J Forensic Sci, July 2009, Vol. 54, No. 4
doi: 10.1111/j.1556-4029.2009.01046.x
Available online at: www.blackwell-synergy.com

Bruce Budowle,[1] *Ph.D.; Anthony J. Onorato,*[1] *M.S.F.S., M.C.I.M.; Thomas F. Callaghan,*[1] *Ph.D.; Angelo Della Manna,*[2] *M.S.; Ann M. Gross,*[3] *M.S.; Richard A. Guerrieri,*[1] *M.S.; Jennifer C. Luttman,*[1] *M.F.S.; and David Lee McClure,*[4] *B.S.*

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.),

[1]FBI Laboratory, 2501 Investigation Parkway, Quantico, VA 22134.
[2]Alabama Department of Forensic Sciences, 2026 Valleydale Road, Hoover, AL 35244.
[3]Minnesota BCA Forensic Science Laboratory, 1430 Maryland Avenue East, St. Paul, MN 55106.
[4]South Carolina Law Enforcement Division, DNA Database Unit, 4416 Broad River Road, Columbia, SC 29210.

*This is publication number 08–04 of the Laboratory Division of the Federal Bureau of Investigation. Names of commercial manufacturers are provided for identification only, and inclusion does not imply endorsement by the Federal Bureau of Investigation.

Received 17 Feb. 2008; and in revised form 30 July 2008; accepted 3 Aug. 2008.

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).

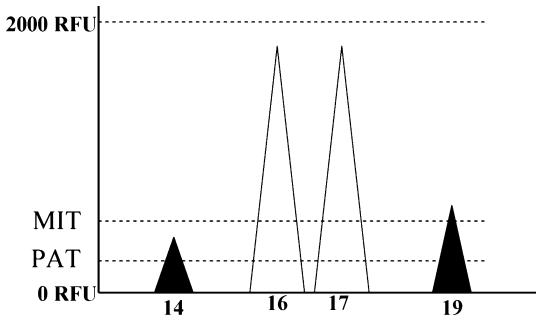

FIG. 1—*Example of two person major and minor mixture profile. The minor contributor has one allele above the PAT but below the MIT and another allele above the MIT.*

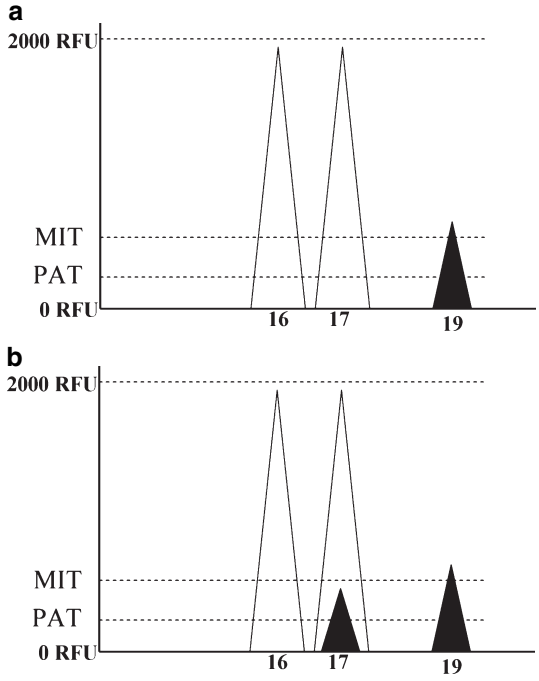

FIG. 2—*Example of a two person major and minor profile. (a) Only one allele is visible for the minor contributor and it is above the MIT. (b) A hypothetical is displayed that the accompanying heterozygous allele on the minor contributor is masked by a major component allele.*

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

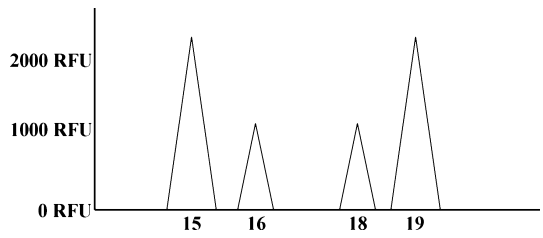

FIG. 3—*Example of a resolvable two person mixture.*

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).

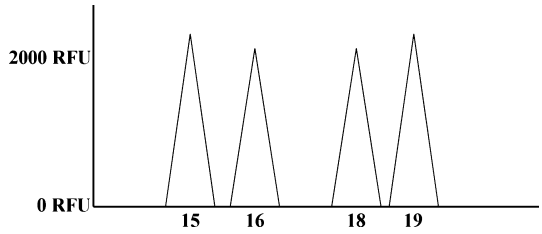

FIG. 4—*Example of an unresolvable two person mixture.*

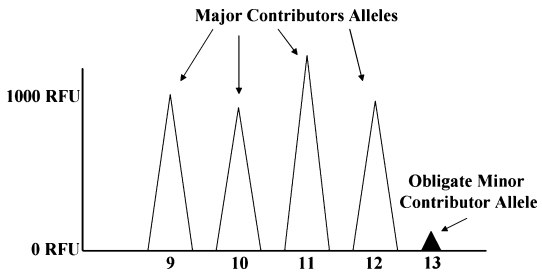

FIG. 5—*An example of an at least three person mixture. Alleles 9,10,11, and 12 are part of the major component and allele 13 is part of the minor component. Resolving the two components is possible because the amount of DNA is in the robust range of the assay.*

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

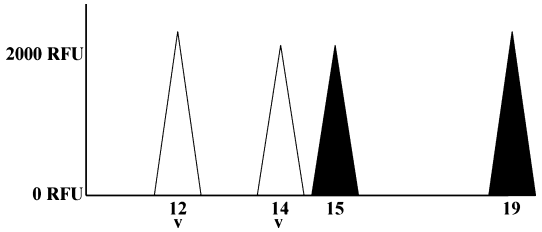

FIG. 6—*An example of a two person mixture that is resolvable because the victim's alleles (12,14) can be subtracted from the profile. The black peaks (15,19) are resolved as a single source component from the unknown contributor.*

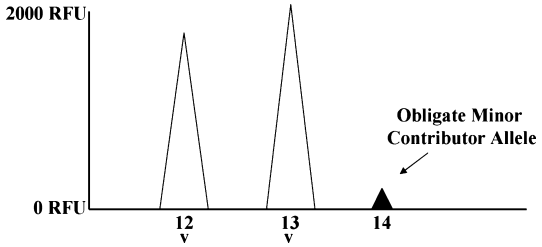

FIG. 7—*An example of a two person mixture where the probative portion is that of the minor contributor. The major alleles (12,13) can be subtracted. Allele 14 is an obligate allele that may be from a contributor of the types 14,14; 12,14; or 13,14.*

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.

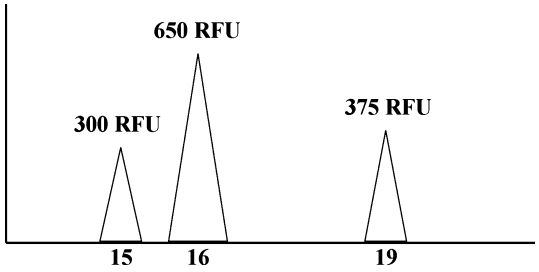

FIG. 8—*An example of a two person mixture where quantitative data supports only two reasonable mixture possibilities.*

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.

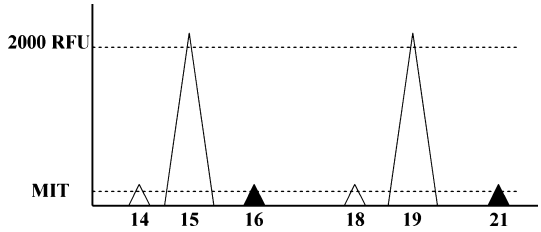

FIG. 10—*An example of an at least two person mixture with a single source major component. The minor contributor is positive for allele 16 and 21. Under the assumption of a single minor contributor, the peaks at the stutter positions (14 and 18) are not considered as possible minor contributor alleles.*

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

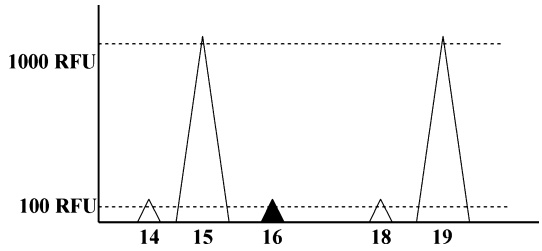

FIG. 9—*An example of an at least two person mixture with a single source major component. The minor contributor is positive for allele 16 and because of its peak height alleles at the stutter positions (14 and 18) also may be considered as possible minor contributor alleles.*

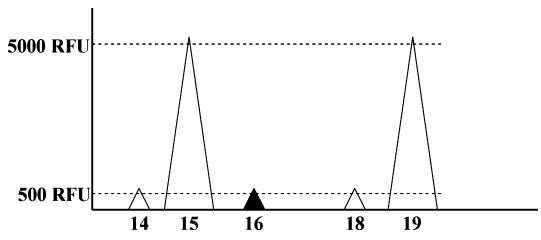

FIG. 11—*An example of an at least two person mixture with a single source major component. The minor contributor is positive for allele 16. The peak height of allele 16 is similar to stutter position peaks (14 and 18). But because the peak heights are in the robust range of the assay, the stutter peaks 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

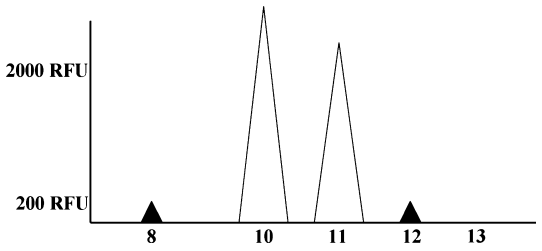

FIG. 12—*Example of a resolvable two person mixture. Only certain genetic profiles can be included: 10,11 for the major contributor and 8,12 for the minor contributor.*

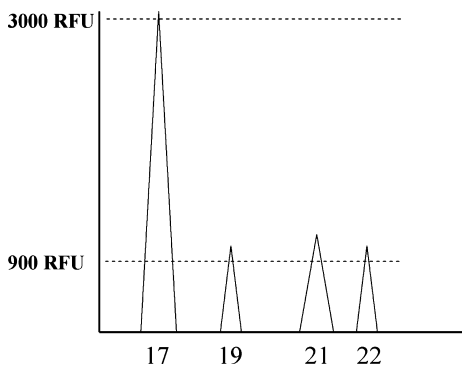

FIG. 13—*An example of an at least two person mixture, based on the presence of only four alleles. However, using quantitative information an interpretation of an at least three person mixture is supported.*

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.

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:

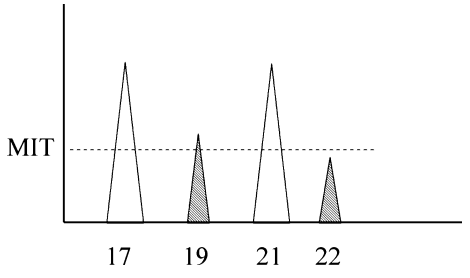

FIG. 14—*Example of two person major and minor mixture profile. The minor contributor (19,22) has one allele of the PAT but below the MIT.*

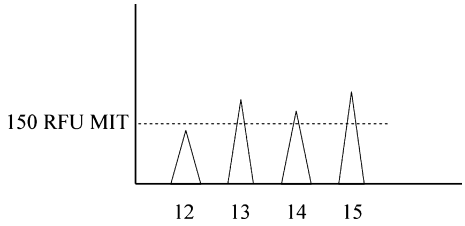

FIG. 15—*Example of an unresolvable two person mixture with one allele having a peak height less than the MIT.*

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:

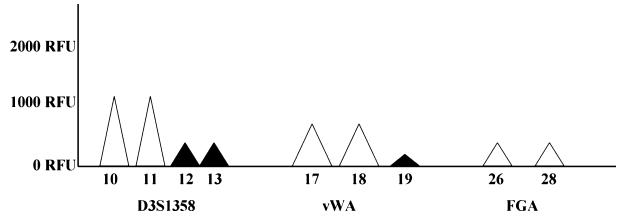

FIG. 16—*Example of two person major and minor mixture profile with fluorescent signal (i.e., peak heights) decreasing from smallest sized locus to the largest sized locus. The minor contributor (black peaks) may have dropped out in the FGA locus.*

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

$$1 - p_i^2$$

$$2p_ip_e + p_e^2$$

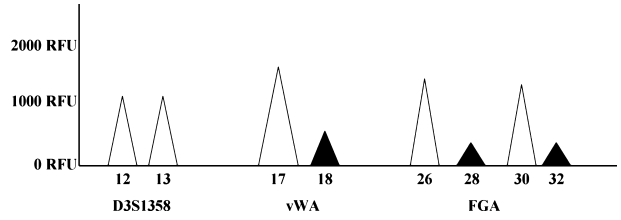

FIG. 17—*Example of two person major and minor mixture profile with fluorescent signal (i.e., peak heights) with no evidence of signal loss from smallest sized locus to the largest sized locus. The minor contributor (black peaks) alleles at the D3S1358 locus can only be 12 and/or 13. Allele dropout is 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.

References

1. Moretti TR, Baumstark AL, Defenbaugh DA, Keys KM, Budowle B. Validation of short tandem repeats (STRs) for forensic usage: performance testing of fluorescent multiplex STR systems and analysis of authentic and simulated forensic samples. J Forensic Sci 2001; 46(3):647–60.
2. Moretti TR, Baumstark AL, Defenbaugh DA, Keys KM, Budowle B. Validation of STR typing by capillary electrophoresis. J Forensic Sci 2001;46(3):661–76.
3. Budowle B, Giusti AM, Waye JS, Baechtel FS, Fourney RM, Adams DE, et al. Fixed bin analysis for statistical evaluation of continuous distributions of allelic data from VNTR loci for use in forensic comparisons. Am J Hum Genet 1991;48:841–55.
4. Brinkmann B. Is the amelogenin sex test valid? Int J Legal Med 2002;116(2):63.
5. Steinlechner M, Berger B, Niederstätter H, Parson W. Rare failures in the amelogenin sex test. Int J Legal Med 2002;116(2):117–20.
6. Thangaraj K, Reddy AG, Singh L. Is the amelogenin gene reliable for gender identification in forensic casework and prenatal diagnosis? Int J Legal Med 2002;116(2):121–3.
7. Holt CL, Buoncristiani M, Wallin JM, Nguyen T, Lazaruk KD, Walsh PS. TWGDAM validation of AmpFlSTR PCR amplification kits for forensic DNA casework. J Forensic Sci 2002;47:66–96.
8. Krenke BE, Viculis L, Richard ML, Prinz M, Milne SC, Ladd C, et al. Validation of male-specific, 12-locus fluorescent short tandem repeat (STR) multiplex. Forensic Sci Int 2005;151:111–24.
9. Wallin JM, Buoncristiani MR, Lazaruk KD, Fildes N, Holt CL, Walsh PS. TWGDAM validation of the AmpFISTR blue PCR amplification kit for forensic casework analysis. J Forensic Sci 1998;43:854–70.
10. Walsh PS, Fildes NJ, Reynolds R. Sequence analysis and characterization of stutter products at the tetranucleotide repeat locus vWA. Nucleic Acids Res 1996;24:2807–12.
11. DNA Advisory Board. Statistical and population genetics issues affecting the evaluation of the frequency of occurrence of DNA profiles calculated from pertinent population database(s). Forensic Sci Commun 2000;2(3): Available at http://www.fbi.gov/hq/lab/fsc/backissu/july2000/dnastat.htm.
12. Devlin B. Forensic inference from genetic markers. Stat Methods Med Res 1992;2:241–62.
13. Evett IW, Weir BS. Interpreting DNA evidence. Sunderland, MA: Sinauer, 1998.
14. Gill P, Brenner CH, Buckleton JS, Carracedo A, Krawczak M, Mayr WR, et al. DNA commission of the International Society of Forensic Genetics: recommendations on the interpretation of mixtures. Forensic Sci Int 2006;160:90–101.
15. Gill P, Brown RM, Fairley M, Smyth M, Simpson N, Irwin B, et al. National recommendations of the Technical UK DNA working group on mixture interpretation for the NDNAD and for court going purposes. Forensic Sci Int Genet 2008;2:76–82.

Additional information and reprint requests:
Bruce Budowle, Ph.D.
FBI Laboratory
2501 Investigation Parkway
Quantico, VA 22135
E-mail: bbudowle@hsc.unt.edu