single person.

The calculation of P_I is independent of assumptions about the number of possible contributors to a stain, the genotypes, and the ethnic origin of persons involved in a given case. It is equivalent to the probability that a randomly selected person is a contributor to the stain [=random man not excluded (RMNE)]. The probability of exclusion $P_E = 1 - P_I$ indicates the probability of excluding a randomly selected person as a contributor to a given stain.

Likelihood ratio

The calculation of the likelihood ratio (LR) is based on the assumption of two mutually excluding hypotheses. This imperatively requires the description of a distinct scenario for a given stain case. Both hypotheses explicitly describe alternative scenarios for the origin of a stain. Each of these hypotheses shall clearly state who contributed to the stain and how many unknown contributors are assumed. Then, a calculation of the likelihood for the occurrence of the DNA profile of the stain is performed based on the assumption of the respective hypotheses: $L(\text{stain}|H)$. The LR

$$\text{LR} = \frac{L(\text{stain}|H_1)}{L(\text{stain}|H_2)}$$

allows the evidential value of a stain to be calculated with reference to a specific person involved in a case, e.g., an accused stain donor.

Given a two-person mixed stain M and that all observed alleles can be explained by the genotype of the victim, G_v, and the genotype of the suspect, G_s, the hypotheses can be formulated as follows:

Hypothesis H_p (view of the prosecution): The stain M originates from the victim V and the suspect S.

Hypothesis H_d (view of the defense): The stain originates from the victim V and from an unknown person U unrelated to the suspect.

$$\text{LR} = \frac{L(M|H_p)}{L(M|H_d)} = \frac{L(M|G_v, G_s)}{L(M|G_v, G_u)}$$

The resulting LR provides a numerical value, which indicates how many times more likely the observed DNA profile is under the assumption of the scenario described in H_p compared to the scenario described in H_d.

Procedures

Calculation for a mixed stain with an unambiguous major component from one person

The conclusion of a major DNA profile from a single contributor in a mixed stain shall only be drawn if a peak height ratio of at least 4:1 (major vs minor component) is observed across all heterozygous DNA systems (see "Definitions" section). In this case, the major DNA profile can be considered equivalent to that of a stain originating from a single person, and all calculations can be performed accordingly.

Calculation based on the LR

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, e.g., the suspect, is part of the minor component of the mixture (see "Definitions" section)]

then the calculation of a LR is appropriate.

Calculation based on probability of exclusion/inclusion

If a major DNA profile cannot be identified based on unambiguous DNA profiles, or if the number of contributors cannot be determined, calculations of the probability of exclusion P_E or the probability of inclusion P_I, respectively, for randomly selected persons is appropriate. Also, the calculation of P_E and P_I is always possible for type A and type B mixtures.

Supplementary recommendations

Further calculations that may result in erroneous interpretations of the evidence shall not be performed (e.g. reporting the genotype frequency of a non-excluded suspect, if the mixed stain does not allow a meaningful biostatistical interpretation).

Validated computer programmes for the calculation of complex mixed stains are available.

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Appendix

Examples of the calculations of P_I and P_E

The probability of inclusion P_I is calculated from the sum of all genotypes of possible stain contributors. In a stain case, where a, b, and c denote the alleles of a DNA system detected in the mixture, the sum of all relevant genotypes can be calculated as follows (assuming that allele frequency data conform to Hardy–Weinberg equilibrium):

$$P_I = a^2 + b^2 + c^2 + 2ab + 2bc + 2ac$$

This term can be simplified using the formula for the binominal distribution:

$$a^2 + b^2 + c^2 + 2ab + 2bc + 2ac = (a + b + c)^2$$

Assuming a frequency of 0.1 for alleles a, b, and c, the following result is obtained:

$$P_I = 0.3^2 = 0.09$$

Thus, it is expected that 9% of a group of randomly selected persons will not be excluded as stain contributors. This is equivalent to one out of 11 randomly selected persons (=RMNE). The probability of exclusion is calculated from the difference

$$P_E = 1 - P_I = 1 - 0.09 = 0.91$$

Thus, it is expected that 91% of a group of randomly selected persons will be excluded as stain contributors. For several DNA systems, S_1, S_2,…, S_n, which are genetically unlinked (i.e., in linkage equilibrium), the general expression of $P_E(S_1, S_2,\ldots, S_n)$ can be derived from the product of the individual inclusion probabilities $P(S_j)$ as follows:

$$P_E(S_1, S_2, \ldots, S_n) = 1 - [P_I(S_1) \cdot P_I(S_2) \cdot \ldots \cdot P_I(S_n)]$$

Examples for the calculation of the LR

Simple scenario

Consider a case with a mixed stain M with three alleles, a, b, and c, composed from a victim and a perpetrator. The victim V has the genotype AB, and the suspect S has the genotype BC. The hypotheses can be given as follows:

> H_p: The stain M originates from the victim V and the suspect S.
> H_d: The stain M originates from the victim V and from an unknown person unrelated to the suspect.

Let us first derive the numerator of the LR. The prosecution claims that the stain can be explained by a combination of the genotypes of the victim and the suspect, as there are no unaccounted alleles. Hence, the numerator results as

$$L(M|H_p) = L(M|G_v, G_s) = 1$$

The defense, however, claims that the suspect has not contributed to the stain. The genotype of the suspect is not relevant since the presence of allele c in the mixture must be explained by the contribution of an unknown person. As allele c may have been contributed either by a person homozygous for allele c or from a person heterozygous for c in combination with allele a or b, the denominator is as follows:

$$L(M|H_d) = L(M|G_v, G_u) = 2ac + 2bc + c^2$$

And, thus, the entire expression is given as

$$\mathrm{LR} = \frac{1}{2ac + 2ab + c^2}$$

Assuming a frequency of 0.1 for alleles a, b, and c, the following result is obtained:

$$\mathrm{LR} = \frac{1}{0.02 + 0.02 + 0.01} = \frac{1}{0.05} = 20$$

The result can be described by the following statement: It is 20 times more likely to observe the DNA profile if the mixed stain originated from the victim and the suspect than if it originated from the victim and an unknown person (who is unrelated to the suspect[3]).

Complex scenario

Let us consider a case with a mixed stain M with four alleles a, b, c, and d found on the victim's clothes. The victim's genotype is EF and, hence, the corresponding alleles e and f are not observed in the stain. Suspect S has genotype AB, but there is no known second person who may have contributed the alleles c and d. The hypotheses can be given as follows:

H_p: Stain M originates from suspect S and an unknown person U.
H_d: Stain M originates from two unknown persons $U1$ and $U2$.

The prosecution claims that the stain can be explained by a combination of the suspect's genotype and a second person with the genotype CD. Hence, the numerator results as

$$L(M|H_p) = L(M|G_s, G_u) = 2cd$$

The defense claims that no genotypes of the contributors are known. Thus, the sum of all possible genotype combinations from two persons $U1$ and $U2$ must be considered for the denominator:

Genotypes $U1$	$U2$	Combined frequency $U2$
AB	CD	$2ab \times 2cd = 4abcd$
AC	BD	$4abcd$
AD	BC	$4abcd$
BC	AD	$4abcd$
BD	AC	$4abcd$
CD	AB	$4abcd$
$L(M\|H_d) = L(M\|G_{U1}, G_{U2}) =$		$24abcd$

After reducing the term and by assuming a frequency of 0.1 for alleles a, b, c, and d, the following result is obtained:

$$\mathrm{LR} = \frac{2cd}{24abcd} = \frac{1}{12ab} = \frac{1}{0.12} = 8.3$$

[3] A familial relationship between S and the unknown stain contributor can be considered for calculating LR. However, the exact degree of relationship must be known.

Thus, it is eight times more likely to observe the DNA profile if the mixed stain originated from the suspect and an unknown person than if it originated from two unknown persons. If two suspects $S1$ and $S2$ with the genotypes AB and CD are considered for the same mixed stain scenario, the hypotheses and, hence, the LR change, as no unknown person remains for H_p:

H_p: Stain M originates from the suspects $S1$ and $S2$.
H_d: Stain M originates from two unknown persons $U1$ and $U2$.

Thus, the numerator of the LR is, again, 1. The term cannot be reduced further and the resulting LR is as follows:

$$\mathrm{LR} = \frac{1}{24abcd} = \frac{1}{0.0024} = 416.7$$

Thus, it is 416 times more likely to observe the DNA profile if the mixed stain originated from suspects $S1$ and $S2$ than if it originated from two unknown persons.

We give the following caveat: Additional hypotheses, which are not discussed here, can be formulated. Depending on the precise scenario, such additional hypotheses may be highly relevant in a given case, such as (a) H_p: the stain originates from $S1$ and $S2$; H_d: the stain originates from $B1$ and U, or (b) H_p: the stain originates from $S1$ and $S2$; H_d: the stain originates from $S2$ and U. Depending on the genotype frequencies of $S1$ and $S2$, the resulting LRs may differ significantly.

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Letter to the Editor

National recommendations of the Technical UK DNA working group on mixture interpretation for the NDNAD and for court going purposes

Abstract

The Technical UK DNA working group comprises representatives from all of the major suppliers of the UK and Ireland who contribute to the UK national DNA database. The group has the following terms of reference:To act as a peer review body.To agree experimental designs, to provide advice to the custodian to facilitate the development of the NDNAD.To support the CJS by the development of a coordinated UK strategy.To be inclusive, rather than exclusive, with regard to the introduction and use of methods.To define best scientific practice.To define guidelines for analysis and interpretation of evidence.To produce guidance that can be used by the UK Accreditation Services (UKAS).The group falls under the European Network of Forensic Science Institutes (ENFSI) umbrella. We will feed back recommendations to the ENFSI group for further discussion in order to facilitate European Policy.

The group recently met in order to consider in detail the ISFG DNA Commission recommendations on the interpretation of mixtures, to place them in the context of the UK jurisdictions.

Keywords: ISFG DNA commission; Mixtures; Technical UK DNA working group

1. Introduction

This group recognises that a diversity of (statistical) results will be achieved that are dependent upon the precise method used in the analysis of a sample for DNA profiling purposes. These statistical differences inevitably result from the efficiency or the sensitivity of the methods used: e.g. extraction protocols, injection times, PCR cycle number, can all contribute to differences in the resultant DNA profile. For a given crime stain, this means that complete or partial profiles may be obtained between laboratories and consequently the statistical results will also differ between laboratories.

However, we do not intend to standardise on particular methodology, neither do we intend to be prescriptive, recognising that all processes are subject to continuous improvement. It is the province of individual laboratories to drive change and to decide their protocols. Rather, our aim is to derive a set of simple guidelines that can be applied to all DNA profiles independent of the method used. Over time it will be necessary to update the recommendations.

Whereas differences in statistical results will still remain between methods and laboratories, the intent is to produce consistency such that different scientists who analyse results for a given DNA profile will produce similar statistical results. Standardisation of interpretation methodology demonstrates peer acceptance, and consequently gives the courts confidence that methods are widely accepted. Our aim is to facilitate peer review via the ENFSI group and the other major scientific bodies.

Key to achieving this is development of guidelines and defining their use. Guidelines are currently applied in association with thresholds. These thresholds are determined experimentally and are specific to each process or method used and may be specific to a particular laboratory. The most important is the 'dropout' threshold. This is applied whenever dropout has to be invoked to support a prosecution hypothesis (H_p) such as suspect alleles = *ab*; crime-stain allele = *a*. The evidence can only be explained under H_p if allele *b* has dropped out. However, in turn, this proposition can only be justified if the survivor allele is small enough such that the probability of dropout is less than one. Conversely, if Pr(*D*) approaches zero then the suspect is excluded since the conclusion must be that the donor is *aa*. The determination of this threshold is derived experimentally. The threshold is a guideline.

The second guideline is in relation to the interpretation of stutters. Here the problem is similar—if the suspect is *aa* and the crime-stain is *ab*, where *b* is in a stutter position, then clearly a consideration is required whether the peak can be a stutter, an allele or both. Again, experimentation is required to determine a 'stutter threshold' that can be used relative to associated guidelines. Stutter thresholds may also be technique dependent.

We have considered the International Society of Forensic Genetics (ISFG) DNA commission recommendations below in order to agree the UK recommendations for DNA reporting and submission of samples to the National DNA database—we have taken into account our 'local' considerations; court-going

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experiences; and appeal court recommendations in arriving at our stated position.

2. Response to the ISFG DNA commission 'recommendations on the interpretation of mixtures [1]'

2.1. Recommendation 1

''The likelihood ratio is the preferred approach to mixture interpretation. The RMNE approach is restricted to profiles where the profile is unambiguous. If the DNA crime-stain profile is low level and some minor alleles are the same size as stutters of major alleles, and/or if dropout is possible, then the RMNE method may not be conservative''.

2.1.1. Response

Conservativeness applies in the 'criminal context' only—civil disputes (such as paternity) should not be biased towards either the complainant or the defendant.

RMNE is a recognised and advocated interpretation method. The likelihood ratio and match probability methods are interchangeable—however, the wording of the match probability is equally acceptable for understanding in court. In addition, a frequency calculation can be used, e.g. ''I have calculated that the chance of observing this combination of DNA markers is about in 1 in X of the UK population'' or ''the chance that a person picked at random from the general UK population would have this combination of DNA markers is about 1 in X''.

If a profile can be identified with confidence from a mixture then the match probability statement may be preferable. A non-exhaustive list of examples is as follows:

(a) There is a major/minor mixture where the major contributor can be easily separated from the minor contributor(s) by virtue of the differences in peak height/area of the alleles.
(b) It may be possible to condition on one contributor, e.g. a victim, and to subtract this profile from the mixture, to leave a single contributor that can be reported separately. The contributors may be even, or major/minor. If the evidential profile is not major then it is inevitable that the conditioned major profile will mask some of the minor contributor alleles. Consequently, if a match probability is reported, some of the minor contributor alleles will be masked by the major contributor. The LR method may be preferred if this is the case.
(c) When conditioning is used to subtract a profile, then this should be made clear in the statement. If conditioning is challenged, then it may be appropriate to recalculate the strength of the evidence using the LR approach. A caveat can be included in the statement to make this point clear.

2.2. Recommendation 2

''Even if the legal system does not implicitly appear to support the use of the likelihood ratio, it is recommended that the scientist is trained in the methodology and routinely uses it in case notes, advising the court in the preferred method before reporting the evidence in line with the court requirements. The scientific community has a responsibility to support improvement of standards of scientific reasoning in the court-room''.

2.2.1. Response

Accepted—albeit we prefer to think in terms of advising the justice system rather than the court or court-room.

2.3. Recommendation 3

''The methods to calculate likelihood ratios of mixtures (not considering peak area) described by Evett et al. and Weir et al. are recommended'' (see [1] for the references cited).

2.3.1. Response

All laboratories in the UK consider peak height/area in their assessments. The formulae are fundamental to all mixture interpretation with or without peak height/area consideration.

2.4. Recommendation 4

''If peak height or area information is used to eliminate various genotypes from the unrestricted combinatorial method, this can be carried out by following a sequence of guidelines based on Clayton et al.'' (see [1] for the reference cited).

2.4.1. Response

Accepted.

2.5. Recommendation 5

''The probability of the evidence under H_p is the province of the prosecution and the probability of the evidence under H_d is the province of the defence. The prosecution and defence both seek to maximise their respective probabilities of the evidence profile. To do this both H_p and H_d require propositions. There is no reason why multiple pairs of propositions may not be evaluated''.

2.5.1. Response

Accepted.

2.6. 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.

2.6.1. Response

Stutters are locus-dependant. The Applied Biosystems SGM plus manual lists maximum experimentally observed stutter sizes per locus (St_{max}) where St_{max} is also utilised as the stutter threshold (described below). It is recommended that laboratories make their own St_{max} determinations since the effects

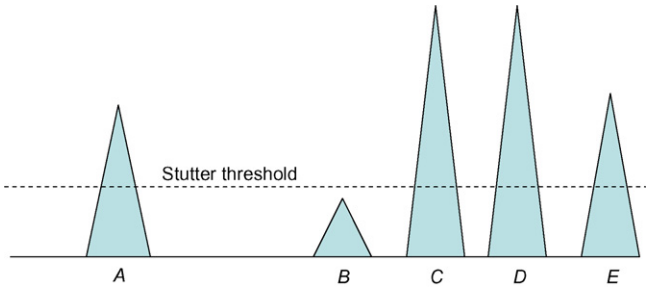

Fig. 1. A two-person mixture with major peaks *C*, *D* and minor peaks *A*, *E*. There is an additional peak present in a stutter position (*B*).

may be technique dependent. It is recommended that St_{max} is evaluated per locus.

We agreed to review stutter guidelines at a subsequent meeting.

2.6.2. How to use stutter guidelines

An evaluation of a mixture proceeds by a preliminary assessment to determine the number of contributors. This may include a consideration of the casework circumstances as well as an examination of the electropherogram (epg). If a simple two-person mixture is apparent, then interpretation can proceed as follows.

In the first example (Fig. 1), we condition on a two-person mixture, assuming that an assessment of the remaining loci justifies this position. Peaks *A* and *E* are minor contributors and are not in stutter positions. Peak *B* is below the stutter guideline (St_{max}), and can therefore be unambiguously designated as a stutter and discounted from the interpretation.

If allele *A* is above the dropout threshold (Fig. 2), and allele *B* is below the dropout threshold and below the stutter threshold, and differences in peak height/area are sufficient to discount the possibility of a heterozygote ($Hb_{obs} < Hb_{min}$) (see appendix for definition of *Hb*) then it may be designated *AA*. If the *C*, *D* allelic combination is unbalanced ($Hb_{obs} < Hb_{min}$) then it may be necessary to include *AC* and *AD* as potential minor contributors in the denominator of a likelihood ratio calculation, as masking may have occurred.

If *A* is low level (Fig. 3), equivalent in size to the stutter peak, then *B* may be an allele, or it may be an allele/stutter composite (contributor is *AB*) or it may be a stutter (the contributor is *AA*). Low-level alleles would usually be below the dropout threshold, hence the *AF* designation would be appropriate

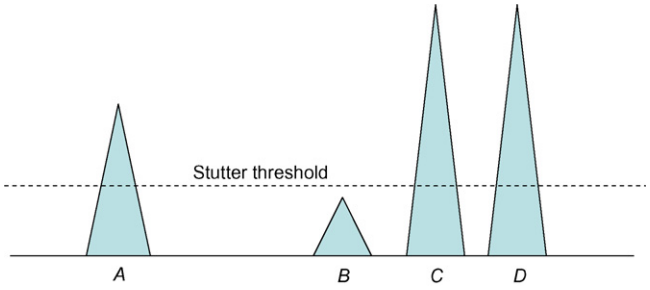

Fig. 2. A two person mixture with major peaks *C*, *D* and minor peaks *A*. There is an additional peak present in a stutter position (*B*).

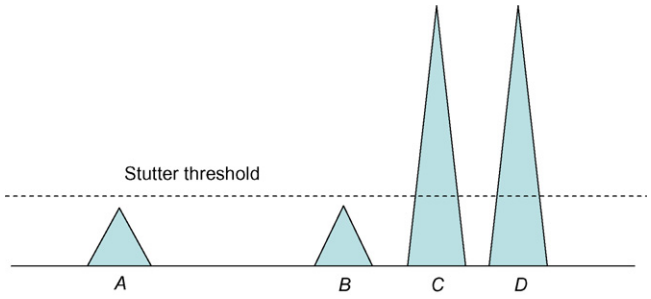

Fig. 3. A two person mixture with major peaks *C*, *D* and minor peaks *A*, *B*, where *B* is in a stutter position.

(see Section 2.7), since this encompasses the possibility of allele *B* in the stutter position. See Appendix A of the ISFG DNA commission report (pp. 96–97) on a method to calculate the likelihood ratio. Provided that the suspect is *AB*, then it is always conservative to compute the likelihood ratio including all possible combinations in the denominator, whereas if the suspect is a homozygote, so that the evidence is only explained if we condition on *B* as a stutter under H_p, then this must *a priori* be demonstrated to be a reasonable proposition—i.e. the size of allele *B* must be less than the stutter guideline (St_{max}) for the given locus. It is always good practice to repeat analyses showing potentially ambiguous results, if this is possible to do.

2.6.3. Characterisation of +4 base stutters

We agreed to review +4 bp stutters, however, we note that their presence often relates to over-amplified samples. Preliminary experimental work suggests that they are low level and generally less then 4% the size of the progenitor allele (Rosalind Brown, personal communication).

Note that −4 bp and +4 bp stutter cannot be distinguished from genetic somatic mutation without experimental work—furthermore, somatic mutations may give rise to peaks that are larger than those caused by stutter artefacts.

2.7. Recommendation 7

"If dropout of an allele is required to explain the evidence under H_p: ($S = ab$; $E = a$) 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 dropout $Pr(D) \approx 0$, then H_p is not supported".

2.7.1. Response

We recommend slight rewording (including underlined below): If dropout of an allele is required to explain the evidence under H_p: ($S = ab$; $E = a$), then the *companion* allele should be small enough (height/area) to justify this (Figs. 4–6).

"Small enough" equates to a peak that is below the predetermined dropout threshold, i.e. Pr(*D*) is more than zero (Fig. 5).

Conversely, 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_p is not supported (Fig. 6).

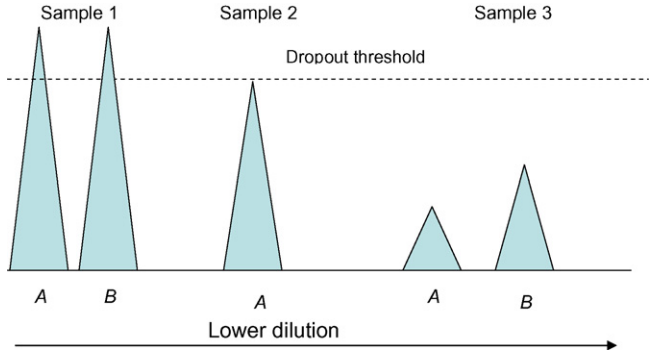

Fig. 4. Results from serial dilutions of the same sample genotype *AB*. The first result (sample 1) shows a locus where both alleles are represented in the profile. One or both of these alleles are above the dropout threshold and consequently are always present in the epg. The second result shows a result where dropout has occurred – the survivor allele is just below the dropout threshold hence this is a rare event, but not impossible. If *A* was just above the dropout threshold we would determine it to be a homozygote *AA* genotype. In the third sample, both alleles are well below the dropout threshold – it is an unambiguous, albeit unbalanced heterozygote. If only one allele was present, then we would have to consider the possibility of dropout of the partner. The same rationale can be applied to any analytical regime, e.g. 28 and 34 PCR cycles.

From the above example: allele *b* may either dropout completely, or it could be present at such low level that a statistical calculation is not supported by Section 2.8 because it is at a level where background noise could be prevalent.

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).

Note that for custodian purposes it is sufficient to unambiguously designate a homozygote locus for databasing purposes provided that it is above the dropout threshold. To apply a statistical analysis, the guidelines provided in this paper will assist to ensure that application of the '*F*' designation is conservative (or nearly so), remembering that care is required only when dropout must be invoked under H_p.

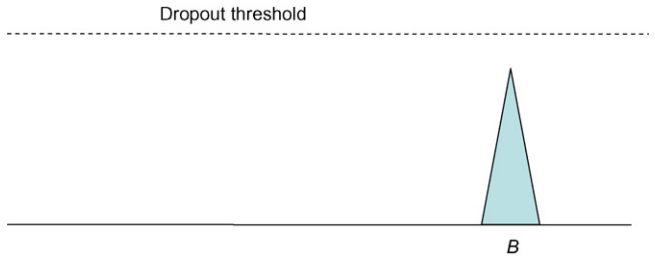

Fig. 5. In this example allele *B* is below the dropout threshold, hence we cannot be confident that it is from a homozygote *BB* individual. It could also be from an individual who is heterozygote, where the missing allele is any other allele. The probability $B|\text{unknown}, H_d$ is $2\Pr(BF)$, where the '*F*' designation is assigned a probability of 1 to take account of the possibility that any allele could have dropped out.

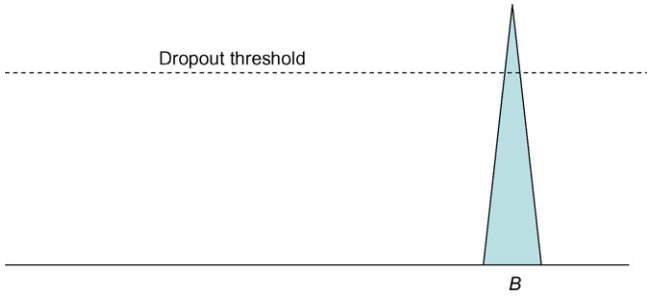

Fig. 6. In this example allele *B* is above the dropout threshold, hence we can be confident that it is from a homozygote *BB* individual. The probability of $B|\text{unknown}, H_d$ is $\Pr(B)^2$.

It is always good practice to repeat analyses with potentially ambiguous results, if this is possible to do. For example, duplication of the test may assist in to determine if dropout is a consideration in the interpretation of the evidence.

2.7.2. Implications of Bates

The appeal court, Bates [2], was asked to consider whether a partial DNA profile was admissible as evidence on the grounds that the DNA profile was incomplete and therefore did not match the defendant at every locus. At two loci (D2 and D8) alleles were missing. The missing alleles were called ''voids'' by the judge. The defence asserted that there was no accepted method to report partial profiles: ''the inability to take account of the potential exculpatory effect of voids invalidates any match probability''.

The Bates ruling specifically examined the implications of reporting a partial DNA profile where some alleles were missing or dropped out and the '*F*' designation was used. The judgement considered:

> ''Such voids are potentially significant because, if the missing allele did not match either of the alleles at that locus of the person under investigation, it would establish conclusively that he (or she) had not provided that sample of DNA. Every partial profile carries within it, therefore, the possibility that the missing information excludes the person under investigation, but there is currently no means of calculating the statistical chances of that being the case''.

The judgement goes on to conclude:

> ''What are the consequences of the impossibility of assigning a statistical weight to the voids? The alternatives are to exclude the evidence entirely or to admit it subject to an appropriate warning to the jury of the limitations of the evidence, and particularly highlighting the fact that although what was found was consistent with Bates' DNA profile, the voids at D2 and D18 in particular may have contained an allele or alleles, the presence of which would have been wholly exculpatory.

In arriving at the correct conclusion it is important to remember that scientific evidence frequently only provides a partial answer to a case. However, the test of admissibility is

not whether the answer is complete, but whether science can properly and fairly contribute to the matter in question. . .''

In the context of our discussions above (especially in relation to a consideration of Section 2.7 when $S = ab$ and $E = a$), we conclude that it is reasonable to assign dropped out alleles or ''voids'' as neutral events provided that the survivor allele is small enough, and below the designated dropout threshold so that the loss of the *b* allele is a reasonably plausible explanation. Appendix B of reference [1] gives a number of worked examples to illustrate this point.

Furthermore, it is advisable to carry out additional work in order to resolve this apparent ambiguity. A 'zoom' of the baseline may reveal the 'missing' allele to be present but sub-threshold? Alternatively, a re-amplification of the DNA extract (if there is sufficient) may reveal the presence of the missing *b* allele.

If both alleles have dropped out at a locus, then there is no information that can be adduced, and this must be regarded as neutral.

2.8. 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 should not be attempted''.

2.8.1. Response

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.

2.9. Recommendation 9

In relation to profiles derived from the amplification of low amounts of template DNA, stochastic effects may limit the usefulness of heterozygous balance and mixture proportion estimates. In addition, allelic dropout and allelic drop-in (contamination) should be taken into consideration of any assessment.

2.9.1. Response

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.

Based on manufacturers guidelines we can define a low-level sample as one that contains ca. <200 pg DNA. At this level we might expect stochastic effects to occur, including:

(a) locus dropout,
(b) allele dropout,
(c) extreme heterozygote imbalance.

These are consequences that are universally observed at 28–34 + PCR cycles. Duplication of the test can aid to interpret profiles with *Hb* imbalance and dropout.

Since the introduction of CE, sub-200 pg amounts of amplifiable DNA can be visualised by multiple methods—where increased cycle number, increased injection time etc (or a combination of the two) can be used to achieve the same effect. We have demonstrated experimentally that some laboratories achieve results from ca. 50 pg of DNA using standard 28 PCR cycles.

Since these consequences are common to all methods of DNA analysis, and are not restricted to 34 cycles, we do not consider the LCN label for 34 cycles work to be useful, or particularly helpful, and propose to abandon it as a scientific concept, because a clear definition cannot be formulated. Rather, our aim is to recommend generic guidelines that can be universally applied to all DNA profiles that are independent of the method utilised. It is important to consider that where the profile is well amplified and fully represented, without allele dropout, then special considerations are not required since interpretation is standard and straightforward.

Therefore, we can easily define a 'conventional' result as one where the alleles are above the dropout threshold (determined by experimentation). Reporting of the locus is normally straightforward because the alleles are unambiguous. The cycle no. used is irrelevant since the dropout threshold may be separately determined for any given protocol.

Conversely, we define a 'low-level' result as one where the alleles are below the dropout threshold. Special considerations are then applied.

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.

Appendix. Guidance note on the use of the heterozygote balance guideline

For a well-amplified heterozygote from good quality DNA >0.5 ng, the heterozgote balance is defined as the proportion of the lower peak height/area divided by the higher peak height/area:

$$Hb = \frac{\text{lower peak height or area}}{\text{higher peak height or area}}$$

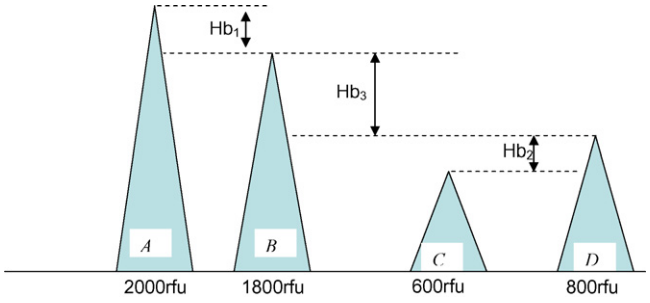

Fig. 7. A typical major(*AB*)/minor(*CD*) mixture.

The distribution of *Hb* generally ranges between ca. 0.5 and 1 for a well-amplified DNA profile. This parameter is used to evaluate DNA profiles. It is particularly useful to determine if mixtures are present and to determine whether respective alleles can be associated with a given contributor.

If a single profile is present, then Hb_{obs} (the observed *Hb*) should be greater than Hb_{min} (the minimum *Hb* from the observed experimental distribution for 'conventional' DNA is usually not less than 0.5—*this parameter may vary between laboratories*).

Consider the mixture in Fig. 7. All of the alleles are above the dropout threshold. Can allele *A* be paired with allele *B* and can allele *C* be paired with allele *D*? $Hb_1 = 1800/2000 = 0.9$; $Hb_2 = 600/800 = 0.8$, i.e. both parameters >0.5 (Hb_{min}). Could alleles *B* and *D* be considered to be from a single contributor? $Hb_3 = 800/1800 = 0.44$, i.e. $Hb_3 < 0.5$. These three calculations provide strong evidence to support the contention that alleles *A* and *B* are a pair of heterozygous alleles from a major contributor and alleles *C* and *D* are a pair of alleles from a minor contributor.

Some care is needed with using the heterozygote balance guideline. As the quantity of DNA declines, then the Hb_{min} also falls, hence it is desirable to understand the relationship between Hb_{min} and the size (height/area) of the respective alleles if this guideline is to be used below 0.5, otherwise, under the defence hypothesis H_d, it is always conservative to include more allelic combinations than necessary in the assessment. To formulate the prosecution hypothesis H_p, it is anti-conservative to include too many combinations here and the opposite applies—if in doubt then do not include the combination. Allele dropout is an extreme form of heterozygote balance and is equivalent to $Hb_{min} = 0$.

Thus, in the above example in Fig. 7, an ultra-conservative assessment would ignore the peak height/area information to formulate the defence hypothesis $\Pr(H_d)$. Suppose that we are evaluating suspect (*S*) and an unknown (*U*) under the prosecution hypothesis (H_p) and two unknown people (U_1 and U_2) under the defence hypothesis (H_d). If the suspect = *AB*, our most conservative evaluation will comprise $2pCpD$ (pC is the frequency of allele *C* in the relevant population) in the numerator (noting that if *A*, *B*, *C*, *D* were all equivalent in peak area then this would still be appropriate). Conversely, under H_d we would include combinations *AB*:*CD*; *AC*:*BD*; *AD*:*BC* (along with reverse options) as viable options using the classic likelihood ratio formulation. The LR = $1/12pApB$.

Given the peak height/area considerations, we can conclude that the major/minor contributors can be separated and consequently the minor contributor can be subtracted from the evidential profile, to allow the major profile to be reported as a match probability. $P_m = 1/2pApB$ which gives a figure that is greater than the LR formulation.

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Mixture Interpretation: Defining the Relevant Features for Guidelines for the Assessment of Mixed DNA Profiles in Forensic Casework*

ABSTRACT: Currently in the United States there is little direction for what constitutes sufficient guidelines for DNA mixture interpretation. While a standardized approach is not possible or desirable, more definition is necessary to ensure reliable interpretation of results is carried out. In addition, qualified DNA examiners should be able to review reports and understand the assumptions made by the analyst who performed the interpretation. Interpretation of DNA mixture profiles requires consideration of a number of aspects of a mixed profile, many of which need to be established by on-site, internal validation studies conducted by a laboratory's technical staff, prior to performing casework analysis. The relevant features include: criteria for identification of mixed specimens, establishing detection and interpretation threshold values, defining allele peaks, defining nonallele peaks, identifying artifacts, consideration of tri-allelic patterns, estimating the minimum number of contributors, resolving components of a mixture, determining when a portion of the mixed profile can be treated as a single source profile, consideration of potential additive effects of allele sharing, impact of stutter peaks on interpretation in the presence of a minor contributor, comparison with reference specimens, and some issues related to the application of mixture calculation statistics. Equally important is using sensible judgment based on sound and documented principles of DNA analyses. Assumptions should be documented so that reliable descriptive information is conveyed adequately concerning that mixture and what were the bases for the interpretations that were carried out. Examples are provided to guide the community. Interpretation guidelines also should incorporate strategies to minimize potential bias that could occur by making inferences based on a reference sample. The intent of this paper is to promote more thought, provide assistance on many aspects for consideration, and to support that more formalized mixture interpretation guidelines are developed.

KEYWORDS: forensic science, DNA analysis, mixtures, STRs, guidelines, interpretation, quality assurance, threshold, validation, peak height ratios, stutter, deconvolution

The interpretation of forensic DNA evidence is a very important part of the analytical process. It requires human processing and experience with the nuances of interpreting evidentiary and reference profiles. In particular, complex DNA mixture profiles at times can present challenges for analysts interpreting the profile(s). However, current mixture interpretation guidelines/requirements within the United States demand only that a mixture interpretation protocol be in place. Such minimal requirements are clearly inadequate and potentially could lead to a wide range of interpretations being carried out. Variations within interpretation guidelines are somewhat acceptable and necessary. But in our experience some approaches are in error, and in some cases good results are being ignored. Because mixed samples can present interpretative challenges, basic assumptions must be stated and well-defined empirical parameters must be established by any laboratory conducting forensic casework (Table 1). Otherwise, incorrect interpretations may arise. Furthermore, due to limited information concerning the nature of any mixture (or single source sample profile for that matter), a laboratory must incorporate strategies within its interpretation guidelines to minimize potential bias that could be influenced by any reference sample analyzed. Preventative measures and sound scientific principles are essential to maintaining fidelity and an objective nature of the conclusions rendered by the forensic scientist. Such practices must be employed by all scientists performing DNA casework analyses. The importance of establishing these quality assurance elements through on-site, internal validation studies to include appropriate mixture studies conducted by a laboratory's technical staff, prior to performing casework analysis using a new technology, cannot be overstated.

The discussion presented herein addresses various scenarios to consider for more defined interpretation guidelines for mixture analysis than currently required by quality assurance standards. The intent is that more formalized mixture interpretation guidelines are developed and assumptions documented so that reliable descriptive information is conveyed adequately concerning that mixture, proper interpretations are carried out, and contextual and confirmation biases are minimized. This document does not evaluate the appropriateness of any specific analytical parameter value (e.g., quantity of target for the PCR, injection time, etc.), re-analysis strategy (e.g., desalting of PCR amplicons, use of multiple detection instruments, use of increased and/or decreased injection times, etc.),

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TABLE 1—*Required elements for forensic laboratory protocols for mixture interpretation.*

Elements relating to mixture interpretation which require validation by forensic laboratories
Distinguish true alleles from nonallelic peaks/artifacts
Stutter peaks
Minus A (−A) peaks
Pull-up peaks
Fluorescent spikes (seen in all four colors)
One color electronic noise peaks (one color spikes)
Off ladder alleles
Dye labeled artifacts
Define appropriate thresholds (where applicable)
PAT
MIT
Saturation/maximum
Determine appropriate peak height ratios for the following:
Maximum stutter peak height values for each locus
Peak height ratios for heterozygous alleles in a single source sample
Peak height ratios for determining major/minor contributors to a mixture
Additional terms which require defined usage
Probative sample
Intimate sample
Subtraction sample
Elimination sample
Match/inclusion
No match/exclusion
Inconclusive
Uninterpretable sample
Resolvable/distinguishable
Unresolvable/indistinguishable
Interpretation of question samples
Question samples must, where possible, be interpreted prior to any comparison to known sample(s)
Criteria used to determine a sample is a mixture
Two or more alleles present at one or more loci
Peak height ratios of heterozygous alleles do not meet peak height ratio values for apparent heterozygous alleles
Peaks in stutter positions that exceed stutter thresholds
Statistical analysis of mixtures
Laboratories must define the use of random match probability for major/minor components of a mixture
Laboratories must define the use of Probability of Inclusion (PI), Probability of Exclusion (PE) or Likelihood
Ratio (LR) for mixtures
Laboratories must define any deviations from their protocol before they can be used
All assumptions must be stated and placed in the case file. They may include the Known sample is expected to be present in a mixture and is used in mixture deconvolution
Using peaks in the stutter position which fall below the stutter guidelines for not excluding

and/or specific threshold value(s) employed by a laboratory. The establishment and assessment of such operational elements are best evaluated through requisite quality control measures developed through well-designed validation studies. The procedures presented below focus solely on nuclear DNA PCR-based short tandem repeat (STR) loci analysis separated and detected on capillary electrophoretic (CE) platforms (i.e., the current methodology in the forensic DNA community).

Identification of Mixed Specimens

The determination of any DNA profile as a mixture first must be based on an evaluation of the profile in its entirety. Some locus-specific phenomena (e.g., stutter, peak height imbalance, tri-allelic patterns, primer mismatches, and differential amplification, etc.) may not permit conclusive allelic or genotype assignments at a given locus or determining whether a locus presents as a single source or a mixture. It would be unsound to focus only on a single locus to the exclusion of the other loci in a profile to determine whether a sample profile supports being a single source or a mixture (or to conclude the minimum number of contributors). A DNA profile is generally considered to be comprised of more than one individual if three or more alleles are present at one or more loci and/or the peak height ratios between a single pair of allelic peaks for one or more loci are below the empirically determined appropriate threshold for heterozygous peak height ratio(s). A laboratory must define within its standard operating protocol (SOP) the specific elements necessary to make reliable allelic and nonallelic peak assignments.

Threshold Values

The use of fluorescent based detection of PCR amplicons affords the analyst quantitative information describing the signal profile (or peaks) present in a given DNA fragment. This quantitative information, expressed as relative fluorescent units (RFU), can be used to establish peak height and/or peak area both of which provide meaningful information for determining what are and what are not interpretable signals. The establishment of thresholds based on fluorescent signals is critical to the proper evaluation of STR typing data because it formalizes the minimum criteria that a PCR product must display for quantitative and/or qualitative evaluation. At a minimum, a peak amplitude threshold (PAT) must be established that operationally defines the minimum peak height in RFU that confidently ascribes a true PCR amplicon peak and when confidence is too low to reliably assign a peak as an allele. The PAT is established to account for the well-recognized stochastic limitations of PCR-based DNA typing systems and effectively sets the lowest peak height value for which a laboratory will operationally treat an instrumental response as the detection of a DNA fragment rather than simple instrument noise. This is not to imply that a given PAT is necessarily equal to the limit of detection (LOD) of an analytical system. While the LOD is the absolute minimal level of analyte that can be expected to routinely result in a positive signal from the analytical system, the PAT may represent a threshold value greater than the LOD by some specified value (e.g., several standard deviation units) to increase the confidence that any given peak at or above this threshold is actually a PCR amplicon. The PAT (of 50 RFUs) used in most U.S. forensic DNA Laboratories is generally higher than the signal noise ratio, but is reasonable given experience with stochastic effects during PCR and potential DNA background levels.

Additionally, a laboratory must establish a match interpretation threshold (MIT). This threshold is necessary for avoiding interpretation where the PCR product is too low such that potential stochastic effects, due to limited template copies or inhibitors, may result in allelic loss or nonreproducible results. The MIT establishes the minimum peak height in RFU that all amplicon peaks at a given locus (or loci) must display to confidently conclude that no genetic components of the interpretive portion of a sample failed to be detected due to the differential PCR amplification of a targeted region(s) of a low copy number template, a degraded sample, or PCR-inhibited sample. Low copy here refers to any sample with too little DNA such that substantial stochastic effects will occur during PCR; typically these are samples that contain 200 pg or less DNA or are compromised in purity or quality. Not all components of a DNA sample will be reliably reproduced when there are substantial stochastic effects during PCR, and this phenomenon will impact on which loci in a profile will be interpreted and which may be deemed inconclusive (see below). While steps can be taken

to maximize the total number of allelic peaks that meet or exceed the MIT (e.g., amplification of a greater template mass) for a given mixed sample, the peak heights of all allelic peaks at a given locus may not exceed the MIT. In such situations where the comparison interpretation is a failure to exclude, the possible stochastic loss of allelic information is addressed in its associated forensic statistic(s) assessment by not including any locus of a profile or component of a profile with an allele that displays a peak height below the MIT in the calculation. Because it is critical that these thresholds be empirically evaluated and established within the laboratory, the PAT and MIT may be implemented operationally as a single threshold value or as two separate peak height thresholds based on the data obtained from a laboratory's own internal validation studies (to include low copy analyses). As an example on how to carry out empirical studies see Moretti et al. (1,2).

Figures 1 and 2 display examples of mixed samples where the use of the MIT and PAT impact on interpretation. In Fig. 1 there is a major and a minor component observed in the profile (see below for discussion of major and minor components). Assume the scientist initially states that there is only one minor contributor to the sample. Two alleles 14 and 19 are detected; they both have peak heights above the PAT. Therefore, it seems reasonable that the type of the minor contributor is 14,19. However, the 14 is below the MIT. It falls within a region where stochastic effects during the PCR are increased. In addition to being masked by the 16 or 17 peaks, it is possible that the 14 may be from a heterozygous profile but its accompanying allele did not amplify and the 19 is from a second minor contributor (of course after looking at all loci in the profile, this scenario may or may not be supported). In this scenario, part of the minor contributor alleles resides between the PAT and MIT. Thus, the minor profile at this locus is not used for increasing the power of the estimate of the rarity of the DNA evidence. It does not mean that the evidence cannot be used to exclude possible suspects. For example, if a suspect's type is 19,20, then he/she cannot be a contributor of the sample, and the interpretation of the comparison is exclusion. Additionally, a 14,19 individual could not be excluded given the profile displayed in Fig. 1. There is an alternate interpretational approach where the allele 19 is recorded and then all individuals carrying the 19 allele either as a homozygote or as a heterozygote would not be excluded. Treating the interpretation in this manner certainly is conservative statistically, but fails to exclude as many individuals as under the assumption that 14,19 is the type of the minor contributor. Since the 19 is above the stochastic level one can be assured that the second allele did not drop out (barring primer binding site mutations that affected amplification).

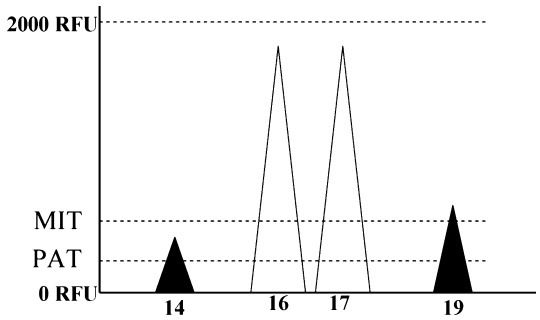

FIG. 1—*Example of two person major and minor mixture profile. The minor contributor has one allele above the PAT but below the MIT and another allele above the MIT.*

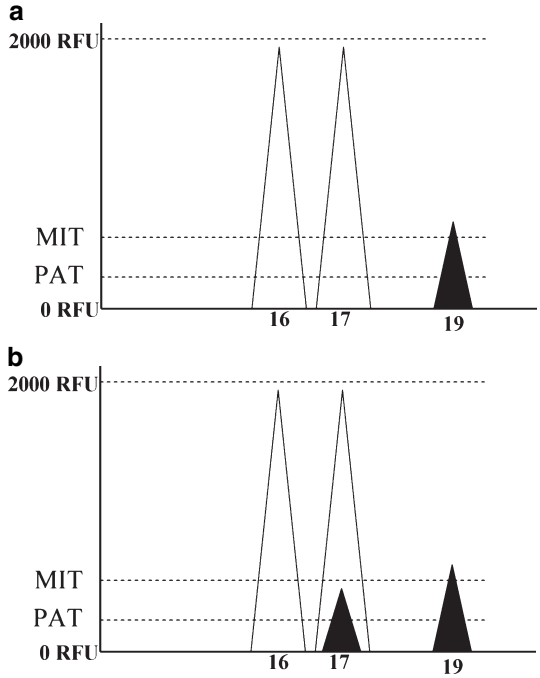

FIG. 2—*Example of a two person major and minor profile. (a) Only one allele is visible for the minor contributor and it is above the MIT. (b) A hypothetical is displayed that the accompanying heterozygous allele on the minor contributor is masked by a major component allele.*

Alternatively, in Fig. 2*a*, there is only one minor allele (allele 19) detected and its peak height is above the MIT. Since no other alleles are observed below the MIT and above the PAT, the profile can be interpreted with the possible types of the minor contributor being 19,19; 16,19; or 17,19. We recognize that there may seem to be an inconsistency between the recommendation for interpretation of the minor contributor profile in Fig. 1 and that for Fig. 2*a*. Clearly allele 19 is above the MIT and can be reliably interpreted in all three scenarios. Indeed, if one assumes only one minor contributor (Fig. 1), then alleles 14 and 19 should derive from the minor contributor. Yet, the minor contributor at this locus would not be used in statistical weight calculations, based on the above recommendation. If the scenario in Fig. 2*b* were a true situation that a minor allele is masked by one of the major alleles and this allele is below the MIT and above the PAT, it would be similar to that presented in Fig. 1. However, we support using the minor profile for statistical calculations in Fig. 2*a*, because all visible alleles would be considered when interpreting the minor contributor (i.e., 16,17,19). Alternatively, for the scenario in Fig. 2*b* a statistical assessment could be made employing the 2p rule at the locus for the minor contributor (3).

One could suggest that the different interpretations in Figs. 1 and 2 could be resolved and not be discordant by instituting only one threshold value for both the PAT and MIT. While certainly a defensible approach, it may not alleviate the issue demonstrated. The two threshold approach recognizes that there is a region between detection of DNA and the robust amplification of DNA, i.e., the stochastic region. Using a single threshold does not alleviate the stochastic issues; they will still occur. If a single threshold is implemented that is similar to that of the PAT, it will still be necessary to recognize that peaks above but near the threshold may be subject to stochastic effects and policies will need to be developed for these profiles. If the single threshold is set higher and similar to that of the MIT, then interpretation issues will still persist.

If peaks at a locus are above and below the higher single threshold, the laboratory will have to address what is deemed inconclusive or conclusive. Thus a single threshold approach will not necessarily eliminate the different results obtained for Figs. 1 and 2 when using the PAT and MIT approach.

The interpretations in Fig. 2 also can be locus dependent. Consider the locus FGA which tends to have the largest size amplicons using the current commercial kits. Additionally, because of the wide range of FGA alleles potentially greater effects of preferential amplification can occur between heterozygous alleles. Thus, it is possible that a large sized FGA allele could drop out when its accompanying heterozygous smaller-sized allele is observed even when there is no apparent effect of dropout at alleles at other loci of similar peak heights. Therefore, one should be cautious when interpreting the minor contributor profile at the FGA locus under the scenario shown in Fig. 2. One could call the locus uninterpretable for the minor contributor when only one minor allele is seen. Alternatively, the laboratory could develop a valid MIT through validation studies such that allele drop out would not be a reasonable interpretation. Or instead the minor contributor could be assessed as carrying the 19 allele (as in Fig. 2), and thus a 19 homozygote or a 19 heterozygote with any other allele (typically a larger sized allele) would not be excluded. All three approaches are valid.

Allelic versus Nonallelic Peak Assignment

The PCR process (or any other enzymatic reaction) is not 100% efficient. As a result, the criteria by which nonallelic peaks, such as stutter and nontemplate directed adenylation, are recognized must be based on internal validation studies. Also, those graphical peaks due to instrumental limitations (e.g., matrix failure, spikes, pull-up) or introduced into the process via one of the reagents (e.g., disassociated primer dye) should be defined. These features must be established empirically under the same conditions by which forensic casework is conducted. Otherwise, the descriptive information generated during validation may not comport with data observed in the course of casework analysis.

Essential to an unbiased assessment of the potential allelic data is making allelic peak assignments for the evidentiary profile(s) prior to conducting any other interpretive or comparative part of the analysis with a reference sample(s). Where possible, the profiles obtained from the evidentiary sample(s) should be interpreted first, then the following should occur: (i) the reference samples interpreted and their allele assignments made; and (ii) the comparisons of the DNA typing results from an evidence item be made with those from any reference sample(s). Thus, the allelic versus nonallelic determinations for the evidentiary profile are not influenced by any conscious or unconscious bias predicated on the DNA profile of the reference specimen(s).

Tri-allelic Patterns

Three allele peaks, although uncommon, can be observed at a locus in a profile and yet be from a single source. Tri-allelic patterns generally present as either a triplet of peaks for which the sum of two of the peaks equals the third (e.g., for the set of allelic peaks 12, 13, and 14, the peak height of 12 is close to that of the sum of the height of peaks 13 and 14) or as a triplet of balanced peak heights. Occurrences of observed tri-allele patterns have been documented at http://cstl.nist.gov/biotech/strbase/tri_tab.htm for all thirteen core Combined DNA Index System (CODIS) STR loci. As of April 3, 2008, 170 tri-allelic patterns had been reported with the following numbers for the 13 CODIS core loci: D3S1358 (n = 6), FGA (n = 22), vWA (n = 19), D8S1179 (n = 11), D21S11 (n = 19), D18S51 (n = 21), D5S818 (n = 4), D13S317 (n = 8), D7S820 (n = 7), D16S539 (n = 8), TH01 (n = 1), TPOX (n = 15), and CSF1PO (n = 7) (Note: the total sample size from which these tri-allelic patterns were drawn is not known; so an estimate of their frequency cannot be made with the data displayed at the website).

For profiles in which three allelic peaks are observed at only one locus and no other loci indicate the presence of a mixture, a single source origin would be the most probable interpretation. Factors such as the number of loci in a profile that display such patterns, and what, if any, other indications of a mixture are present (i.e., heterozygous peak height imbalance) must be considered. Tri-allelic patterns at a locus from a single source occur infrequently. Therefore, the presence of two or more loci presenting tri-allelic patterns should be given serious consideration as a potential mixture. The conclusion that a three peak pattern observed in an evidentiary specimen is a true tri-allelic condition and not an indication of a mixed sample should be made on a sample-by-sample basis.

While conclusions regarding the allelic nature of individual peaks should be done prior to the interpretation of reference samples, the indication of a tri-allelic pattern (or other genetically based variation such as a primer binding site mutation) in a reference sample may support otherwise less likely interpretations of the profile. Regardless, any conclusion made as to the inclusion or exclusion of the reference individual as a potential source of the evidence DNA should be based on the shared alleles between the two profiles. In fact, the presence of a matching three allele pattern at any locus is strong ancillary evidence that the two samples may have originated from the same source. While the rarity of a matching tri-allelic pattern within an otherwise determined single source DNA profile has not been generally used by us to modify the random match probability calculated for such an inclusion, one could use the locus statistically based on the number of tri-allelic patterns seen for the particular locus in a sample population data set (with some sampling correction). Either approach would be acceptable.

Similar reasoning can be applied to mixed DNA profiles. For example, a mixed sample that displays no more than four allelic peaks at all of the loci of the multi-loci profile is most consistent with having originated from two individuals. Given a mixed sample that is consistent with having originated from a minimum of two individuals at all loci except at one locus at which five allelic peaks are observed, one possible interpretation is that the mixture originated from two individuals one of which displays a three peak pattern at this locus. The presence of a matching reference profile that shares a matching three peak pattern at this locus can not be excluded as a source even if one were to proffer that possibly three people may comprise the profile.

Reporting of Mixed Specimens

Estimation of the Minimum Number of Contributors

Once a specimen is determined to contain DNA from more than one individual, the minimum number of DNA contributors to that mixture should be estimated. A conclusion with regard to the minimum number of contributors to a mixture can provide important quantitative information that may help to convey something of the general nature of the DNA typing results obtained from a given sample. As such, a conclusion with respect to the minimum number of contributors to a mixture should be routinely included in a report and should be used as a general statement to introduce the

detection of a mixture for a given specimen. Generally, an estimate of the minimum number of contributors is based on the locus that exhibits the greatest number of allelic peaks. As an example, if at most five alleles are detected at one or more loci of a multi-loci profile, the DNA typing results are consistent with having arisen from three or more individuals (although a tri-allelic pattern could be present infrequently). A statement that conveys this observation may be:

The STR typing result for specimen Q1 is a mixture of DNA from three or more individuals.

or

The STR typing results for specimen Q1 indicate the presence of DNA from at least three individuals.

One caveat to this strategy is the following scenario: a mixed profile possesses five alleles at only one locus and there are no more than four alleles at all other loci. Two hypotheses may be considered: (i) the profile is comprised of at least three contributors; or (ii) the five allele pattern is the result of a two person contribution and one of the contributors carries a three allele profile. This is not common. But if it is a consideration, then one could institute a policy that five alleles must be observed at two loci before issuing the above statements. Regardless, these interpretations should be described in the interpretation guidelines. An estimation of the minimum number of contributors to a mixture should not be construed as designation of an absolute number of individuals that must have contributed to a mixed specimen. Additionally, it does not imply that a mixture of three individuals could not possibly appear to be a mixture comprised of only two individuals (i.e., have at most four allelic peaks at all loci). While the true number of contributors to a mixture can be made with high probability, a conclusive determination can not be made of the number of contributors to the profile. Rather, this estimation is provided to describe the fewest number of individuals who must have contributed to a mixture. Well-established statistical calculations for mixtures (see below) subsequently can accommodate the uncertainty in the absolute number of contributors.

For multiplex systems that include the amelogenin sex-typing locus, a profile comprised of more than one individual based on the STR typing results can be concluded to contain male DNA if the sample exhibits (i) both an "X" and a "Y" allelic peak at or above the empirically established PAT; (ii) only a "Y" allelic peak at or above the PAT; (iii) is positive using a Y chromosome (male) specific quantitation assay; or (iv) is positive for Y STR loci. An example statement can be:

Based on the typing results from the amelogenin locus (for sex determination), male DNA is present in the DNA obtained from specimen Q1.

or

The DNA profile from specimen Q1 is a mixture of DNA from at least two individuals. The amelogenin result indicates that at least one of these individuals is male.

A mixed DNA profile that exhibits an "X" allelic peak above the MIT and the absence of a "Y" allelic peak in many cases can be concluded to be consistent with the presence of female DNA. A statement can be:

Based on the typing results from the amelogenin locus (for sex determination), female DNA is present in the DNA obtained from specimen Q1.

Confidence is greater in the above gender inferences in mixtures when predicated on the presence of a Y amelogenin peak. The Y amelogenin region may not amplify during the PCR in a low percentage of males due to deletions or primer binding site mutations (4–6). The same could occur for the X homologous region, although the likelihood of drop out may be lower. While inferences for the presence of male and female contributors are most reliably made when a Y peak is detected, there may be some situations with null Y amelogenin male profiles that can be interpreted as male in origin. Consider a differential extraction of sexual assault evidence where two profiles are obtained—one in the female fraction and a different one in the male fraction—and neither demonstrates a Y peak. It may be inferred that the profile from the male fraction is likely to be male in origin and null for the amelogenin Y peak. Follow up analyses with Y STRs or a Y specific quantitation assay could confirm that the profile is from a male donor.

Conclusions concerning the number of contributors to a mixed specimen based solely on the relative peak heights of the amelogenin "X" and/or "Y" allelic peak are at best limited. The assignment of sex type to individual contributors to a mixture might be made in some two person mixtures such as: (i) one contributor is male and one is female and the female contributor is unequivocally a major component and the male is a minor component; and (ii) where both contributors are of the same gender, particularly if they are females. Should such determinations be made, the assumptions and justifications necessary for conclusions to be rendered must be defined in the SOP to ensure uniform application of such interpretive elements across analysts in the same laboratory and documented in the case notes or report. Additional methodologies, such as Y STR typing, may be useful for rendering conclusions concerning the number of contributors and/or sex typing of individual contributors to a mixture.

Types of Interpretable Mixtures

Resolving Components of a Mixture

A resolvable (or distinguishable) mixture is a DNA typing result from a mixed sample for which alleles can be attributed to a single source(s). This is possible when differing amounts of DNA are donated to the specimen typically by two individuals, thus resulting in major and minor contributions (Fig. 3). All loci for which DNA typing results are obtained (to include the amelogenin locus) must be considered in distinguishing contributors. However, an interpretation of the STR typing results as resolvable (for the major or minor contributors of a mixture) may be limited to only some loci.

Elements within a SOP should describe the criteria for defining what constitutes a major and/or minor contributor in a mixed specimen, and these criteria should be based on the data from internal laboratory validation studies. At a minimum, locus peak height ratios (PHR) should be defined to assign alleles to a major and/or minor contributor type(s). The PHR thresholds may be established

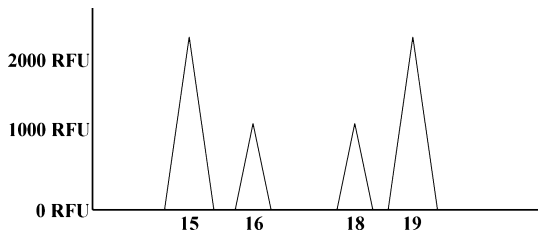

FIG. 3—*Example of a resolvable two person mixture.*

as (i) a single empirically determined value, (ii) a series of locus-specific values, (iii) a series of peak height dependent values, or (iv) a series of locus-specific values across multiple peak height ranges. Typically, empirically established PHR threshold values range from 60% to 70%. At a locus for which a contributor is deemed to be heterozygous, the alleles attributable to that contributor must be the only pair of peaks present that meet allelic PHR value at that locus. If the contributor is deemed to be homozygous at a locus, then that allelic peak, displaying the greater(est) peak height, cannot be accompanied by another peak that meets the PHR threshold value, if no additive effects can explain the height of that peak.

Due to the possibility that the minor contributor's alleles may be masked by the major contributor at some loci and thus such alleles may not be detectable, deconvolution of the minor contributor profile to a single source may be possible at only those loci where heterozygote alleles are unequivocal or quantitation data support only one possible profile for that contributor.

Unresolvable/Indistinguishable Mixtures

An unresolvable (or indistinguishable) mixture is a DNA typing result for which the alleles detected cannot be attributed unequivocally to a single source(s). This usually occurs when similar amounts of DNA are contributed to the specimen by multiple donors (Fig. 4) (or as described above for a minor contributor) and at least one of the profiles cannot be attributed to a known donor, e.g., from the epithelial fraction of a vaginal swab (see below on subtracting profiles). Such unresolvable mixtures may reside within different categories of contributors (that may be present within a single locus or a profile). Those mixtures for which predominant and/or minor components can be identified may have unresolvable contributors at the major contribution, the minor contribution, or both. For example, for a locus at which the alleles 9,10,11,12,13 are detected with respective peak heights of 1000, 900, 1200, 950, and 200 RFU, the alleles can be segregated into two groupings (Fig. 5).

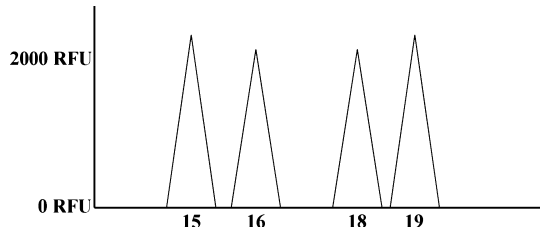

FIG. 4—*Example of an unresolvable two person mixture.*

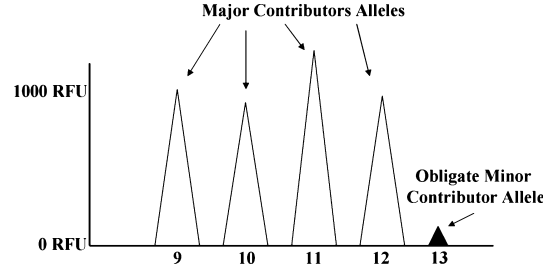

FIG. 5—*An example of an at least three person mixture. Alleles 9,10,11, and 12 are part of the major component and allele 13 is part of the minor component. Resolving the two components is possible because the amount of DNA is in the robust range of the assay.*

This separation of alleles 9,10,11, and 12 into one group—major component—is warranted since the PHR threshold is met for all allelic peaks at or near 1000 RFU (to include the allelic pair 10,11 [75%], which is the pairing of the major component alleles that displays the greatest difference in peak heights). These four alleles are from at least two contributors and they constitute an unresolvable mixture. Because of allelic masking, the most that can be determined with respect to the minor contribution is that allele 13 is an obligate minor contributor allele. For this example allele 13 has a peak height above the MIT; therefore the minor contributor may be homozygous for allele 13 or heterozygous in combination with any of the other visible alleles 9,10,11,12. The most plausible explanation for the number of minor contributors should be based on the data at all loci comprising the profile and in some complex mixtures it may not even be possible to determine this.

While every effort should be made to reliably draw typing information from mixed samples, some mixtures, after having been subjected to the interpretation strategies described above, may not lend themselves to interpretation using a laboratory's prescribed procedures. Although not always, these tend to be three or more person mixtures where quantitative deconvolution becomes more complex. The weight of these complex mixtures can be assessed by estimating the Probability of Exclusion or Inclusion or with consideration of the number of contributors (when possible) by the likelihood ratio (see below). Alternatively, at times and depending on the complexity, such mixtures may yield DNA typing information only for exclusionary purposes; they should then not be used for inclusionary/statistical assessments. An example statement can be:

The STR typing results for specimen Q1 indicate the presence of DNA from three or more individuals. The DNA profile obtained from specimen Q1 does not satisfy the Laboratory's inclusionary reporting criteria and therefore may be utilized only for exclusionary purposes. Based upon the STR typing results, specimen K1 is excluded as a potential contributor to the mixture of DNA obtained from specimen Q1.

Deduced Single Source Profiles from Mixtures

An evidence item taken directly from an identified anatomical location (e.g., vaginal swab, oral swab, fingernail clippings, etc.) and/or a piece of intimate apparel (i.e., undershirts, panties, bra, etc.) typically will yield DNA from the individual from which the evidence item was taken. In such circumstances, any DNA typing results that are consistent with the individual of origin reasonably can be subtracted from the mixed profile to attempt to further deduce the profile (or obligate alleles) of other contributors. Where possible, those sample types from which known contributor profile information can be subtracted should be defined within the SOP and documented in case notes and the report to promote uniform treatment of such items among forensic scientists within the same laboratory. For example, consider a vaginal swab (submitted as evidence as part of a sexual assault kit) with a mixture result of alleles 12,14,15,19 at a locus (Fig. 6) and consistent with a two person mixture. If alleles 12 and 14 are attributed to the victim, they can be subtracted from the mixture result, thus leaving the 15,19 alleles to be assigned to the unknown individual. If sharing of alleles between the known donor and another individual is possible, any designation of the unknown individual's alleles at a given locus must be based on supportable quantitative differences in peak heights due to the potential additive effects of shared allelic peaks; otherwise only obligate alleles can be unequivocally assigned to the unknown contributor. For example, consider a vaginal swab and

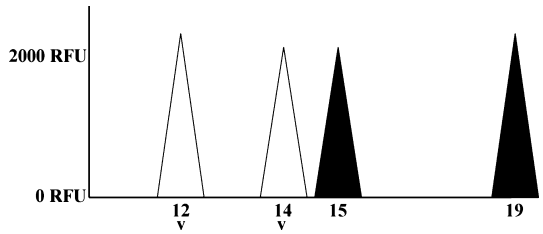

FIG. 6—*An example of a two person mixture that is resolvable because the victim's alleles (12,14) can be subtracted from the profile. The black peaks (15,19) are resolved as a single source component from the unknown contributor.*

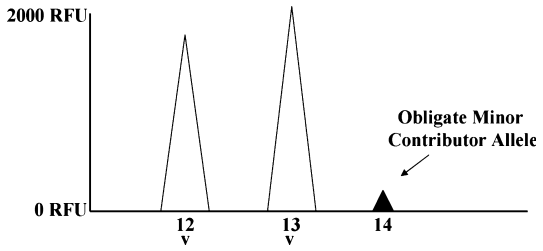

FIG. 7—*An example of a two person mixture where the probative portion is that of the minor contributor. The major alleles (12,13) can be subtracted. Allele 14 is an obligate allele that may be from a contributor of the types 14,14; 12,14; or 13,14.*

the alleles 12,13,14 at a locus with respective peak heights of 1800, 2000, and 200 RFU, which are all above the MIT (Fig. 7). If the DNA typing results from the victim's reference sample are 12,13 then the 14 allele is definitively assigned to the unknown contributor. The possible types for the unknown contributor to this mixture are either 12,14; 13,14; or 14,14. The genotype for the unknown contributor cannot be further deduced at this locus.

This subtraction approach also can be used when another known individual can be reasonably expected to have contributed biological material to the mixed specimen (e.g., consensual sex partners, etc.). For such elimination samples, the accounting strategies given for the subtraction of the DNA typing results also may be applied where possible (to both the victim and consensual individual). Additionally, a similar approach can be applied to evidentiary items from which DNA is isolated by a differential extraction. Because a differential extraction procedurally divides an individual sample into the sperm (male) and epithelial (female) fractions, the accounting strategies given may be applied to a mixed result obtained from either the female and/or male fractions. In such situations, the single source or major contributor typing results from one fraction (i.e., male or female) can be used to deduce information from its complementary fraction.

There may be scenarios not described herein where subtraction is legitimate for determining obligate foreign alleles. If subtraction is used, the assumptions and reasons justifying the use of the approach must be described and documented.

Considerations in Evaluating Mixtures

Additive Effects of Allele Sharing

The ability to assess a given mixture (i.e., deduce a single source profile from an intimate item, deconvolute a resolvable/distinguishable mixture, or determine the potential contributing genotypes to an unresolvable/indistinguishable mixture) diminishes as the number of contributors to a mixture increases. The greater the number of contributors to a mixture, the more allelic overlap is expected across a mixture due to the sharing of alleles among contributors. This sharing is expected given the allele frequency distributions of particularly common alleles in the population for the 13 CODIS STR loci.

The consequence of this sharing is that an allelic peak in a mixture may be from multiple copies of an allele from various donors (i.e., multi-copy allelic peak) as opposed to two copies from a single source homozygote or a single copy contribution from a part heterozygote of a single contributor. Because in such situations the specific contribution from each individual contributor cannot be determined reliably, allelic attributions must be based on the relative peak heights observed across all of the allelic peaks detected. This is generally done by accounting strategies that rely on legitimate simple subtractions of suspected single-copy allelic peak heights from the heights of possible multiple-copy allelic peaks.

Multiple single-copy peaks may or may not have recognizable corresponding heterozygous partner alleles contained within a potential multiple-copy allelic peak at a locus. For example, consider a mixed single locus profile of 15,16,19 with corresponding peak heights of 300, 650, and 375 RFU (Fig. 8). Given a minimum of two contributors, application of PHR expectations would be consistent with a homozygous contributor of 16,16 mixed together with a heterozygous 15,19 individual. However, application of simple peak height quantitation would also yield the possibility of a mixed specimen consistent with being from two heterozygous individuals 15,16 and 16,19, respectively.

While the strategy of deconvolving the above example into two possible scenarios can explain the evidence, as the number of contributors to a mixture increases (thus increasing the number of allelic copies possibly represented in an allelic peak of potential multiple-copy origin), applicability quickly is lost for assigning specific genotypes either directly or indirectly through subtraction (i.e., assembling a contributor based on allelic information not assigned [directly] to other contributors). This loss of effectiveness is in part due to the result of the slightly unequal amplification of two allelic peaks of a heterozygous profile in any PCR (generally 60–70% or higher with appropriate PCR template quantity). As the number of possible allelic copies increases in a multi-copy allelic peak, the uncertainty surrounding the peak height contribution of any individual partner allele of a specific heterozygous profile is confounded by the uncertainty associated with amplification of the other partner alleles contributing to that peak.

The point here is that technology does have limitations and over-interpretation should be avoided.

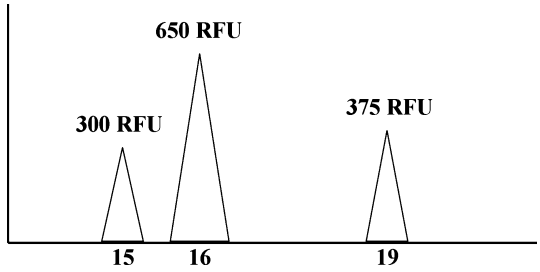

FIG. 8—*An example of a two person mixture where quantitative data supports only two reasonable mixture possibilities.*

Stutter Peaks versus Potential Minor Contributor Alleles

Most nonallelic products occur at low levels and thus present as peaks of low height. Stutter peaks, observed at all of the forensically employed STR loci, are the most prevalent of the nonallelic products. They typically are one repeat smaller in size than their true parent allele products and generally are 5–20% of the peak height of the parent allelic peak. While not problematic for interpretation of a single source profile, stutter peaks can complicate the interpretation of mixture profiles in those situations where a minor contributor's allelic peaks are of similar heights to that of stutter peaks.

For mixtures in which minor contributor allele peaks are similar in height to that of the stutter peaks, a peak in a stutter position may be (i) only a stutter peak, (ii) only an allelic peak, or (iii) overlapping allelic and stutter peaks. Resolving these three possible scenarios is based principally on the height of the peak in the stutter position, its relationship to the stutter percentage thresholds established through internal validation studies, and the peak heights of a minor contributor(s). On average for a heterozygote pair of alleles, the smaller allele tends to have a greater peak height than the larger allele, although not always. However for the stutter peaks, the percent stutter increases with increasing allele length (7–10), and thus may complicate interpretive additive affects of stutter and an allele. If a peak at a stutter position has a peak height exceeding the stutter threshold (and the allele peaks are in the linear response range of the analytical system), that peak should be designated as an allele. However, it is possible that a peak at the stutter position can exceed the stutter peak height threshold and still be only stutter (either as an attribute of that allele or due to signal saturation or stochastic effects). Confidence in assigning the peak as an allele increases as the peak height increases beyond the stutter threshold. If a peak is at or below the stutter threshold, it may be designated a stutter peak; however, the peak should also be considered as a possible allelic peak that may have arisen from the minor contributor, if the minor contributor peaks have similar peak heights. Should a peak in a stutter peak position meet the stutter threshold, but be concluded to be an allelic peak, all stutter peaks must then be treated as potential allelic peaks (Fig. 9). An exception would be where the stated assumption is that there is only one minor contributor and a heterozygous pattern can be unequivocally assigned to the minor contributor (Fig. 10). Treating stutter peaks as potential alleles in this circumstance reduces the potential of analyst bias by not allowing the typing results obtained from the reference sample(s) to have an impact on the interpretation of stutter versus allele. Additionally, considering all potential stutter peaks in the same manner on a per comparison basis (regardless of the number of references samples being compared at the same or different times) ensures that all statistical estimates rendered are conditioned on the DNA typing results obtained from the evidence and that they are not modified by the DNA types of the reference samples.

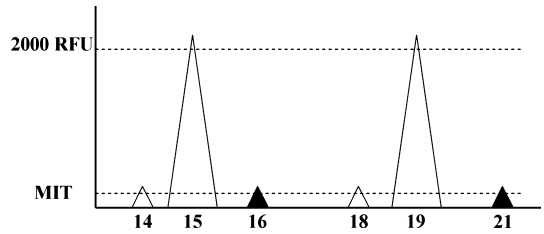

FIG. 10—*An example of an at least two person mixture with a single source major component. The minor contributor is positive for allele 16 and 21. Under the assumption of a single minor contributor, the peaks at the stutter positions (14 and 18) are not considered as possible minor contributor alleles.*

Not all stutter peaks and minor contributor scenarios would fall under the above discussion. The above discussion focused on situations where the peak heights are relatively close to the MIT threshold. For situations where there is ample signal such that the stochastic effects on stutter and minor contributor allele peaks are less of an impact (i.e., the robust range of the assay), then quantitative data can be used to eliminate peaks that would be solely stutter. For example, consider a locus profile with five peaks of which alleles 15 and 19 have RFU around 5000 and thus are from one major contributor (i.e., interpreted as a single source) (Fig. 11). The three minor peaks "14,16,18" have peak heights around 500 RFU. The 16 allele is an obligate minor contributor allele. Assume here only one minor contributor for this example. Because the peak heights are in the robust range of the assay, it is unlikely that alleles 14 and 18 are stutter plus an allele. The most plausible interpretation is that alleles 14 and 18 are solely stutter. The minor contributor can be 16,16; 15,16; or 16,19.

Comparison with Reference Specimens

Based on a forensic comparison between an evidentiary mixed specimen and a reference sample, three possible conclusions can be reached: exclusion, inclusion, or inconclusive. An SOP must contain definitions of these potential conclusions and descriptions of the data that must be present in support of any one of these conclusions.

Generally, upon comparison of the DNA profile obtained from a reference specimen with that from a mixed specimen, an exclusion is declared when the reference specimen has alleles that are not observed in the evidence and these unobserved alleles cannot be

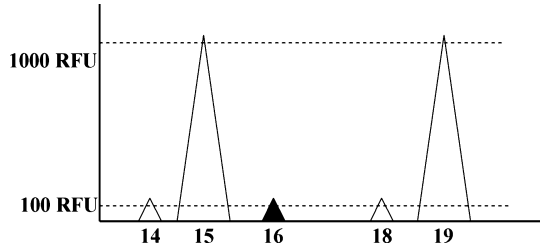

FIG. 9—*An example of an at least two person mixture with a single source major component. The minor contributor is positive for allele 16 and because of its peak height alleles at the stutter positions (14 and 18) also may be considered as possible minor contributor alleles.*

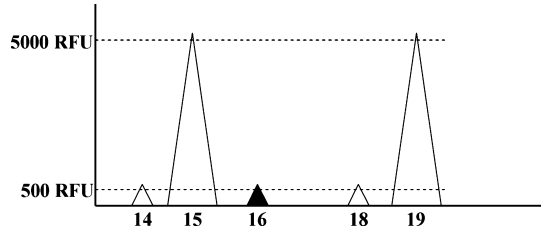

FIG. 11—*An example of an at least two person mixture with a single source major component. The minor contributor is positive for allele 16. The peak height of allele 16 is similar to stutter position peaks (14 and 18). But because the peak heights are in the robust range of the assay, the stutter peaks may not have to be considered as possible minor alleles.*

due to degradation within the evidence sample. Simply, the known individual cannot be a part contributor of the mixed profile. An exclusionary conclusion can be stated as follows:

Based on the STR typing results, the source of specimen K1 is excluded as a potential contributor to the mixture of DNA obtained from specimen Q1.

In contrast, an inclusion is declared when the genetic results obtained from a mixture is such that the reference sample(s) can not be excluded as a part contributor(s) of the mixed profile. In other words, barring degradation or signal loss, all the alleles observed in the reference sample are identified as part of the mixed profile. Such a conclusion is based both on qualitative (i.e., simple presence or absence of alleles) and quantitatively derived possible genotypes at specified loci of the evidence of which the reference profiles share. The assessment should include the formation of potential genotypes for major/minor components using established heterozygous PHR values. For example, a locus displays the alleles 8,10,11,12 with respective peak heights of 200, 2500, 2230, and 180 RFU (Fig. 12). The contributing genotypes for a two person mixture would be 8,12 and 10,11. Then when comparing a suspect's profile, an individual with the genotypes 8,10; 8,11; 10,12; and 11,12 could be reasonably expected to be excluded as a contributor of the evidence profile.

While it may not always be possible to determine the specific genotypes at a locus for a given mixture, a simple comparison based on the alleles present in a mixture can be expected to be possible for most mixtures for which allelic results are obtained. As an example, consider the mixed profile (Fig. 13) in which (i) a minimum of two individuals is indicated based on the number of allelic peaks present and (ii) based on an established heterozygous PHR threshold these results are consistent with a single homozygous major contributor and at least two minor contributors. However, another possibility is that there are three contributors and the highest peak is a composite of three doses of an allele shared by all three contributors. The three contributors all could be heterozygous with the following types: 17,19; 17,21; 17,22. In this case, all individuals that can be part contributors to this mixed profile cannot be excluded (to include many other genotypes not described above). Of course other loci for the mixed profile might assist in supporting a general interpretation of which scenario is favored.

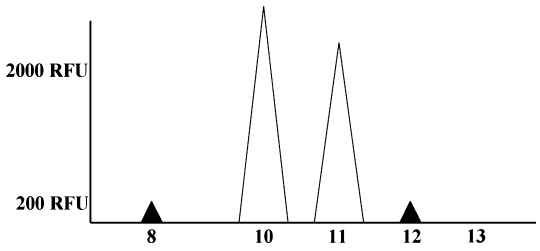

FIG. 12—*Example of a resolvable two person mixture. Only certain genetic profiles can be included: 10,11 for the major contributor and 8,12 for the minor contributor.*

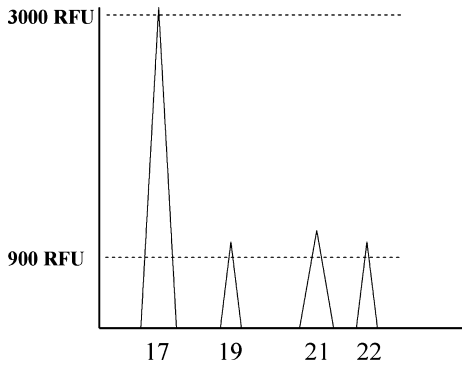

FIG. 13—*An example of an at least two person mixture, based on the presence of only four alleles. However, using quantitative information an interpretation of an at least three person mixture is supported.*

Alternatively, consider the profile (Fig. 14) in which (i) based on the number of allelic peaks, a minimum of two individuals is supported and (ii) based on PHR values, these results are consistent with a single heterozygous major contributor and a heterozygous minor contributor. Assuming a two person mixture scenario, the major contributor can be treated as a single source sample. With respect to the minor contributor, this locus is not used for statistical purposes because one of the potential alleles is below the MIT (even though alleles 19 and 22 reasonably explain the minor contributor as being a heterozygote). While not generally used by our laboratories, alternatively, it is defensible to use allele 19 and not allele 22 for a statistical assessment and employ the 2p rule at the locus for the minor contributor (3). An inconclusive call can be divided into two categories: (i) those profiles that are unsuitable for comparison (other than for exculpatory purposes); and (ii) an interpretation where the profile or portion of a profile is not used for statistical purposes such as for any locus of an indistinguishable mixture when any potentially attributable allele to a single contributor(s) is below the empirically established MIT.

For an indistinguishable mixture, all allelic peaks for all possible contributors are considered collectively for purposes of determining the loci to be used subsequently for statistical purposes. If any allelic peaks, at a locus in which a major component cannot be distinguished (such as equal contributions from two donors) and one or more allele peak heights are less than the MIT, the locus is not used for statistical purposes (alleles that fall below the PAT are inconclusive for interpretation or can be considered negative). For example, at a given locus, the MIT is 150 RFU and alleles 12,13,14,15 with peak heights 140, 160, 155, 165, respectively, are detected (Fig. 15). The locus is not used for assessing statistical weight of the evidence, and the alleles could be used only for exclusionary purposes. Consider a comparison of the two reference samples 12,13 and 14,15 with the 12,13,14,15. Neither reference specimen could be excluded but this locus would not be used for performing a mixture statistics calculation. In such an indistinguishable mixture, if all loci exhibit one or more allelic peaks that are less than the MIT, then no statistical calculations are made for the profile. A general statement that describes the reason for no application of statistics or no inclusionary result should be included in the report:

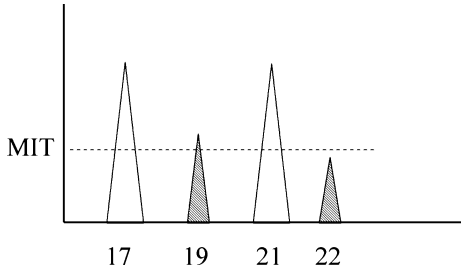

FIG. 14—*Example of two person major and minor mixture profile. The minor contributor (19,22) has one allele of the PAT but below the MIT.*

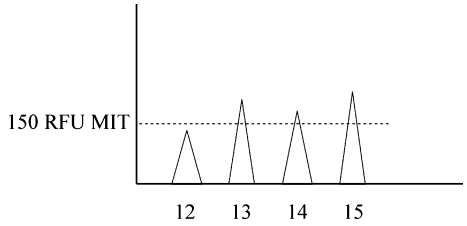

FIG. 15—*Example of an unresolvable two person mixture with one allele having a peak height less than the MIT.*

The DNA profile obtained from specimen Q1 does not satisfy the Laboratory's inclusionary reporting criteria and therefore may be utilized only for exclusionary purposes. Based upon the STR typing results, specimen K1 is excluded.

or

The DNA profile obtained for specimen Q1 does not satisfy the Laboratory's inclusionary reporting criteria and therefore may be utilized only for exclusionary purposes. These results will be maintained by the Laboratory for possible future comparisons.

Now, consider a mixed profile (similar to Fig. 14 but instead all peak heights are around the MIT) in which (i) based on the number of allelic peaks present a minimum of two individuals is supported and (ii) based on heterozygous peak height values these results are consistent with two heterozygous contributors. Assuming a minimum of a two person scenario based on all loci in the profile, a reference specimen observed to contain an allele not detected in this result could be excluded as a potential contributor to this mixture.

Consider the mixture results for three loci where amplicon size increases from left to right (Fig. 16). Based on the general amplification efficiencies attributed to low copy templates and possible degradation or amplification efficiency, the potential loss of a minor contributor's alleles at the largest locus in this series (the right hand portion) would have to be considered together with the possibility that the minor contributor's alleles are masked at this locus. In this scenario, there is only one minor contributor observed. At locus D3S1358 alleles 12 and 13 are from the minor contributor, and thus the minor contributor is a 12,13 heterozygote. At the vWA locus allele 19 is an obligate allele from the minor contributor who can be either a 19,19 homozygote or a 17,19 or 18,19 heterozygote. At the FGA locus alleles 26 and 28 are present. The minor contributor alleles are either masked by alleles 26 and/or 28 or reasonably the alleles of the minor contributor may have dropped out. Because both possibilities must be entertained when interpreting the evidence profile, the FGA locus should be considered inconclusive before any comparisons are made. A conclusion can be:

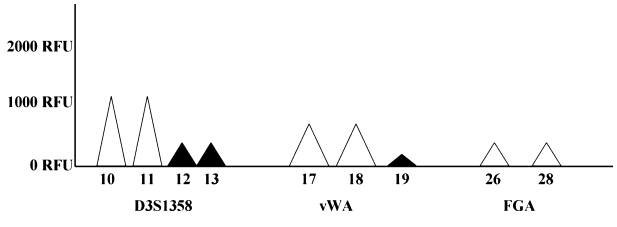

FIG. 16—*Example of two person major and minor mixture profile with fluorescent signal (i.e., peak heights) decreasing from smallest sized locus to the largest sized locus. The minor contributor (black peaks) may have dropped out in the FGA locus.*

The STR typing results for specimen Q1 indicate the presence of DNA from two or more individuals. It is noted that the sources of specimen K1 and K2 cannot be excluded as potential contributors of the major (and minor) component of the DNA obtained from specimen Q1.

Under the scenario in Fig. 16 described above, the minor contributor was the probative profile. In contrast, if the major component was the probative part of the profile, then the FGA locus could be used and the major contributor would be interpreted the same as a single source profile.

Conversely, given the profile in Fig. 17, the minor contributor alleles are unequivocally identified at the FGA locus. At the D3S1358 locus only two alleles are observed—12 and 13. In this scenario, the most plausible interpretation is that the minor contributor alleles at locus D3S1358 are masked, and the type of the minor contributor can only be a 12,12; 12,13; or 13,13. A reference sample containing other alleles at the D3S1358, say an 8,9 type would be excluded as a part contributor of the evidence profile. Allele dropout due to degradation does not increase from large to small size amplicons; therefore it is entirely reasonable to interpret the profile as suggested.

Calculation of Probability of Inclusion/Probability of Exclusion

Once a suspect's reference profile is compared with a mixture profile and an interpretation of inclusion is obtained, then the significance of the evidence needs to be conveyed to the fact finder. There are two approaches available for rendering an estimate (11). One approach, the probability of exclusion (PE), conveys how often a random person would be excluded as a part contributor of an observed mixture. In the strictest application of the PE, the calculation is based on the alleles in the mixture with no consideration of quantitative data (thus all possible genotypes that could be part contributions to the mixture). The PE does not require any assumptions or estimates of the number of contributors that comprise the mixture. Other than the requisite that the suspect (or in some cases the victim) cannot be excluded, the profile of the suspect is not considered in the calculation. The calculation of the PE is straightforward (12). Essentially, the sum of the frequencies of the alleles present in the mixture is p_i. Then, $1 - p_i = p_e$ where p_e is the sum of the frequencies of the alleles not observed in the mixed profile. Using the binomial expansion, either of the following formulas can be used to calculate the PE

$$1 - p_i^2$$

$$2p_ip_e + p_e^2$$

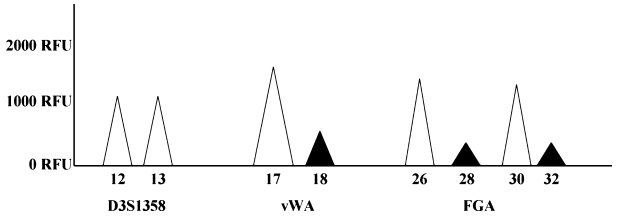

FIG. 17—*Example of two person major and minor mixture profile with fluorescent signal (i.e., peak heights) with no evidence of signal loss from smallest sized locus to the largest sized locus. The minor contributor (black peaks) alleles at the D3S1358 locus can only be 12 and/or 13. Allele dropout is not a plausible explanation for minor contributor alleles in this profile.*

For a distinguishable mixed specimen with an interpretable major and/or minor contributor or a derived profile (i.e., a mixed sample from which the allelic information from the specimen source is considered to facilitate identification of the unknown allele profile), a combined multi-locus random match probability calculation should be performed for the major contributor in accordance with a laboratory's established procedures for single source profiles and where possible for the minor contributor.

For an indistinguishable mixture, the PE calculation is more appropriate than the single source calculation. All of the alleles at a locus (or subset of alleles if separated into components) must meet the MIT, or that locus can not be included as a part of the PE mixture calculation. If any allele in a mixed specimen is below the MIT, except if major and/or minor contributions are being declared, that locus must not be used for statistical purposes; however, it should be used for exclusionary purposes where possible.

The other statistical approach, the likelihood ratio (LR), provides statistical support for postulated hypotheses on the origin of the mixture by comparing the probabilities of a given observation under the two different hypotheses. For the two (mutually exclusive) hypotheses, say H_1 and H_2, the LR is the ratio of probabilities of observing the same data under H_1 and H_2, giving

$$LR = Prob.(Data/H_1)/Prob.(Data/H_2)$$

When the LR <1, the DNA data are less well supported by H_1, compared with H_2; when the LR = 1, the DNA data are equally well supported by H_1 and H_2; and when the LR >1, the DNA data are better supported by H_1, compared with H_2.

For example, H_1 may be that the two identified suspects are the sources of the mixture and all their alleles explain all the alleles that comprise the mixture. In contrast, H_2 may be that the two identified suspects are not the sources of the mixture and two unknown unrelated individuals are the source. Under this scenario the probability of the evidence given H_1 is 1 and the probability of the evidence given H_2 essentially is the probability of inclusion under a prescribed number of contributors. While the formal logic for calculating the LR is provided elsewhere (13), we stress that every effort should be made to provide the best estimate of the number of contributors. It is not in the best interest of the defense to suggest unreasonable number of contributors; usually this will increase the LR favoring the prosecution's position.

Even with the simplistic and less powerful analysis provided by the PE (compared with the LR), there are situations where additional clarification is needed. One is where some loci present as distinguishable and some present as indistinguishable mixtures. Thus, some loci may be able to be deconvolved into single source loci and some may not. When such occurs, to follow the strict approach for calculating the PE, it is not recommended to combine single source and mixture calculations for estimating the rarity of the mixture profile. Primarily, we are concerned that such a combined calculation could be construed as a simple combined multi-locus random match probability (i.e., a quantitative deconvolution of the entire profile into single source loci). Also, if a single source profile were heterozygous at a locus (for example a 17,20 type) and treated as such, it would only consider the one genotype. However, under the PE a homozygous 17 and homozygous 20 should be added to the calculation. Consider a mixture profile where it is possible to deconvolve the two person mixture at four loci and at nine loci the mixtures are indistinguishable. Single source calculations should be done for the four loci and the PE can be calculated for the 13 loci. The estimate that is rarer can be reported. There may be other statistical approaches for such composite single source and indistinguishable mixed profiles that we have not considered; we raise the issue and present one approach so that the community is aware of potential ambiguities.

Full Accounting of Allelic Data

Mixed specimens for which multiple reference specimens are included as potential contributors should be evaluated for whether or not all of the DNA typing results obtained from the mixed specimen are accounted for by the multiple matching references samples. When such a full accounting is made, the analyst can provide this observation in the report. An example statement is:

The STR typing results for specimen Q1 indicate the presence of DNA from at least two individuals. The sources of specimens K1 and K2 cannot be excluded as potential contributors to this mixture. It is noted that the sources of K1 and K2 can account for all of the DNA typing results obtained from specimen Q1.

A full accounting of the alleles observed in a mixture conveys that a mixture displays a minimum number of individuals and that the individuals found to be included contributors of the mixture do, in fact, account for all of the allelic information obtained from the mixture. In this way this mixture is one for which a set of known individuals has been identified whose DNA profiles combined would yield the results obtained from an evidentiary sample. However, it is important to note that this statement does not imply that because these matching individuals can account for all of the results obtained from a mixed sample, that they, by extension, can be the only two individuals who could do so. Care must be taken not to portray such a result as being an establishment of source attribution. Proper statistical calculations should be provided that are commensurate with the results obtained**.**

Conclusions

A standardized mixture interpretation protocol is not recommended or possible. There are myriad ways that mixed profiles may present and all possibilities could never be prescribed. Additionally, protocols may be developed that have different degrees of conservatism and this should not be construed as disagreement within the field. However, the aspects of mixture interpretation described herein should be considered as requisites to be included in any documented mixture interpretation guidelines. Thus, any qualified forensic scientist would be able to understand the process that is advocated within a laboratory and to evaluate any specific case interpretation for its validity.

The ISFG recommendations (14) gave some basic considerations for mixture interpretation. We provide more guidance to consider for establishing mixture interpretation guidelines. Gill et al. (15) recently addressed some of the same aspects of mixture interpretations that are provided herein in response for clarification of the ISFG recommendations. In general we agree with the recommendations of Gill et al. that are: (i) when possible peak height/area should be included in mixture interpretation; (ii) stutter position peaks at similar peak height/area as that of obligate minor contributor alleles should be considered as potential alleles in the interpretation and statistics calculation; and (iii) a stochastic threshold (termed "dropout threshold") should be defined.

Gill et al also recognized that the Probability of Inclusion (termed "RMNE"), which is 1 – PE, is a recognized and advocated statistical method, and we concur. They also recommend that even if the LR is not used, the calculation should be included in case

notes and advise the court of the LR results. We support that forensic scientists should be trained to calculate either statistical approach; but do not support that the LR is a preferred method that must be captured in the notes. It is clear that the significance of some mixtures may not be easily calculated using the LR, such as some mixtures with three or more contributors. Instead we support the position of the DAB (11): "Rarely is there only one statistical approach to interpret and explain the evidence. The choice of approach is affected by the philosophy and experience of the user, the legal system, the practicality of the approach, the question(s) posed, available data, and/or assumptions. For forensic applications, it is important that the statistical conclusions be conveyed meaningfully. Simplistic or less rigorous approaches are often sought. Frequently, calculations such as the random match probability and probability of exclusion convey to the trier of fact the probative value of the evidence in a straightforward fashion. Simplified approaches are appropriate, as long as the analysis is conservative or does not provide false inferences. Likelihood ratio (LR) approaches compare mutually exclusive hypotheses and can be quite useful for evaluating the data. However, some LR calculations and interpretations can be complicated, and their significance to the case may not be apparent to the practitioner and the trier of fact." Also the DAB stated "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." This is a more balanced position and is more practical for addressing the various mixture profiles that may be encountered. It is better to use what is best determined to be meaningful for assessment and/or for communication by a laboratory. However, what ever is used must be clearly documented in the SOP and any assumptions impacting the calculation should be recorded.

Lastly, we strongly urge caution with mixture interpretation with any low copy number (LCN) typing. The interpretation guidelines described above do not apply to LCN typing. Additional analytical measures beyond routine typing protocols are taken to increase amplicon yield from LCN samples. By its nature LCN typing typically analyzes samples that fall below the stochastic threshold. Peak height ratios and allele dropout thresholds cannot be instituted for such samples. Indeed, most peaks from LCN samples should be below a dropout threshold. In order to obtain reliable interpretations, it is imperative that analysts recognize when they are working with LCN samples, define what modifications they make to their protocols to obtain detectable amplified product, and develop more strict interpretation protocols than provided herein.

Documenting the minimum number of contributors of a mixed specimen and stating appropriate assumptions ensures that the nature of the mixture is fully communicated in the report. While accurate, a statement in a report describing a mixture as indicating the presence of DNA from more than one individual when more clarity can be conveyed lacks the precision to provide a sense of what it is that an analyst observed as a part of the analysis. In itself such a statement may have reduced investigative lead value. As much as it is the responsibility of the forensic scientist to not overstate the significance of a test result, an equally important tasking is that an analyst should not ignore defendable conclusions in a mistaken effort to be "conservative." Conclusions so "conservative" that they strip away supportable elements of their meaning (i.e., grossly understate) are effectively rendered inaccurate and are no less unsuitable for reporting than an inaccurate over-statement of a conclusion.

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