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Characterizing rates of allelic dropout and the impact on estimating the number of contributors

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Thesis

**CHARACTERIZING RATES OF ALLELIC DROPOUT AND
THE IMPACT ON ESTIMATING THE NUMBER OF CONTRIBUTORS**

by

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ABSTRACT

Forensic analysis of a deoxyribonucleic acid (DNA) profile includes determining if DNA from a known person should be considered as a likely contributor to the biological evidence. Prior to making this determination, the number of contributors (NOC) of the DNA sample is considered. It is important to take multiple factors into account when estimating the NOC, including stutter, baseline noise, and peak imbalance as these can affect the number of peaks observed at each locus. Allelic dropout can also have an impact on the number of peaks observed. Dropout occurs when an allele is not detected due to technical, biochemical, or sampling issues, and predominantly affects the level of ambiguity associated with low-template DNA interpretation. As the NOC to a sample may be underestimated in the presence of dropout, it is essential to reasonably predict the probability that an allele has dropped out.

This work has two aims: first, to evaluate different characterizations of allelic dropout rates and, second, to determine the impact allelic dropout has on estimating the NOC to a DNA sample. Two different types of dropout characterization were examined – ‘indirect’ models based on observed peak heights and ‘direct’ models using observed dropout frequencies of single-source calibration data. The indirect models predicted allelic dropout based on the peak height distribution of the data at a specific target amount and locus using a fitted or non-fitted cumulative Gaussian curve. For the direct

models, a logistic or exponential regression of the observed dropout frequencies versus target amount for each locus was used to predict dropout rates.

The impact that allelic dropout has on estimating the NOC was assessed by varying the probability of dropout ($\text{Pr}(D)$) in simulated mixtures with up to six contributors in the presence or absence of a major contributor. Simulations for the short tandem repeat (STR) loci consistent with the AmpF ℓ STR $\text{\textcircled{R}}$ Identifiler $\text{\textcircled{R}}$ Plus (Applied Biosystems $\text{\textcircled{R}}$, Foster City, CA) and GlobalFiler TM (Applied Biosystems $\text{\textcircled{R}}$, Foster City, CA) amplification kits were completed to explore the impact additional polymorphic loci have on estimating the NOC. The NOC for each profile was determined using the maximum allele count (MAC) method.

An exponential or logistic regression of observed frequencies of dropout ($\text{Fr}(D)$) was found to be an appropriate characterization of allelic dropout rates. In general, the peak height based methods overestimated dropout at higher target levels and underestimated it at lower target amounts. The underestimation suggests that other factors beyond detection and polymerase chain reaction (PCR) variation contribute to dropout. Across all loci, the $\text{Fr}(D)$ increased as target amount decreased and as molecular weight increased.

Estimating the actual NOC using MAC was found to be unreliable for mixtures with greater than three contributors or with one or more minor contributors present at low levels. While a high level of dropout did not affect correctly identifying two-person mixtures, it greatly increased the number of misidentifications with three or more contributors. The number of misidentifications was reduced for mixtures when 21 STR

loci plus amelogenin were used to evaluate the NOC. These higher accuracies were frequently attributable to the highly polymorphic locus SE33. The presence or absence of a major contributor did not appear to substantially affect the results. Forensic laboratories using MAC to determine the NOC of mixed samples should be aware of the tendency to underestimate the NOC using this method. It is also important to understand the impact that allelic dropout has on correctly estimating the NOC. The probability that allele dropout may have occurred in a sample should be considered when evaluating the NOC that explains the evidentiary profile.

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LIST OF ABBREVIATIONS

AT	Analytical threshold
CE	Capillary electrophoresis
CODIS	Combined DNA Index System
DNA	Deoxyribonucleic acid
Fr(D)	Frequency of dropout
LR	Likelihood ratio
LT	Low-template
MAC	Maximum allele count
MLE	Maximum likelihood estimator
ng	Nanogram
NOC	Number of contributors
PCR	Polymerase chain reaction
pg	Picogram
Pr(D)	Probability of dropout
RFU	Relative fluorescent unit
STR	Short tandem repeat
SWGDM	Scientific Working Group on DNA Analysis Methods

1. CHARACTERIZING RATES OF ALLELIC DROPOUT

1.1 Introduction

During forensic deoxyribonucleic acid (DNA) analysis, a DNA profile obtained from an item of evidence may be compared to profiles obtained from persons of interest. Depending on the inferred genotypes, a person may be classified as included or excluded as a possible contributor to the sample. Many processes or software programs that infer the possible unknown genotypes exist and most commercially available systems require an assumption on the number of contributors (NOC). Multiple signal interferences can affect the number and intensity of observed peaks, which are considered when estimating the NOC. Examples of signal interferences include, but are not limited to, stutter, baseline noise, and imbalance in allele signal. Allelic dropout can also have an impact on the number of peaks observed. Allelic dropout occurs when an allele is not detected due to technical, biochemical, or sampling issues [1-3] and affects the level of ambiguity associated with low-template (LT) DNA interpretation. For example, the assigned NOC may be underestimated if dropout has occurred. As a result, incorrect genotypes may be inferred, which in turn, affect the reported conclusion. If underestimation occurs, a suspect may be wrongly ‘excluded’ as a possible contributor if their genotype at a locus is a,b while only allele a is detected in the evidence. Alternatively, the locus may unnecessarily be disregarded during interpretation, or the likelihood ratio (LR), which is a measure of the ‘match-strength’, may be affected. It is therefore essential to accurately predict the probability that dropout may have occurred in a sample, and to understand the extent to which dropout impacts the interpretation of a DNA profile. Since levels of

dropout increase as the target mass of a sample decreases, understanding the impact is particularly important when analyzing LT samples.

1.1.1 Challenges Associated with Low-Template DNA Interpretation

Samples with a high DNA concentration were typically analyzed, processed, and interpreted when DNA typing was first developed [4]. Since then, laboratory methods have continually become more sensitive, allowing for samples with low DNA quantities to be typed [5]. Complex mixtures with LT components are difficult to interpret due to the occurrence of stochastic effects, including heterozygote peak imbalance, increased incidences of stutter, allele dropout, and allele drop-in [6]. Several studies have investigated the challenges associated with LT DNA interpretation [4-13], while other publications have focused on the development of guidelines for LT DNA profiling for specific amplification kits [14-15].

Benschop et al. evaluated the difficulty of interpreting complex LT DNA mixtures through an assessment of mock cases [4]. Mixtures were prepared using known amounts of high quality DNA extracts of unrelated or related individuals and amplified four times to create consensus profiles; alleles must be present in at least two of the profiles in order to be considered a ‘consensus’ allele. Reporting officers at the Netherlands Forensic Institute individually analyzed the profiles provided – the four replicate profiles, the consensus profile, the reference profiles of assumed contributors, and a profile of a potential suspect – and determined if the suspect was included or excluded as a possible contributor to the samples. One case consisted of one major (150 picograms, pg) and two

minor contributors (30 pg, 6 pg). Due to severe allelic dropout of the minor contributors, both the maximum number of alleles at each locus and peak height ratios failed to indicate the presence of a third contributor for three of the four replicate profiles. Another case was a mixture of four donors (300 pg, 30 pg, 30 pg, 30 pg). Approximately 15-56% of non-shared alleles for all three minor contributors dropped out, making it difficult to correctly estimate the NOC. Only the consensus profile exhibited the characteristics of a four-person mixture. This descriptive study demonstrates the difficulties associated with LT DNA interpretation. Allele sharing and the dropout of alleles belonging to the minor contributor(s) can lead to an inaccurate assessment of the NOC to a sample and, thus, inaccurate genotype inferences for each contributor.

1.1.2 Allelic Dropout

An extreme form of heterozygote peak imbalance [11], allelic dropout is defined by the Scientific Working Group on DNA Analysis Methods (SWGDM) as “the failure to detect an allele within a sample or failure to amplify an allele during PCR”, the polymerase chain reaction [2]. Peaks above the analytical threshold (AT) are considered true peaks, either artifacts or alleles, as they can reliably be distinguished from background noise. While balanced heterozygote peaks and no dropout are typical of samples with high signal-to-noise, samples with low starting quantities of DNA are characterized by increased peak imbalance and dropout [11]. Figure 1 illustrates the scenarios that can potentially arise at a locus with a known genotype a,b .

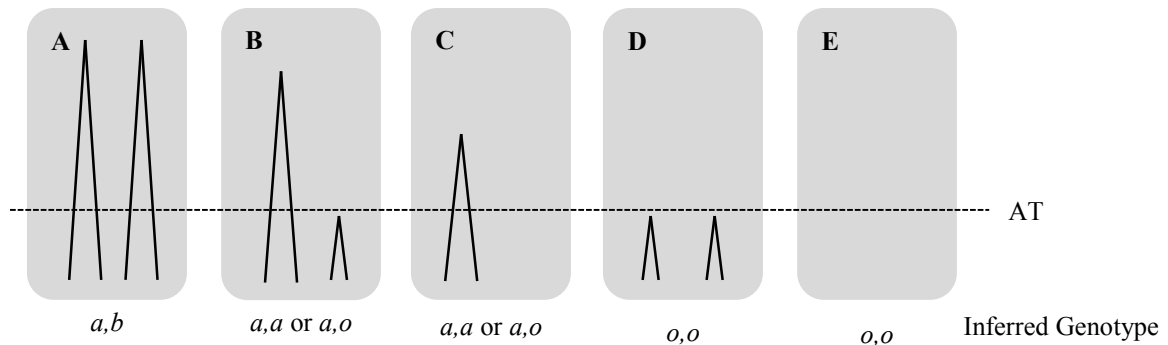


Figure 1. Possible scenarios obtained during LT DNA evidence processing and analysis. (A) No dropout is observed. (B) Dropout of allele b , which is observed but below the AT. The genotype a,a or a,o (for other allele, not observed) can be inferred. (C) Complete dropout of allele b due to the absence of the allele during PCR. Again, the genotype a,a or a,o can be inferred. (D) Locus dropout because neither allele surpasses the AT. (E) Complete locus dropout.

1.1.3 Overview of STR Analysis

Studies by Hedell et al. and Timkin et al. concluded that allele dropout occurs due to a combination of preferential amplification and pre-PCR selection, with pre-PCR selection being the dominant factor [16, 17]. An overview of the entire process of short tandem repeat (STR) analysis is described by Gill et al. beginning with the extraction of DNA from a sample, preparing an aliquot of the extract for PCR, PCR itself, and the visualization of alleles via capillary electrophoresis (CE) [18]. Figure 2 demonstrates that an allele will only be amplified if the aliquot of the extract taken for PCR contains a copy of the allele. Further, if the aliquot does not contain a sufficient number of copies, the signal of the amplified allele will fall below the AT, resulting in the allele dropping out.

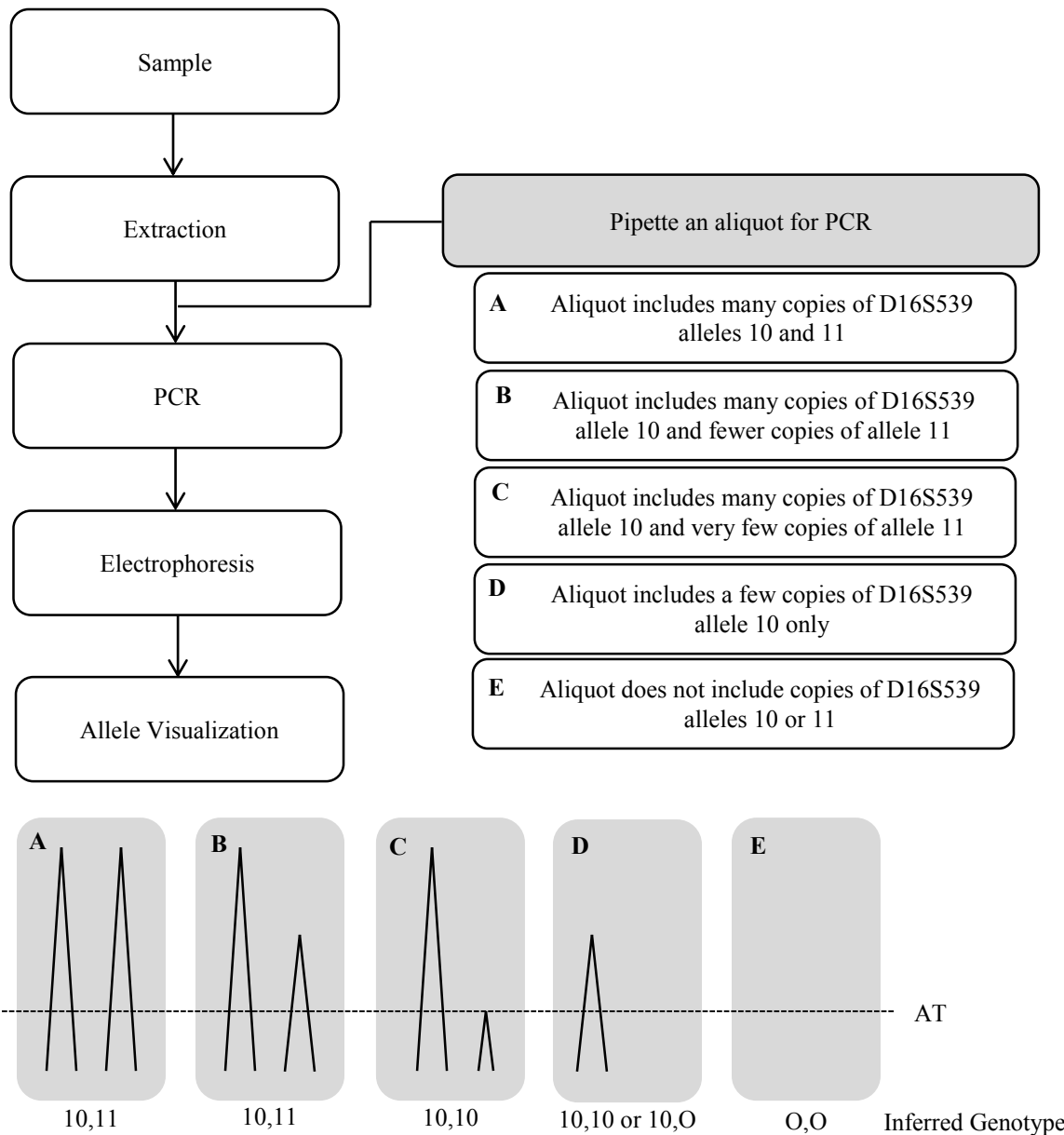


Figure 2. An overview of DNA analysis and interpretation for a representative locus, D16S539 and genotype 10,11. (A) Equal peak heights are visualized for an aliquot containing equal copies of alleles 10 and 11. When the aliquot includes more copies of allele 10 than allele 11, a higher peak height for allele 10 is observed with the peak height of allele 11 above (B) or below (C) the AT depending on the number of copies of allele 11 present in the aliquot. (C) With allele 11 falling below the AT, an incorrect genotype of 10,10 is inferred. (D) If the pipette does not pick up any copies of allele 11, the aliquot will contain copies of allele 10 only and allele 11 will not be amplified. The genotype 10,10 or 10,O is inferred. (E) Complete locus dropout is observed if the aliquot does not include any copies of alleles 10 and 11. Thus the genotype O,O is inferred.

1.1.4 Impact of Allelic Dropout on Estimating the Number of Contributors

The true NOC to a forensic profile can never be known, however, when analyzing a profile analysts must make a reasonable assumption by examining all aspects of the profile [19]. The maximum allele count (MAC) of a profile is a common method used to determine the lower bound on the NOC that can explain the observed set of alleles. However, the MAC result may not reflect the actual NOC [19]. A number of factors can make it difficult to accurately assess the true NOC to a sample, including the presence of multiple low-level contributors or degraded DNA, allele sharing, and allelic dropout [20]. If dropout has occurred in a sample, the NOC may be underestimated as dropout can lead to a decrease in the number of observed alleles. The impact that allelic dropout has on the NOC is investigated in the second part of this work.

1.1.5 Current Guidelines for Assessing Dropout

The DNA commission of the International Society of Forensic Genetics provides recommendations on the interpretation of mixtures [3]. Recommendation 7 states that in order to explain the evidence with the dropout of an allele, then the allele that is present must have a small enough peak height to justify this interpretation. For example, if the evidence contains allele a at locus l and the suspect's genotype at locus l is a,b then allele a must have a peak height that allows for the potential of a dropped out sister allele, where the height criterion is established prior to interpretation, presumably during validation studies. Further, Recommendation 8 states that a peak with height similar to background noise should not be considered a true allele. In addition, the probability that

dropout may have occurred should be reflected in any statistical calculation. The DNA commission includes three examples that outline how the probability of dropout ($\text{Pr}(D)$) can be incorporated into the statistical calculation and shows how the LR varies when the $\text{Pr}(D)$ ranges from 0.1 to 0.9. However, no guidance on accurately determining the $\text{Pr}(D)$ is provided.

The SWGDAM Interpretation Guidelines for Autosomal STR Typing also provide a basic definition of dropout and provide a means, through the application of a stochastic threshold, to evaluate whether an allele has dropped out [2], but again, no guidance on accurately determining the $\text{Pr}(D)$ is provided. Different statistical formulae used to represent the strength of inclusion are also included in the guidelines but in the standard application of one of the formulae, the combined probability of inclusion and exclusion (CPI/CPE), no statistical calculation should be performed at loci where allele dropout is suspected. Furthermore, despite a description of the LR, no examples on incorporating the $\text{Pr}(D)$ into the calculation are provided. One must refer to outside sources for additional information. Several studies have investigated how to estimate the $\text{Pr}(D)$, and these are discussed in the section below.

1.1.6 Allelic Dropout Models

Some dropout models condition the $\text{Pr}(D)$ on the average peak height of a profile, which is taken as a proxy for the amount of DNA present in a sample, or on the peak height of an observed allele [1, 16, 21-24]. A logistic regression to model allelic dropout was first introduced by Tvedebrink et al. and Gill et al. in 2009 [1, 24]. Based on one or

more variables, a logistic regression can illustrate the probability that a binary event has or has not occurred [25]. Tvedebrink et al. conditioned $P(D)$, the probability of dropout, on ' \hat{H} ', the sum of the observed peak heights divided by the number of observed alleles, where a homozygous allele is counted twice [1]. The logistic model has one explanatory variable, \hat{H} , such that

$$P(D|\hat{H}) = \frac{\exp(\beta_0 + \beta_1 \log \hat{H})}{1 + \exp(\beta_0 + \beta_1 \log \hat{H})} = \begin{cases} P(D|H), & \text{Non - shared het allele} \\ P(D|2H), & \text{Non - shared hom allele,} \\ P(D|H^{(1)} + H^{(2)}), & \text{shared het allele} \end{cases}$$

where β_0 and β_1 are regression coefficients, the $P(D|\hat{H})$ decreases as \hat{H} increases, and $P(D|\hat{H} = 0) = 1$. The probability of allelic dropout was found to be locus dependent with high molecular weight loci exhibiting more dropouts than low molecular weight loci when an AT of 50 relative fluorescent units (RFU) was used. Tvedebrink et al. later expanded this approach by allowing for varying number of PCR cycles [22]. The model developed by Gill et al. differs from the model above with the $\Pr(D)$ conditioned on the peak height of the present allele rather than a proxy for the total amount of DNA present in a sample [24].

Another logistic regression model for allelic dropout was developed in 2015 by Hedell et al [16]. This model allowed for varying CE injection times, PCR cycles, DNA input amounts, amplicon lengths, markers, and fluorescent dyes. The aim of this study was to evaluate the impact amplicon length, STR marker, and fluorescent label have on the $\Pr(D)$. The basis of the model developed by Hedell et al. is given below:

$$\text{logit}(p_i) = \log\left(\frac{p_i}{1-p_i}\right) = \alpha + \beta_d \log(d_i),$$

where p_i is the $\text{Pr}(D)$, α is the intercept, β_d is the regression parameter, and d_i is the estimated amount of DNA.

Dropout rates are also dependent on the AT. A study by Lohmueller et al. in 2014 characterized patterns of allelic dropout for ATs of 30 and 50 RFU [23]. More dropouts were observed at an AT of 50 RFU compared to 30 RFU. Tvedebrink et al., Gill et al., and Hedell et al. all used an AT of 50 RFU in their studies [1, 16, 22, 24]. Although it is suspected that lower dropout rates would have been observed had an AT of 30 RFU been chosen, the logistic model would probably hold. Overall, these models demonstrate that the $\text{Pr}(D)$ increases as the amount of DNA or observed peak heights decreases, is higher for large molecular weight loci, and may be locus dependent.

The purpose of the present study is to evaluate different characterizations of allelic dropout rates and determine an appropriate model. As the effects of allelic dropout are most prevalent in LT or degraded DNA samples, it was of interest to base the model on LT target amounts ranging from 0.008 to 0.25 nanograms (ng). Two different types of dropout characterization were examined – ‘indirect’ models based on observed peak heights, and ‘direct’ models using observed dropout frequencies of single-source calibration data.

1.2 Methods

1.2.1 Sample Preparation

Previously prepared samples were analyzed in this study. Briefly, 96 samples of known genotype were amplified using the AmpF ℓ STR $\text{\textcircled{R}}$ Identifiler $\text{\textcircled{R}}$ Plus (Applied Biosystems $\text{\textcircled{R}}$, Foster City, CA) kit at seven LT target amounts: 0.25, 0.125, 0.063, 0.047, 0.031, 0.016, 0.008 ng. The Identifiler $\text{\textcircled{R}}$ Plus amplification chemistry provides genetic information on the following STR loci: D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, FGA, and AMEL, the sex-determining locus [26]. Amplified products were then analyzed on the ABI 3130 Genetic Analyzer (Applied Biosystems $\text{\textcircled{R}}$, Foster City, CA) using a 3 kilovolt (kV) injection for 10 seconds.

1.2.2 Data Filtering

The electropherograms were analyzed in GeneMapper ID-X v 1.1.1 (Applied Biosystems $\text{\textcircled{R}}$, Foster City, CA) using the Local Southern Method [27] with no AT applied. All of the non-allele peak information was filtered from the exported data based on the known genotypes. The peak height of homozygous alleles was divided by two as it was assumed that each allele contributed equally to the observed peak height. Alleles that were not observed were assigned a peak height of zero. The data were sorted by target mass and locus.

1.2.3 Calculating Observed Frequencies of Dropout

Alleles with peak heights greater than or equal to 1 RFU were considered detected alleles. The observed frequency of dropout (Fr(D)) was calculated for each locus at the seven different target masses using the following equation:

$$\frac{\text{\# of non-detected alleles}}{\text{\# of expected alleles}} \quad (\text{Equation 1}).$$

For example, the dropout frequency for D8S1179 at an input amount of 0.008 ng is 0.223 as 37 of 166 expected alleles were not detected. Homozygous loci were not considered in the dropout frequency calculation since it would not be possible to determine if both alleles were present or one allele dropped out and only the second allele was observed.

1.2.4 Calculating Probabilities of Dropout

Allelic dropout models evaluated in this study are summarized in Figure 3 on page 16.

1.2.4.1 Model 1 – Fitted Cumulative Gaussian

The first two models investigated the ability to predict allelic dropout based on the peak height distribution of the single-source calibration data at a specific target amount and locus. A cumulative peak height histogram was created using the auto-binning function provided in IGOR Pro v 6.2 (WaveMetrics, Inc., Portland, OR). The bin width was calculated via Scott's Method [28], a method for random samples of normally distributed data:

$$3.49 * s * N^{-1/3} \quad (\text{Equation 2}),$$

where s = standard deviation and N = number of data points. Heterozygote and homozygote peak heights were used in the creation of the histogram. The mean (μ) and standard deviation (σ) of each histogram were computed by fitting the data to a cumulative Gaussian curve:

$$f(x) = 0.5 \left[1 + \operatorname{erf} \left(\frac{x - \mu}{\sqrt{2\sigma^2}} \right) \right] \quad (\text{Equation 3}),$$

where

$$\operatorname{erf}(z) = \frac{2}{\sqrt{\pi}} \int_0^z e^{-t^2} dt \quad (\text{Equation 4})$$

[29-31]. The $\operatorname{Pr}(D)$ at each target amount and locus was then calculated by determining the value of the curve at $x = 1$,

$$\operatorname{Pr}(D) = f(1) \quad (\text{Equation 5}),$$

as any peak below 1 RFU was considered to have dropped out.

1.2.4.2 Model 2 – Non-Fitted Cumulative Gaussian

Similar to Model 1, Model 2 determined dropout probabilities based on the peak height distribution of the single-source calibration data at each target amount and locus. Again, a cumulative peak height histogram was created using the auto-binning function provided in IGOR Pro v 6.2 following Scott's Method [28] with heterozygote and homozygote peak heights used in the creation of the histogram. Differing from Model 1, the mean (μ) and standard deviation (σ) were derived from the single-source calibration data with

$$\mu = \bar{x} = \frac{\sum x}{n} \quad (\text{Equation 6})$$

and

$$\sigma = s = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}} \quad (\text{Equation 7}),$$

where x is each data point and n is the number of data points [32]. These derived values were used as the coefficients in the cumulative Gaussian curve equation described above for Model 1 (Equation 3). Thus, Model 2 is essentially a non-fitted cumulative Gaussian curve as the coefficients were derived directly from the single-source calibration data rather than a curve fitted to the histogram. As with Model 1, the Pr(D) for each target amount and locus was calculated using Equation 5.

1.2.4.3 Model 3 – Logistic Regression

The last two models for calculating dropout probabilities were based on the observed Fr(D) of the single-source calibration data rather than the peak height distributions. Since a logistic regression has previously been found to successfully characterize allelic dropout [1], a logistic regression was used to characterize the observed Fr(D) of the calibration data versus target amount per locus for Model 3. For each locus, the observed Fr(D) was plotted against the seven target amounts. The data were then fitted with a logistic regression in IGOR Pro v 6.2 according to the following equation:

$$f(x) = \frac{1}{1 + e^{-(a+bx)}} \quad (\text{Equation 8}),$$

where the coefficients of the function, a and b , were determined by regression [33]. For any target amount, x ,

$$\Pr(D) = f(x) \quad (\text{Equation 9}).$$

1.2.4.4 Model 4 – Exponential regression

An exponential regression for characterizing the $\Pr(D)$ was explored. As in Model 3, the observed $\text{Fr}(D)$ was plotted against the seven target amounts for each locus. The data were then fitted with an exponential regression in IGOR Pro v 6.2 according to the following equation:

$$f(x) = ae^{bx} \quad (\text{Equation 10}),$$

where the coefficients of the function, a and b , were determined by regression [34]. As with Model 3, the $\Pr(D)$ for each target amount and locus was calculated using Equation 9.

1.2.5 Model Comparison

The estimated dropout probabilities for each model were compared against the observed dropout frequencies in order to determine which was an appropriate characterization of allelic dropout. To evaluate each model, the common logarithm of the observed $\text{Fr}(D)$ and the calculated $\Pr(D)$ was computed. The difference between the two values was calculated,

$$\log(\text{Fr}(D)) - \log(\Pr(D)) \quad (\text{Equation 11}).$$

A difference of zero or close to zero for each locus and target amount indicates a model that characterizes the data well. A summary of allelic dropout models evaluated in this study is given in Figure 3.

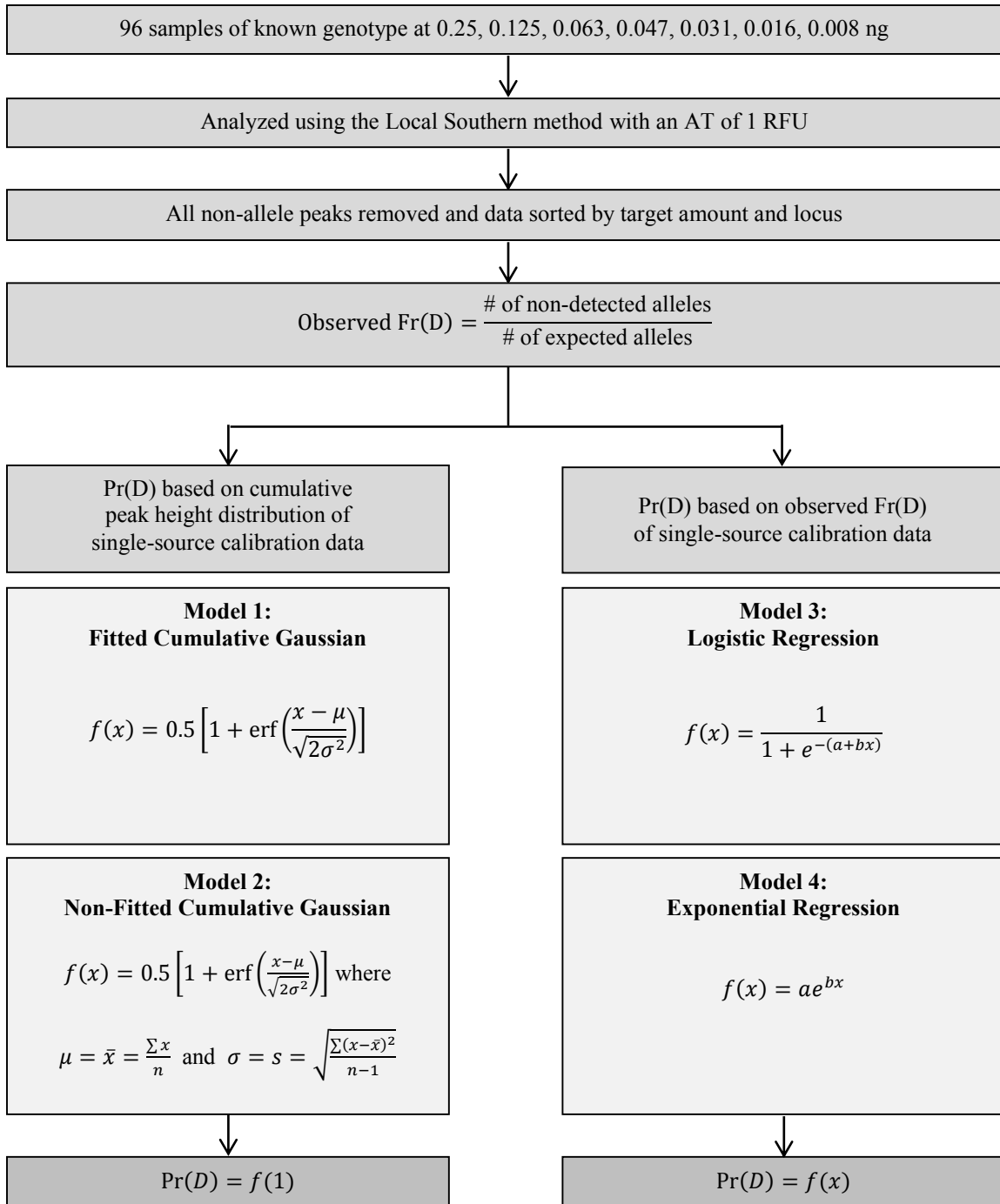


Figure 3. A summary of allelic dropout models evaluated in this study.

1.3 Results

1.3.1 Evaluation of Observed Dropout Frequencies

Before an appropriate model of allelic dropout rates was determined, the observed Fr(D) across all tested target amounts was evaluated. Overall, as the target amount decreased from 0.25 ng to 0.008 ng, the Fr(D) increased from 0.000 to 0.299. Approximately 40 cells of DNA is equivalent to 0.25 ng and approximately one cell is equivalent to 0.008 ng. No dropout was observed at 0.25 ng. At 0.125 ng, approximately 20 cells, 2 of 16 loci exhibited dropout. At 0.063 ng, approximately 10 cells, 8 of 16 loci exhibited dropout. At 0.047 ng, approximately 5 cells, 11 of 16 loci exhibited dropout. At 0.031 ng, 0.016 ng, and 0.008 ng, dropout was observed at all loci. At 0.008 ng, the minimum frequency was 0.210 at D5S818 and the maximum was 0.391 at CSF1PO. No more than 39.1% of alleles dropped out at approximately one cell's worth of DNA. Figure 4 shows the average Fr(D) across all loci. The minimum, maximum, and mean Fr(D) are given in Table 1.

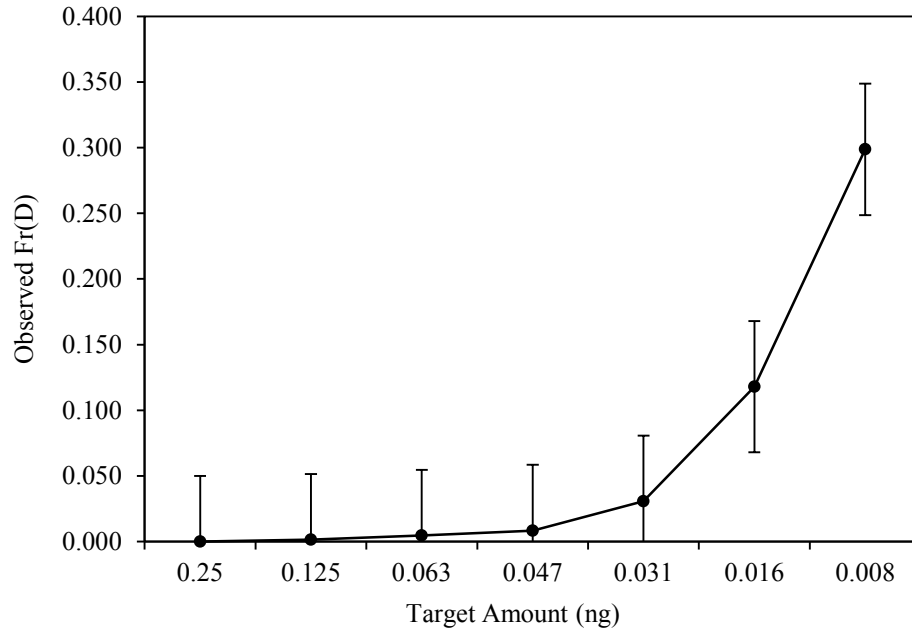


Figure 4. The average observed Fr(D) \pm 1 standard deviation across all loci (—●—).

Table 1. A summary of the mean \pm 1 standard deviation, minimum, and maximum observed Fr(D) for all loci at seven LT amounts.

Target Amount (ng)	Mean (1 SD)	Minimum	Maximum
0.25	0.000 (0.000)	0.000	0.000
0.125	0.001 (0.004)	0.000	0.017
0.063	0.005 (0.006)	0.000	0.016
0.047	0.008 (0.008)	0.000	0.023
0.031	0.031 (0.015)	0.008	0.065
0.016	0.118 (0.043)	0.034	0.202
0.008	0.299 (0.054)	0.210	0.391

In addition to observing a larger rate of dropout at lower target amounts, high molecular weight loci tended to exhibit a higher Fr(D) as compared to low molecular weight loci. On average, the Fr(D) was approximately 0.024 higher. D8S1179, D3S1358, TH01, D19S433, vWA, Amelogenin, and D5S818 are loci less than approximately 200 base pairs in length, or low molecular weight. High molecular weight loci, or loci greater than approximately 200 base pairs, include D21S11, D7S820, CSF1PO, D13S317, D16S539, D2S1338, TPOX, D18S51, and FGA [26, 35]. The disparity between the low and high molecular weight loci also increased as the target amount decreased. At 0.125 ng, approximately 0.3% more dropout was observed for the high molecular weight loci as compared to the low molecular weight loci, while approximately 8% more dropout was observed at 0.008 ng. Figure 5 illustrates the relationship between dropout rates and molecular weight. The mean and standard deviation are given in Table 2.

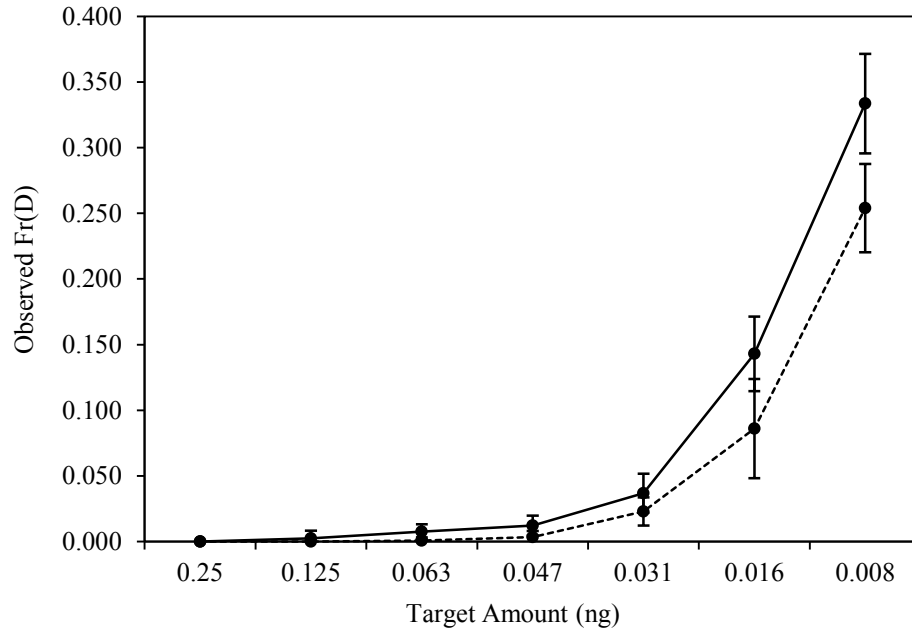


Figure 5. The average observed Fr(D) \pm 1 standard deviation for low (---●---) and high molecular weight loci (—●—).

Table 2. The mean \pm 1 standard deviation, minimum, and maximum observed Fr(D) for low (< 200 bp) and high (> 200 bp) molecular weight loci.

Target Amount (ng)	Molecular Weight	Mean (1 SD)	Minimum	Maximum
0.25	low	0.000 (0.000)	0.000	0.000
0.25	high	0.000 (0.000)	0.000	0.000
0.125	low	0.000 (0.000)	0.000	0.000
0.125	high	0.003 (0.006)	0.000	0.017
0.063	low	0.001 (0.002)	0.000	0.006
0.063	high	0.008 (0.006)	0.000	0.016
0.047	low	0.004 (0.004)	0.000	0.009
0.047	high	0.012 (0.007)	0.000	0.023
0.031	low	0.023 (0.011)	0.012	0.044
0.031	high	0.037 (0.015)	0.008	0.065
0.016	low	0.086 (0.038)	0.034	0.145
0.016	high	0.143 (0.028)	0.103	0.202
0.008	low	0.254 (0.034)	0.210	0.302
0.008	high	0.334 (0.038)	0.250	0.391

1.3.2 Evaluation of Dropout Models Based on Peak Height Distribution

The first two models used to characterize the $\text{Pr}(D)$ were based on the peak height distribution of the single-source calibration data at each target amount and locus. Cumulative peak height histograms were produced for both models. For Model 1, the mean and standard deviation of the histogram were computed by fitting the data with a cumulative Gaussian curve. For Model 2, these values were derived directly from the single-source calibration data. The $\text{Pr}(D)$ was then calculated by determining the value of the corresponding curve at $x = 1$, as any peak below 1 RFU was considered to have dropped out. The peak height distribution at each target amount for a representative locus, D16S539, is shown in Figure 6. As the target amount decreases, the peak heights and their spread decrease. Figure 7 shows the cumulative peak height histogram for D16S539 at 0.25 ng and 0.008 ng with the fitted (Model 1) and non-fitted (Model 2) Gaussian curves. The average residual of each model is given in Table 3. Both visually and based on the average residual, Model 1 is a better fit of the data compared to Model 2. However, the dropout rate is overestimated at 0.25 ng (dropout probability of 0.017 versus an observed dropout frequency of 0) and underestimated at 0.008 ng (dropout probability of 0.226 versus an observed dropout frequency of 0.319).

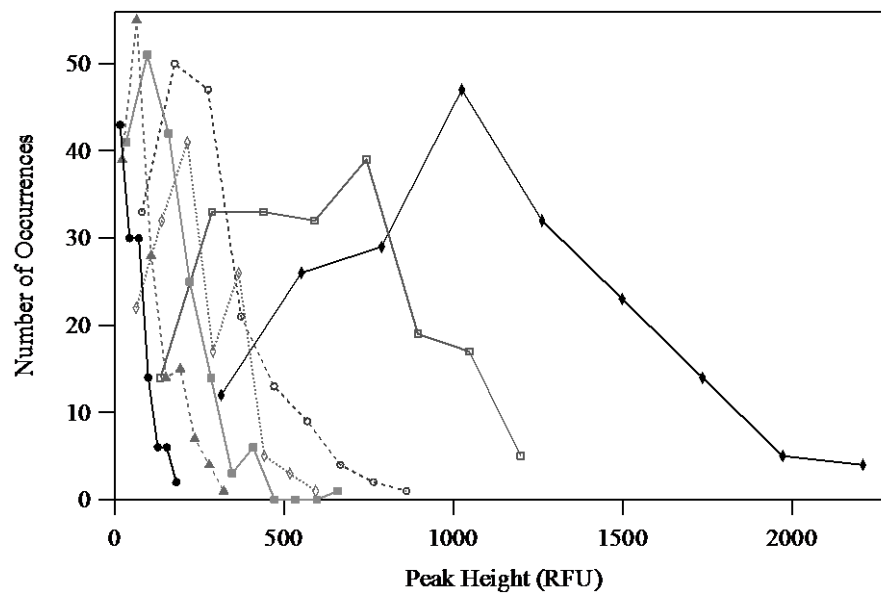


Figure 6. The peak height distribution of the single-source calibration data for D16S539 at 0.25 ng (—◆—), 0.125 ng (—□—), 0.063 ng (—○—), 0.047 ng (—◊—), 0.031 ng (—■—), 0.016 ng (—▲—), and 0.008 ng (—●—).

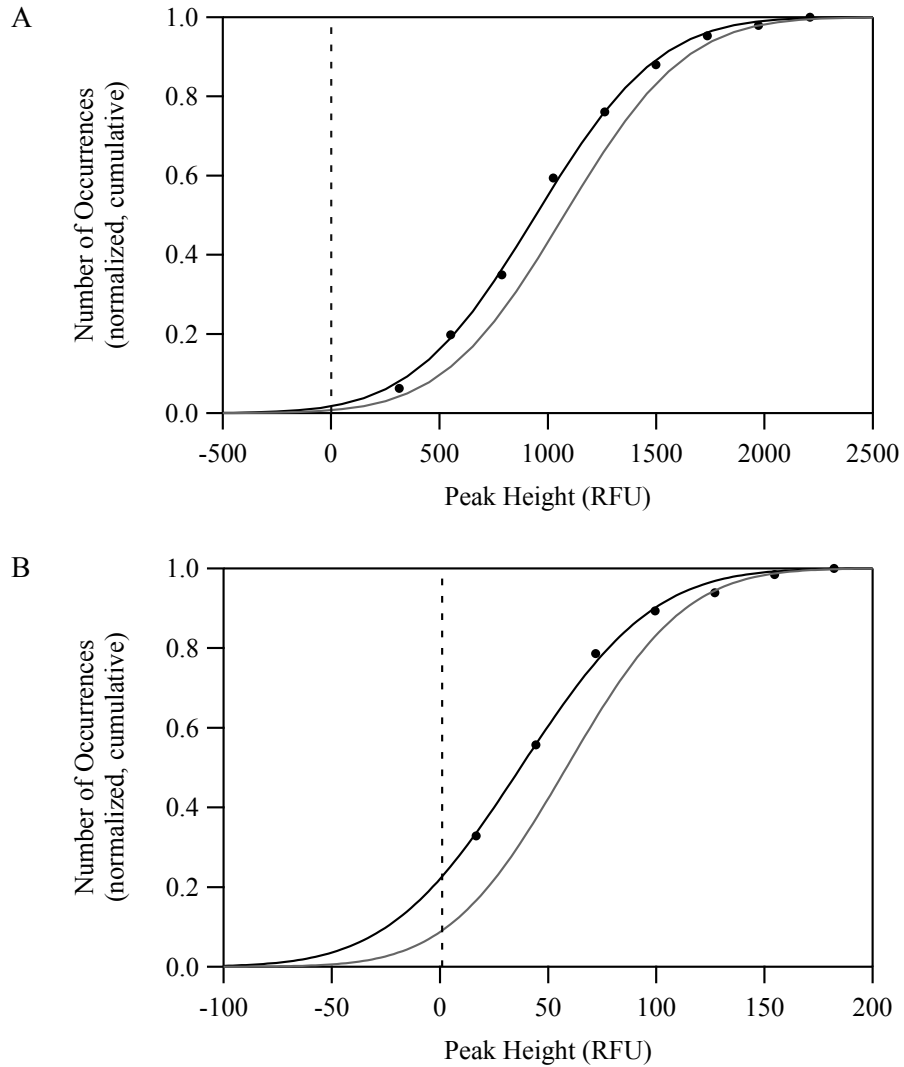


Figure 7. The cumulative peak height histogram (●) for D16S539 at (A) 0.25 ng and (B) 0.008 ng with a fitted (Model 1, —) and non-fitted (Model 2, - -) Gaussian curve. The Pr(D) is equal to the value at $x=1$ (---).

Table 3. The average residual across peak heights of Models 1 and 2 for the locus D16S539 at 0.25 ng and 0.008 ng.

Model	0.25 ng	0.008 ng
1	-0.003	-4.36E-03
2	0.056	8.12E-02

Overall, the probabilities of dropout calculated by both Model 1 and Model 2 increased linearly from a target amount of 0.25 ng to 0.008 ng, and Model 1 produced dropout probabilities greater than Model 2 at all target levels. The average $\text{Pr}(\text{D})$ for Model 1 ranged from 0.014 at 0.25 ng to 0.173 at 0.008 ng, increasing by approximately 0.029 between each target amount (Figure 8). Greater dropout probabilities were generated by high molecular weight loci compared to low molecular weight loci (Figure 9).

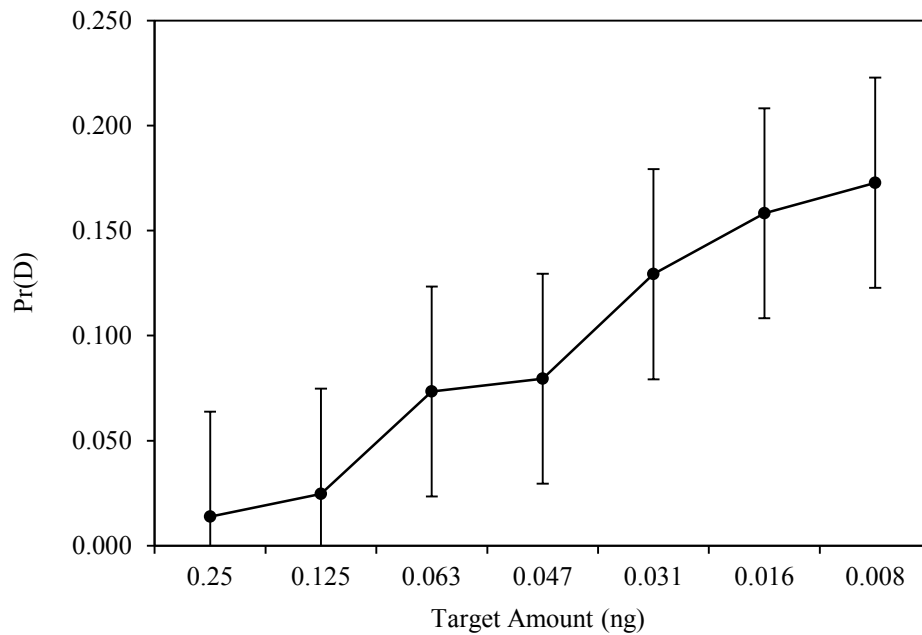


Figure 8. The average $\text{Pr}(\text{D}) \pm 1$ standard deviation using Model 1 across all loci (—●—).

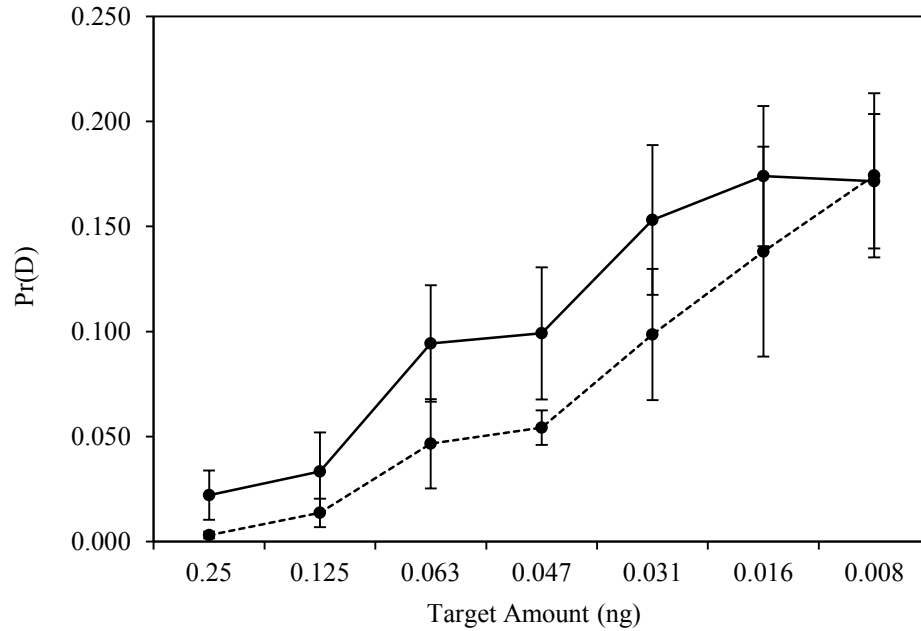


Figure 9. The average $\text{Pr}(D) \pm 1$ standard deviation using Model 1 for low (---●---) and high molecular weight loci (—●—).

For Model 2, the average $\text{Pr}(D)$ ranged from 0.006 at 0.25 ng to 0.079 at 0.008 ng, increasing by approximately 0.013 between each target amount. The same trends as Model 1 were observed with Model 2; high molecular weight loci resulted in higher dropout probabilities than low molecular weight loci. These results are summarized in Figures 10 and 11.

In general, Models 1 and 2 overestimated dropout at higher target amounts and underestimated it at lower target amounts. The underestimation suggests that other factors beyond detection and PCR variation contribute to dropout, as suggested by Hedell et al., Timken et al., and Gill et al. [16-18]. Across all loci, the $\text{Pr}(D)$ increased as target amount decreased and molecular weight increased.

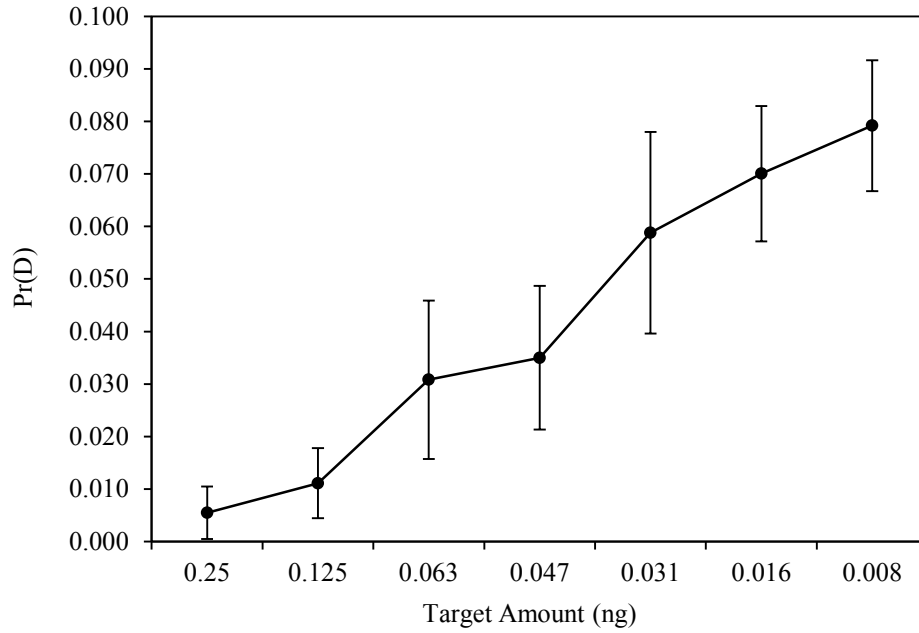


Figure 10. The average $\text{Pr}(D) \pm 1$ standard deviation using Model 2 across all loci (—●—).

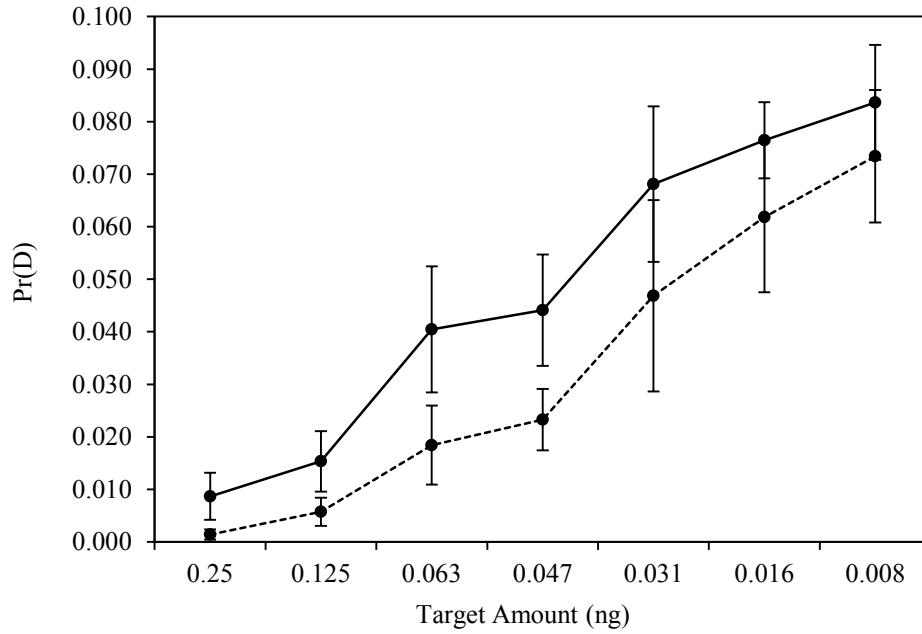


Figure 11. The average $\text{Pr}(D) \pm 1$ standard deviation using Model 2 for low (---●---) and high molecular weight loci (—●—).

1.3.3 Evaluation of Dropout Models Based on Observed Frequencies

Models 3 and 4 evaluated modeling the rates of allelic dropout with a logistic and exponential regression, respectively. The regression models are based on the observed $Fr(D)$ of the single-source calibration data. Figure 12 shows the $Fr(D)$ at all target amounts with logistic and exponential regressions for a representative locus, D16S539. Both models have an average residual close to zero (Table 4). The regression coefficients a and b with one standard deviation are listed in Table 4. Figure 13 shows the average $Pr(D)$ across all loci in addition to the average across low and high molecular weight loci for both Models 3 and 4. The similarity between the curves indicates the two models are similar. As the target amount decreases the $Pr(D)$ increases and the $Pr(D)$ is always higher for high molecular weight loci compared to low molecular weight loci.

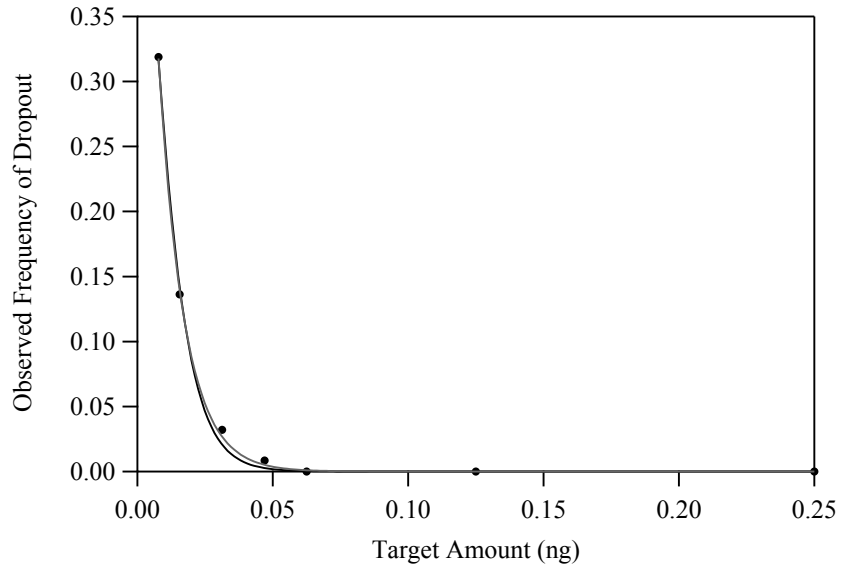


Figure 12. The observed $Fr(D)$ for D16S539 (●) at seven LT target amounts with a logistic (---) and exponential (—) regression.

Table 4. The coefficients, a and b , ± 1 standard deviation and the average residual of Models 3 and 4 for D16S539 across all target amounts.

Model	a (1 SD)	b (1 SD)	Average Residual
3	0.254 (0.0791)	-131 (6.82)	1.73E-03
4	0.723 (0.0197)	-105 (2.78)	7.21E-04

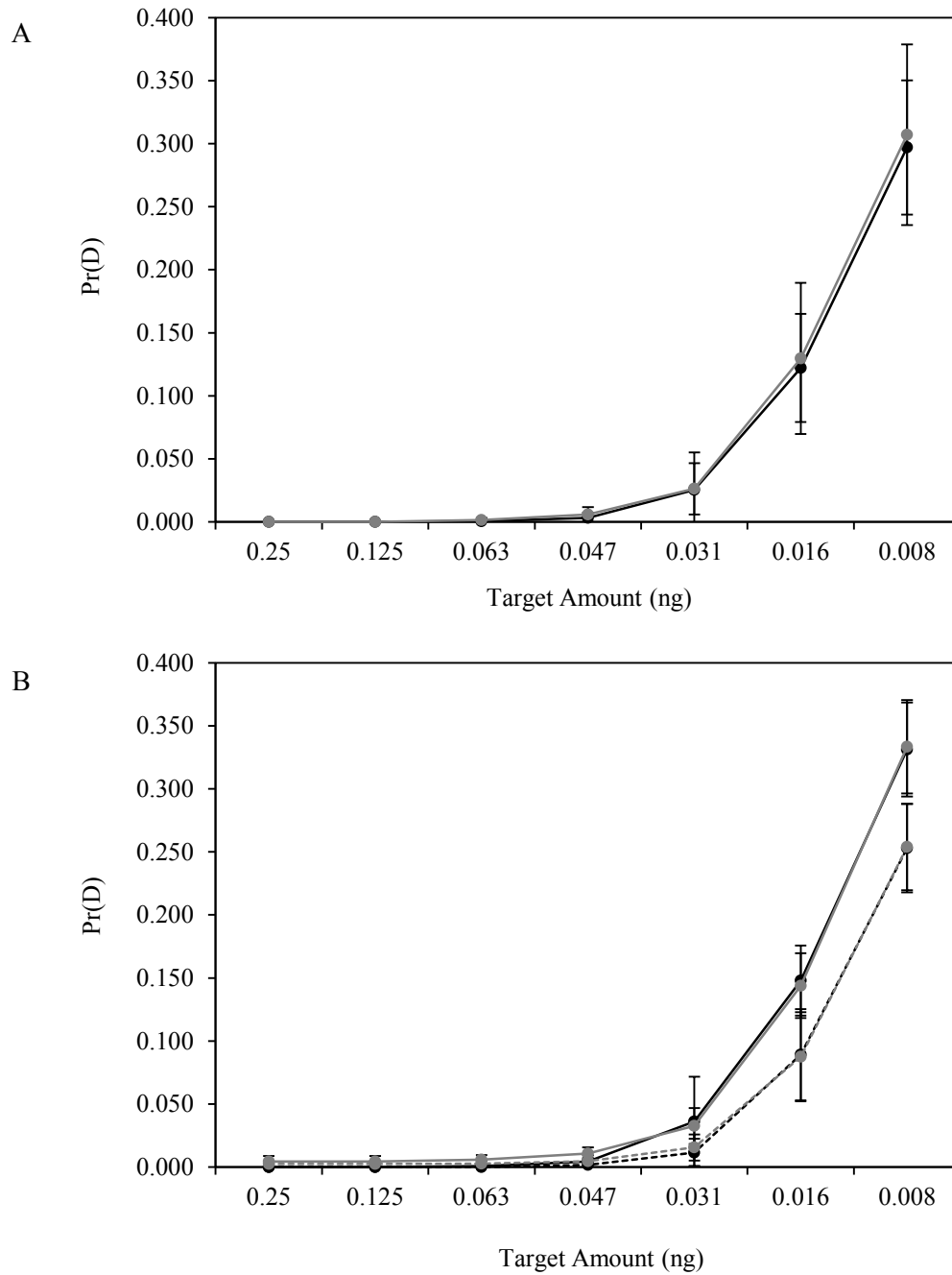


Figure 13. The average $\text{Pr}(D) \pm 1$ standard deviation for Models 3 (—●) and 4 (---●); (A) across all loci and (B) for low molecular weight loci (---●) and high molecular weight loci (—●).

1.3.4 Modeling Dropout

Rather than directly comparing the observed Fr(D) and the calculated Pr(D) at each locus and target amount in order to determine a reasonable characterization of allelic dropout, the common logarithms of the values were compared for each model. This method was used because the Pr(D) was not exactly zero at some loci. For example, in Table 5, the Pr(D) at 0.25 ng calculated with Model 3 is 7.250×10^{-15} while the Fr(D) was 0.000. Since the common logarithm of zero is undefined, it was estimated to be equal to -31 as the minimum Pr(D) for all models was 1.02×10^{-31} for Amelogenin at 0.25 ng. A difference of zero or close to zero for each locus and target amount indicates a model that fits the data well. A positive difference between the logarithm of the observed frequency and the estimated probability indicates a tendency to underestimate dropout, while a negative difference indicates a tendency towards overestimation. At a representative locus, D16S539, Models 3 and 4 were found to be equally appropriate models of dropout (Table 5). For example, at 0.008 ng, the observed Fr(D) was 0.319 and the Pr(D) estimated with Models 3 and 4 was 0.316 and 0.318, respectively. The difference between the logarithms of 0.319 and 0.316 is 0.003 and the difference between the logarithms of 0.319 and 0.318 is 0.001. These values signify overall good characterizations of dropout at 0.008 ng as the values are close to zero. Recall this difference was 0.148 and 0.545 for Models 1 and 2, respectively. Figure 14 shows the logarithm of the Fr(D) and the Pr(D) calculated with all models for D16S539 and Table 6 gives the differences between the values.

Table 5. The observed frequencies of dropout and resultant dropout probabilities calculated with Models 1, 2, 3, and 4 for D16S539. Highlighted dropout probabilities signify the most accurate approximation of the observed Fr(D) at each target amount.

Target (ng)	Observed	Model 1	Model 2	Model 3	Model 4
0.25	0.000	0.017	0.007	7.25E-15	2.72E-12
0.125	0.000	0.005	0.016	9.67E-08	1.40E-06
0.063	0.000	0.104	0.050	3.53E-04	0.001
0.047	0.008	0.074	0.027	0.003	0.005
0.031	0.032	0.157	0.081	0.021	0.027
0.016	0.136	0.209	0.085	0.143	0.140
0.008	0.319	0.226	0.091	0.316	0.318

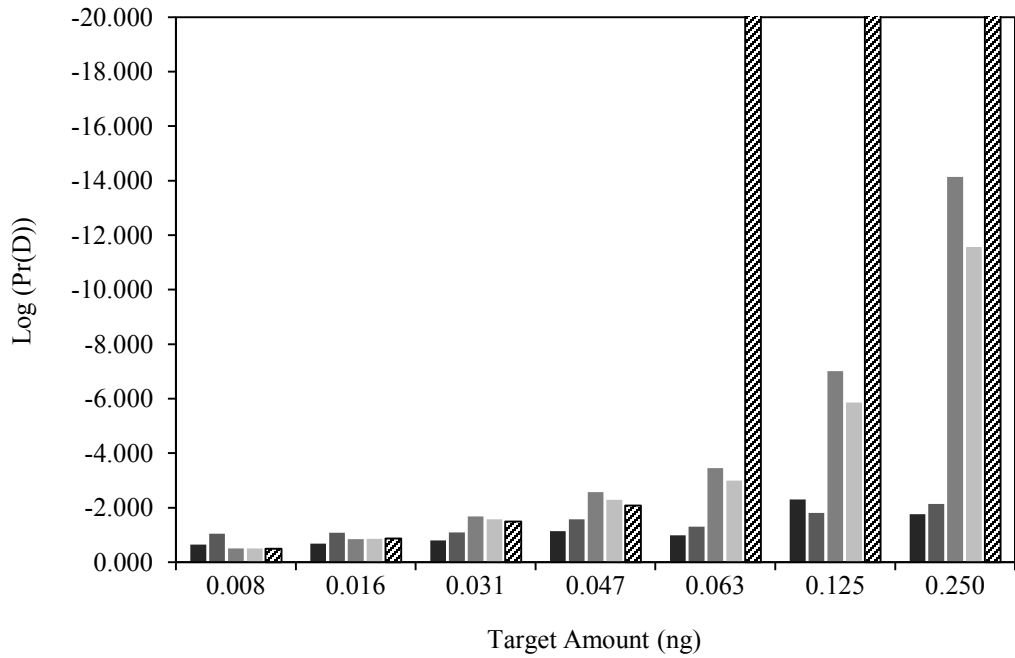


Figure 14. The common logarithm of the observed frequencies of dropout (z) and resultant dropout probabilities calculated with Models 1 (■), 2 (■), 3 (■) and 4 (■) for D16S539.

Table 6. The difference between the common logarithm of the observed Fr(D) and the resultant dropout probabilities for D16S539. A difference of zero or close to zero indicates a model that characterizes the data well. Highlighted cells signify the most accurate approximation of the observed Fr(D) at each target amount.

Target (ng)	Model 1	Model 2	Model 3	Model 4
0.25	-29.2	-28.9	-16.9	-19.4
0.125	-28.7	-29.2	-24.0	-25.1
0.063	-30.0	-29.7	-27.5	-28.0
0.047	-0.940	-0.503	0.498	0.217
0.031	-0.692	-0.402	0.189	0.077
0.016	-0.186	0.207	-0.020	-0.011
0.008	0.148	0.545	0.003	0.001

Across all loci, differences close to zero were produced by both Models 3 and 4 (Figure 15 and Table 7), indicating that an exponential or logistic regression of the Fr(D) is an appropriate characterization of allelic dropout.

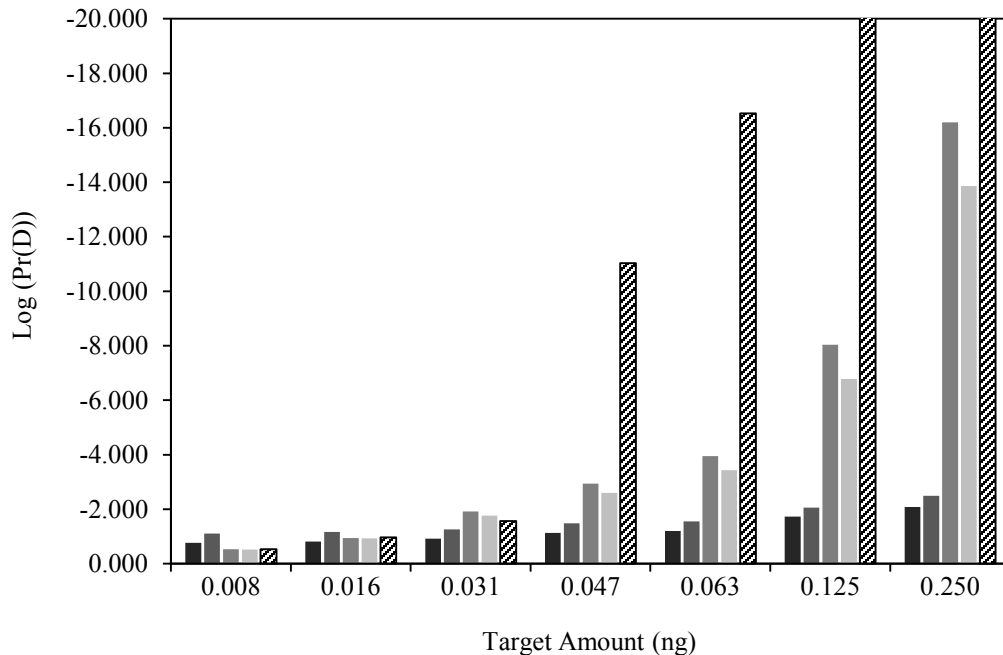


Figure 15. The common logarithm of the observed frequencies of dropout (hatched) and resultant dropout probabilities calculated with Models 1 (black), 2 (grey), 3 (dark grey) and 4 (light grey) across all loci.

Table 7. The difference between the common logarithm of the observed Fr(D) and the resultant dropout probabilities across all loci. A difference of zero or close to zero indicates a model that characterizes the data well. Highlighted cells signify the most accurate approximation of the observed Fr(D) at each target amount.

Target (ng)	Model 1	Model 2	Model 3	Model 4
0.25	-28.9	-28.5	-14.8	-17.1
0.125	-25.6	-25.3	-19.3	-20.6
0.063	-15.3	-15.0	-12.6	-13.1
0.047	-9.90	-9.54	-8.09	-8.42
0.031	-0.653	-0.308	0.355	0.198
0.016	-0.145	0.200	-0.017	-0.031
0.008	0.239	0.575	0.002	-0.008

1.4 Discussion

This study shows that the relationship between target amount and the frequency of allelic dropout can be modeled using a logistic or an exponential regression. A model based on peak height distribution to predict rates of allelic dropout was found to be less accurate than a model based on observed dropout frequencies. Across all loci, the $Fr(D)$ increased as the target amount decreased and with increasing molecular weight.

Using the distribution of observed peak heights of single-source calibration data to model allelic dropout was investigated prior to using observed dropout frequencies since it would be a relatively simple way for a forensic laboratory to determine dropout probabilities for their current amplification kit. Fitting the peak height distribution with a cumulative Gaussian curve was found to overestimate dropout at higher target levels and underestimate dropout at lower target levels (Figure 15). If detection and PCR variation alone contributed to the occurrence of allelic dropout, these tendencies would not have been observed, suggesting that other factors beyond these are responsible for dropout; possibly pre-PCR sampling effects. The potential for alleles to drop out must exist before the amplification process during volume transfer and/or extraction. This corroborates the findings of Hedell et al. and Timken et al. who also conclude that allele dropout occurs due to a combination of pre-PCR selection and preferential amplification when evaluating allele dropout patterns [16-17]. Since the peak height based model of allelic dropout was found to be inaccurate, alternative characterizations were explored.

Conditioning dropout on target amount was investigated as this could be considered the most direct way to characterize allelic dropout. The data was regressed

with a logistic curve as the use of a logistic regression to model allele dropout has been evaluated in several studies [1, 16, 21-24]. Given the similarity of logistic and exponential curves, an exponential regression was also explored as an alternative method to characterize allelic dropout rates.

Logistic regression is a standard way to estimate the probability of a binary event based on one or more variables [25]. Thus, the probability that dropout did or did not occur can be estimated with a logistic regression. Using a logistic regression to model allelic dropout rates was first introduced by Tvedebrink et al. [1] and Gill et al. [24] in 2009, and several additional studies have further investigated this method [16, 21-23]. However, differing from the present study, the logistic regression was not based directly on observed allele dropout frequencies of known single-source samples. Rather, peak height was used as an indicator of the amount of DNA present in a sample. To investigate how to characterize allelic dropout, Tvedebrink et al. prepared high quality samples with no contamination or degradation ranging from 24.6 to 410 pg for single-source samples and 328 to 528 pg for mixture samples [1]. Alleles in stutter positions of true alleles were not included in the analysis in order to avoid complications of masked dropouts. An analytical threshold of 50 RFU was applied. The $\text{Pr}(D)$ was conditioned on ' \hat{H} ', the sum of observed peak heights divided by the total number of observed alleles with homozygote alleles considered as two alleles. They found a logistic regression to be an appropriate characterization of allelic dropout, with high molecular weight loci exhibiting more dropouts than low molecular weight loci and the $\text{Pr}(D)$ decreasing as the observed peak height increased. The work of Gill et al. differed from that of Tvedebrink

et al. by conditioning the probability of allelic dropout on the height of the present allele instead of the average of all peak heights [24]. Despite conditioning the allelic dropout models in the present study on observed dropout frequencies rather than observed peak heights, similar conclusions to Tvedebrink et al. and Gill et al. can be drawn.

Models are continually improved upon and their performance evaluated in order to make them as accurate as possible. The model by Tvedebrink et al. described above was further expanded to allow for varying numbers of PCR cycles [22]. They also developed a simulation tool that mimics the pre-PCR generating process, including pipette and aliquot sampling, to explore impacts of laboratory alterations on signal. A range of input amounts, 15 to 500 pg, and an analytical threshold of 50 RFU was again used. The results of the two approaches were found to be very similar, which provided additional support for the logistic regression model. Further support was provided by Haned et al. who tested the robustness of the logistic model by simulating the process of generating a DNA profile [21].

Direct comparison between the results of this study to previous studies is challenging as an AT was set much lower at 1 RFU, compared to most studies, which use an AT of 50 RFU or 30 RFU. Allelic dropout has previously been demonstrated to be influenced by the AT [23]. Lohmueller et al. characterized patterns of allelic dropout in single-source samples using ATs of 30 and 50 RFU. Similar to the model developed by Tvedebrink et al., the $Pr(D)$ was conditioned on a proxy for the amount of DNA in a sample and estimated with a logistic regression. Less dropout was observed when the AT was set lower at 30 RFU. As the AT is lowered, the amount of dropout continues to

decrease, as was seen in the present study with all peaks above 1 RFU analyzed. Hedell et al. developed models describing allele dropout patterns to assess the impact amplicon length, STR marker, and fluorescent label have on the risk for allelic dropout [16]. At an AT of 50 RFU and input amount of 8 pg, they found that allele dropouts leveled off around 75%. A similar result would have been obtained in the present study if an AT was applied at 50 RFU (72%) instead of applying no AT (30%).

Despite differences in the methods of all of these studies, the conclusions are consistent – the $Pr(D)$ is locus dependent, increases with decreasing input amount, and increases with increasing fragment length. In addition, Hedell et al. noted differences in dropout rates between the fluorescent labels and suggested this could be related to amplification efficiency and/or the levels of released fluorescence [16]. Dropout may also be influenced by the amplification kit chemistry and instrument platform.

The purpose of a model is to explain how various parameters affect a particular phenomenon and predict its behavior. Irrespective of how a model for allelic dropout is developed, it should accurately characterize the probability that dropout may have occurred in a forensic DNA sample. While a logistic regression to model dropout was first introduced by Tvedebrink et al. and Gill et al. in 2009 for forensic purposes, this model and research in this area has since been expanded and more data evaluated. Alternative models have also been proposed. Generally, dropout can be conditioned on input amount directly, on a proxy for the amount of DNA, or on the peak height of an allele whose sister allele has dropped out.

Regardless on the condition, general trends are consistent and it is important to understand the implications those conclusions have on the end goal of forensic DNA analysis – presenting findings in court. One important step in the interpretation of a DNA profile is determining the NOC. Several factors must be considered when making this determination, including allelic dropout. The probability that dropout may have occurred in the sample cannot be accurately assessed without an appropriate model of dropout rates. The impact that allelic dropout has on estimating the NOC is discussed in the next section.

Modeling dropout rates is one of many steps that occur when evaluating forensic DNA evidence, but it is an essential step. The research presented herein has evaluated alternative methods than have previously been developed to characterize allelic dropout rates and has found both a logistic and an exponential regression to be appropriate models, for samples containing as little as one cell's worth of DNA. Additionally, these results further support the positive correlation between dropout rates and the molecular weight of loci. It also corroborates previous findings that suggest that allele dropout is locus-dependent.

1.5 Conclusions

This research aimed to evaluate different models of allelic dropout and determine an appropriate characterization. No dropout was observed at 0.25 ng and, on average, the $Fr(D)$ was less than 0.05 between 0.125 and 0.031 ng. The $Fr(D)$ increased to about 0.1 at 0.016 ng. The maximum dropout frequency (0.4) occurred at 0.008 ng, approximately one cell's worth of DNA. Data indicate that an exponential and a logistic regression of observed frequencies of dropout are both appropriate characterizations of allelic dropout. In general, Models 1 and 2 overestimated dropout at higher target amounts and underestimated it at lower target amounts. The underestimation suggests that other factors beyond detection and PCR variation contribute to dropout. Across all loci, the $Fr(D)$ increased as target amount decreased and the molecular weight increased. To conclude, because it is a known and characterized phenomenon, the probability that dropout may have occurred in a DNA sample should be taken into account when estimating the NOC and inferring genotypes.

2. THE IMPACT OF ALLELIC DROPOUT ON ESTIMATING THE NUMBER OF CONTRIBUTORS

2.1 Introduction

In forensic DNA analysis, a profile obtained from an item of evidence is compared to profiles obtained from persons of interest. The presence of a mixture and an assumption of the NOC to the sample are typically evaluated before attempting to compare the mixture to a known, or determine the possible genotype combinations [3, 36]. When estimating the NOC, it may be necessary to take multiple interfering factors, such as stutter, baseline noise, peak imbalance, and allelic dropout into account as these can affect the number of peaks detected at each locus.

2.1.1 Recognizing a Mixture

Mixed profiles consist of DNA originating from two or more individuals. One or two alleles present at all loci and expected peak height ratios, typically greater than 60%, for all heterozygous loci are indicative of a single contributor [2, 37]. If greater than two alleles are observed at a locus, and peak height ratios fall below the expected peak ratio value, a mixture of more than one individual may be present [2, 37]. Figure 16 is a schematic that illustrates various scenarios that may be encountered. Though Figure 16 does not encompass all possible scenarios, it provides an illustration of some commonly encountered scenarios. The likelihood of detecting a mixture improves as the number of loci and degree of polymorphism for each locus in an STR kit increases [37].

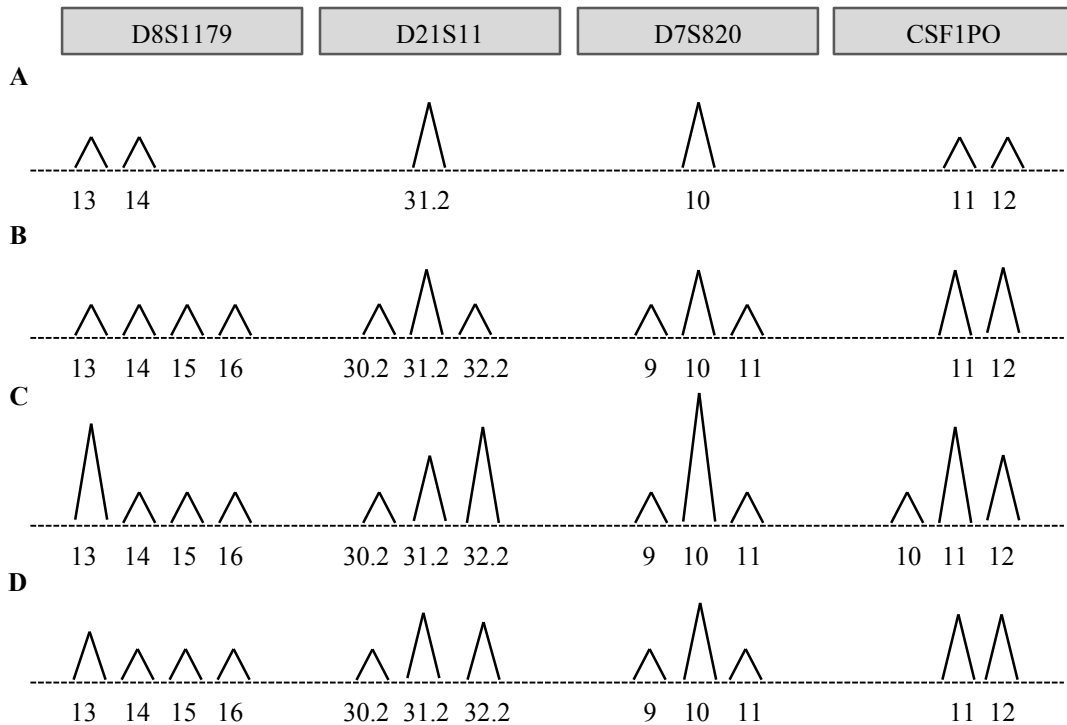


Figure 16. Recognizing a mixture and estimating the NOC. (A) Single-source sample. One or two alleles observed at each locus and balanced peak heights suggest this is a single-source profile. (B) Two-person mixture. The presence of more than two alleles at three loci and unbalanced peak heights at D21S11 and D7S820 indicate a mixture and suggests the presence of two contributors. (C) Three-person mixture. The presence of more than two alleles at every locus indicates a mixture. The higher peak height of allele 13 at D8S1179 may be indicative of the presence of a third contributor, despite the absence of more than four alleles at a locus. Based on the MAC, this profile would be classified as a two-person mixture. (D) Three-person mixture. Despite having the same contributors as mixture (C), the presence of a third contributor in this profile is difficult to detect. Due to the presence of a low-level contributor with dropout, this profile may be misidentified as a two-person mixture.

2.1.2 Estimating the Number of Contributors

Since the true NOC to a forensic profile is unknown, analysts must examine all aspects of the profile to make a reasonable assumption regarding the NOC [19]. The MAC method (or a combination of MAC and peak height ratio) is typically used to determine the lower bound on the NOC that can explain the detected set of alleles. Simply put, the MAC method involves counting the number of alleles at each locus,

dividing the maximum observed by two, and rounding up [19]. While MAC determines the minimum NOC that explains the observed profile, it may not be reflective of the actual NOC [19]. There are a number of signal interferences that can make it difficult to accurately assess the actual NOC to a sample. Examples include allele dropout due to the presence of multiple low-level contributors or degraded DNA [38]. Allele sharing is also a known complicating factor [20, 39-41]. For example, Buckleton et al. investigated the probability of observing x alleles for simulated mixtures of two, three, and four contributors [39]. For the AmpF ℓ STR $\text{\textcircled{R}}$ SGM Plus TM PCR amplification kit (Applied Biosystems, Foster City, CA) loci, about 3% of three-person mixtures exhibited four or fewer alleles at all loci studied. When they evaluated four-person mixtures, about 66% exhibited six or fewer alleles for the SGM Plus TM loci. Coble et al. extended this work by exploring the uncertainty in the NOC in the proposed new Combined DNA Index System (CODIS) set [40]. They reported that within the new dataset, approximately 43% of four-person mixtures appeared as a three-person mixture for Caucasian allele frequencies. This number of misidentifications dropped to 16% when the highly polymorphic locus SE33 was added to the dataset. Other work on the subject has also been published by Paoletti et al. [20] and Haned et al. [41]. Results from these studies suggest that while MAC may perform well for two- and three-person mixtures, it is not a reliable estimator of the actual NOC to a high-order complex DNA sample.

2.1.3 Impact of Allelic Dropout

Though a number of studies that detail the impact of allele sharing on the ability to accurately estimate the NOC are available, few studies that evaluate the impact of both allele sharing and dropout are available. The preliminary work of Perez et al. evaluates the combined effect by examining the total number of observed alleles for 728 two-, three-, and four-person mixtures, with a template amount ranging from 10 pg to 500 pg [38, 42-43]. For high-template samples (150 pg to 500 pg), two-person mixtures exhibited 35 to 50 alleles, three-person mixtures exhibited 38 to 64 alleles, and four-person mixtures exhibited 51 to 72 alleles. For low-template samples (10 pg to 150 pg), two-person mixtures exhibited 27 to 54 alleles, three-person mixtures exhibited 42 to 66 alleles, and four-person mixtures exhibited 53 to 75 alleles. Allelic dropout caused some three-person mixtures to appear as two-person mixtures and some four-person mixtures to appear as three-person mixtures. Based on the results of this work, recommended allele count thresholds for NOC groupings – two-persons, two- to three-persons, three-persons, three- to-four persons, and four-persons – were established. As suggested by the aforementioned study, the NOC to a sample may be underestimated if dropout has occurred as it can lead to a decrease in the number of observed alleles. Therefore, it is essential to understand the extent to which dropout impacts estimating the NOC. To that end, this study investigates the impact that both allelic dropout and allele sharing has on assessing the NOC to a mixture for loci which are represented in the AmpF ℓ STR $\text{\textcircled{R}}$ Identifiler $\text{\textcircled{R}}$ Plus and GlobalFiler TM (Applied Biosystems $\text{\textcircled{R}}$, Foster City, CA) amplification kits. To accomplish this, mixtures with and without a major contributor for

up to six contributors were simulated with the probability of allelic dropout ranging from 0 to 0.4. A maximum dropout rate of 0.4 was chosen because it roughly estimates the probability of sampling zero copies of DNA, assuming the Poisson distribution,

$$P(x; \lambda) = \frac{\lambda^x e^{-\lambda}}{x!} \quad (\text{Equation 12}),$$

when the average is $\lambda = 1$, or when one cell's worth of DNA is present,

$$P(x = 0; \lambda = 1) = \frac{1}{e} = 0.37 \quad (\text{Equation 13}).$$

This choice is consistent with previous studies that show that for LT samples, random sampling of the DNA molecules or alleles are a major component of peak signal imbalance and, in extreme cases, dropout [17-18, 21-22, 44]. Further, Stenman et al. suggest that in a solution containing evenly distributed molecules, the probability of the presence of a defined number of molecules in an aliquot of the solution can be calculated according to the Poisson distribution [44]. Similarly, studies by Gill et al. and Tvedebrink et al. have shown that allelic dropout is affected by the pre-PCR sampling process [18, 22]. The maximum dropout rate of 0.4 was also chosen for this work because empirical studies in this laboratory show that the observed frequency of dropout for a set of 96 samples amplified using 8 pg of DNA and the 29-cycle AmpF ℓ STR $\text{\textcircled{R}}$ Identifiler $\text{\textcircled{R}}$ Plus chemistry with an AT of 1 RFU resulted in dropout rates ranging from 0.21 to 0.39 between the 15 STR loci (data not shown).

The simulation tool, GGETIt, which is written in Visual Basic for Applications using Microsoft $\text{\textcircled{R}}$ Excel $\text{\textcircled{R}}$ (Microsoft Corporation, Redmond, WA), will be available for download on Boston University's DNA Mixture website,

<http://www.bu.edu/dnamixtures/>. GGETIt was used to generate the 1.5 million simulated samples utilized in this work.

2.2 Methods

2.2.1 GGETIt Development/Algorithm

To simulate mixtures for this study, GGETIt was developed using VBA in Microsoft® Excel®. GGETIt simulates profiles with STR loci (and amelogenin) consistent with the Identifiler® Plus or GlobalFiler™ amplification kits for up to six contributors based on allele frequencies in the population. Y-STR's were not simulated. Users can input desired allele frequencies, the true NOC to the mixtures, the number of profiles they wish to generate, and the Pr(D) of each contributor (Figure 17).

A

Number of Contributors	
Number of Profiles	

Enter Dropout Probabilities

Generate Profiles

B

Number of Contributors	2
Number of Profiles	100

Enter Dropout Probabilities

C

Number of Contributors	2
Number of Profiles	100
Pr(D) for Contributor 1	
Pr(D) for Contributor 2	

Generate Profiles

D

Number of Contributors	2
Number of Profiles	100
Pr(D) for Contributor 1	0
Pr(D) for Contributor 2	0.4

Figure 17. GGETIt user input. (A) The observed user inputs and buttons when GGETIt is opened. (B) After entering the true NOC to the mixtures and the number of profiles they wish to generate, users would click the *Enter Dropout Probabilities* button. (C) Users can now enter the dropout probability for each contributor. (D) Users would next click the *Generate Profiles* button to generate 100 true two-person mixtures with a Pr(D) of 0 for contributor 1 and 0.4 for contributor 2.

The allele frequency table lists all possible alleles for each locus, allowing the users to input their desired frequencies into the highlighted cells (Figure 18). After entering the frequencies, users would click on the *Update Allele Table* button, which updates the cumulative frequency column for each locus.

Locus	Allele	Frequency	Cumulative Frequency
D8S1179			
	4	0.000	
	5	0.000	
	6	0.000	
	7	0.000	
	8	0.020	0.020
	9	0.013	0.034
	10	0.105	0.138
	11	0.067	0.206
	12	0.152	0.357
	13	0.332	0.690
	14	0.188	0.878
	15	0.090	0.968
	16	0.028	0.996
	17	0.004	1.000
	18	0.000	
	19	0.000	
	20	0.000	




Figure 18. The allele frequency table for a representative locus, **D8S1179**. Users input their desired frequencies into the highlighted cells and then click on the *Update Allele Table* button.

During the simulation, alleles for each STR locus are chosen by generating a random number from a uniform distribution between 0 and the sum of the allele frequencies, and assigning the allele that corresponds to that random number. The sum of the allele frequencies may be one or greater than one if a minimum allele frequency is applied, according to the $5/2N$ rule, where N is the sampled number of individuals, as suggested by the National Research Council [45-46]. For the amelogenin locus, the first

allele chosen is always ‘X’ since both males and females have one X chromosome; the second allele is chosen based on the process previously described. The profile S_C of each contributor C is a sequence of unordered pair of alleles. This representation allows for a simple model of allele expression without taking into account signal intensity [47]. Thus, we have:

$$S_C = (\{A_{C,L,1}, A_{C,L,2}\})_{L=1}^n \quad (\text{Equation 14}),$$

where, for $i = 1, 2$, $A_{C,L,i}$ is the i th allele of contributor C at locus L ; and n is the number of loci (16 for the Identifiler® Plus simulation and 22 for the GlobalFiler™ simulation). Using this representation for an individual profile, a mixture M_C created by the set of contributors \mathcal{C} can be expressed using the following equation:

$$M_C = \left(\bigcup_{C \in \mathcal{C}} \{A_{C,L,1}, A_{C,L,2}\} \right)_{L=1}^n \quad (\text{Equation 15}).$$

A mixture profile with all contributed alleles detected would result if no dropout was assumed; this is considered a ‘pristine mixture’ profile and is analogous to samples with a 1:1 mixture ratio and input mass of greater than 0.5 ng [47]. To account for instances where lower amounts of DNA are amplified, varying allelic dropout rates, up to 0.4, were factored into the simulations. Once the alleles are generated, the simulation applies dropout based on the user defined dropout probabilities for each contributor. This is accomplished by Bernoulli trial. A random number uniformly selected from 0 and 1, $d_{C,L,i}$ is generated for each allele $A_{C,L,i}$. The allele $A_{C,L,i}$ ‘drops out’ if

$$d_{C,L,i} \leq \Pr(D)_C, \quad (\text{Equation 16})$$

where $\Pr(D)_C$ is the user defined dropout probability for contributor C . After applying dropout, alleles that have not dropped out remain in black text while red text indicates alleles that have dropped out. The simulation produces the profiles for each contributor, color-coded to delineate the alleles per contributor that did (red) and did not (black) dropout. It also produces the ‘observed allele output’ and calculates the minimum NOC using the MAC of the ‘observed allele output’. The ‘true profiles’ represent the known genotypes of each contributor in the mixture. The ‘observed allele output’ is representative of the alleles that would be detected in the electropherogram. To generate this output, alleles in red text and duplicate alleles are filtered. Figure 19 shows a representative locus exhibiting allelic dropout. In this example, the second contributor, Person2, is homozygous for allele 14 at the locus D8S1179. Based on the user inputs in Figure 17, if the random number 0.15 was generated for Allele1 for this contributor, this instance of the allele would drop out since the defined $\Pr(D)$ is 0.4. In order for the ‘observed allele output’ to show the complete dropout of allele 14, both Allele1 and Allele2 would have to dropout. Representative ‘true profiles’ and ‘observed allele outputs’ for the Identifiler® Plus and GlobalFiler™ simulations are shown in Figures 20 and 21.

A	Profile 1	Person1		Person2	
		Allele1	Allele2	Allele1	Allele2
	D8S1179	11	12	14	14

B	Profile 1	Person1		Person2	
		Allele1	Allele2	Allele1	Allele2
	D8S1179	11	12	14	14

C	Profile 1	Allele1	Allele2	Allele3
		D8S1179	11	12

Figure 19. A representative locus where there are two contributors and some allelic dropout. (A) Known genotypes of Person1 and Person2 for the locus D8S1179 prior to the simulation applying dropout. (B) Person2's Allele1 drops out since the random number generated by the simulation (0.15) is less than the Pr(D) the user assigned for this contributor (0.4). The red text indicates the allele has dropped out. (C) The 'observed allele output' for this locus.

Profile 1	A				B			
	Person1		Person2		Allele1	Allele2	Allele3	Allele4
	Allele1	Allele2	Allele1	Allele2				
D8S1179	11	12	14	14	11	12	14	
D21S11	30	32.2	28	29	28	30	32.2	
D7S820	11	10	10	11	10	11		
CSF1PO	12	9	10	10	9	10	12	
D3S1358	16	16	15	16	15	16		
TH01	8	6	8	6	6	8		
D13S317	12	8	11	12	8	12		
D16S539	11	12	9	11	9	11	12	
D2S1338	18	20	19	17	17	18	19	20
D19S433	13	14	15	15.2	13	14		
vWA	19	17	19	19	17	19		
TPOX	10	9	12	11	9	10		
D18S51	18	17	15	16	16	17	18	
Amel	X	X	X	X	X			
D5S818	12	11	12	12	11	12		
FGA	21	20	24	25	20	21	24	25

Figure 20. Representative ‘true profiles’ and ‘observed allele output’ for the STR loci consistent with the Identifiler® Plus kit for a two-person mixture with dropout. (A) ‘True profiles’ for each contributor. Black text indicates alleles that have not dropped out, and red text indicates alleles that have dropped out. (B) Dropped out and duplicate alleles are filtered from the ‘true profiles’ to generate the ‘observed signal output’.

Profile 1	A				B			
	Person1		Person2		Allele1	Allele2	Allele3	Allele4
	Allele1	Allele2	Allele1	Allele2				
D3S1358	14	18	14	16	14	18		
vWA	18	15	18	17	15	17	18	
D16S539	13	12	9	12	12	13		
CSF1PO	13	10	10	12	10	13		
TPOX	11	11	8	9	9	11		
Amel	X	Y	X	Y	X	Y		
D8S1179	12	14	14	10	12	14		
D21S11	29	30	29	30	29	30		
D18S51	17	14	15	16	14	16	17	
D2S441	14	11	10	10	10	11	14	
D19S433	14	12	14	14	12	14		
TH01	9.3	9.3	7	8	8	9.3		
FGA	23	19	24	25	19	23	24	25
D22S1045	17	15	16	15	15	16	17	
D5S818	12	11	11	11	11	12		
D13S317	11	11	10	11	11			
D7S820	10	11	8	9	9	10	11	
SE33	30.2	19	19	26.2	19	30.2		
D10S1248	14	14	13	13	14			
D1S1656	16.3	16	15	13	15	16	16.3	
D12S391	17.3	19	18	17	17.3	18	19	
D2S1338	24	18	21	17	18	21	24	

Figure 21. Representative ‘true profiles’ and ‘observed allele output’ for the STR loci consistent with the GlobalFiler™ kit for a two-person mixture with dropout. (A) ‘True profiles’ for each contributor. Black text indicates alleles that have not dropped out, and red text indicates alleles that have dropped out. (B) Dropped out and duplicate alleles are filtered from the ‘true profiles’ to generate the ‘observed signal output’.

2.2.2 Mixture Simulations

Two-, three-, four-, five-, and six-person Identifiler® Plus and GlobalFiler™ mixtures with no major contributor, one ‘moderate’ major contributor, and one ‘substantial’ major contributor were generated with the simulation. Allele frequencies

used in the simulations were taken from the Caucasian population data in the GlobalFiler™ User Guide [48]. The Pr(D) of the minor contributor(s) ranged from 0 to 0.4 for each mixture ratio. Dropout probabilities for the major contributor varied based on the minor contributor and were chosen to represent different mixture ratio scenarios. In essence, these mixtures represent a series of minor(s)/major mixtures with high- to low-template levels. As an example, the mixture ratio containing a minor contributor where approximately one cell's worth of DNA is amplified, will have a Pr(D) of 0.4 and a moderate major contributor where there are three cells amplified will have a Pr(D) of 0.1. Similarly, if the total template level is large enough then neither the minor nor major contributor is expected to exhibit allele dropout. This is represented by 'Scenario 1' in Figure 22, which summarizes all simulated mixtures. For every n contributor combination and Pr(D) scenario, 10,000 profiles were simulated. A total of 750,000 profiles were generated for each amplification kit. Though other signal interferences, such as noise and stutter, exist and are expected to impact the ability to infer the actual NOC, only the impact of allelic dropout and allele overlap is evaluated in this study.

(1) No Major Contributor	(2) Moderate Major Contributor	(3) Substantial Major Contributor
<u>Contributor combinations</u> 2 contributors, $a:a$ 3 contributors, $a:a:a$ 4 contributors, $a:a:a:a$ 5 contributors, $a:a:a:a:a$ 6 contributors, $a:a:a:a:a:a$	<u>Contributor combinations</u> 2 contributors, $b:a$ 3 contributors, $b:a:a$ 4 contributors, $b:a:a:a$ 5 contributors, $b:a:a:a:a$ 6 contributors, $b:a:a:a:a:a$	<u>Contributor combinations</u> 2 contributors, $c:a$ 3 contributors, $c:a:a$ 4 contributors, $c:a:a:a$ 5 contributors, $c:a:a:a:a$ 6 contributors, $c:a:a:a:a:a$
<u>Pr(D) scenarios for each n contributor combination</u> Scenario 1: $a = 0$ Scenario 2: $a = 0.1$ Scenario 3: $a = 0.2$ Scenario 4: $a = 0.3$ Scenario 5: $a = 0.4$	<u>Pr(D) scenarios for each n contributor combination</u> Scenario 1: $a = 0, b = 0$ Scenario 2: $a = 0.1, b = 0$ Scenario 3: $a = 0.2, b = 0$ Scenario 4: $a = 0.3, b = 0.05$ Scenario 5: $a = 0.4, b = 0.1$	<u>Pr(D) scenarios for each n contributor combination</u> Scenario 1: $a = 0, c = 0$ Scenario 2: $a = 0.1, c = 0$ Scenario 3: $a = 0.2, c = 0$ Scenario 4: $a = 0.3, c = 0$ Scenario 5: $a = 0.4, c = 0$

Figure 22. A summary of simulated mixtures with no major contributor (1), one moderate major contributor (2) and one substantial major contributor (3). For every n contributor combination and Pr(D) scenario, 10,000 profiles were simulated (total of 750,000 profiles). The Pr(D) of the minor contributor (a) ranged from 0 to 0.4 for each contributor combination, and the Pr(D) of the major contributor (b , moderate major; c , substantial major) varied based on the minor contributor.

2.3 Results

Overall, the number of profiles for which the observed minimum NOC was misclassified increased as the Pr(D) and the NOC increased. The percentage of misclassifications also increased from three-contributor to four-contributor mixtures for both kits. Fewer misclassifications were observed with GlobalFiler™ than Identifiler® Plus simulated mixtures for each condition, which was frequently attributable to the highly polymorphic locus SE33. This locus is not a part of the Identifiler® Plus kit [26]. The results of all Identifiler® Plus and GlobalFiler™ simulations are summarized in Figures 23 and 24.

Further, it is noted that many four-, five-, and six-person mixtures were misclassified as three-person mixtures. Over all the 750,000 Identifiler® Plus simulations in this study, 375,434 resulted in an NOC designation of three. Of these, 113,547 samples were actual three-contributor mixtures. Table 8 summarizes the output for all samples, the corresponding MAC determination, and the number of samples containing n contributors that comprised the output set. The next sections evaluate each mixture condition separately.

Table 8. A summary of the MAC determination for 750,000 Identifiler® Plus (A) and GlobalFiler™ (B) samples, and the corresponding number of samples containing n contributors (NOC $_n$) that comprised the output set.

A

MAC Determination	Total Number of Profiles	NOC ₂	NOC ₃	NOC ₄	NOC ₅	NOC ₆
1	127	126	1	0	0	0
2	192,072	149,874	36,452	5,226	485	35
3	375,434	0	113,547	124,485	86,567	50,835
4	176,114	0	0	20,289	61,996	93,829
5	6,245	0	0	0	952	5,293
6	8	0	0	0	0	8

B

MAC Determination	Total Number of Profiles	NOC ₂	NOC ₃	NOC ₄	NOC ₅	NOC ₆
1	22	22	0	0	0	0
2	166,035	149,978	15,245	794	18	0
3	260,145	0	134,755	83,917	31,635	9,838
4	240,577	0	0	65,289	96,650	78,638
5	77,437	0	0	0	21,697	55,740
6	5,784	0	0	0	0	5,784

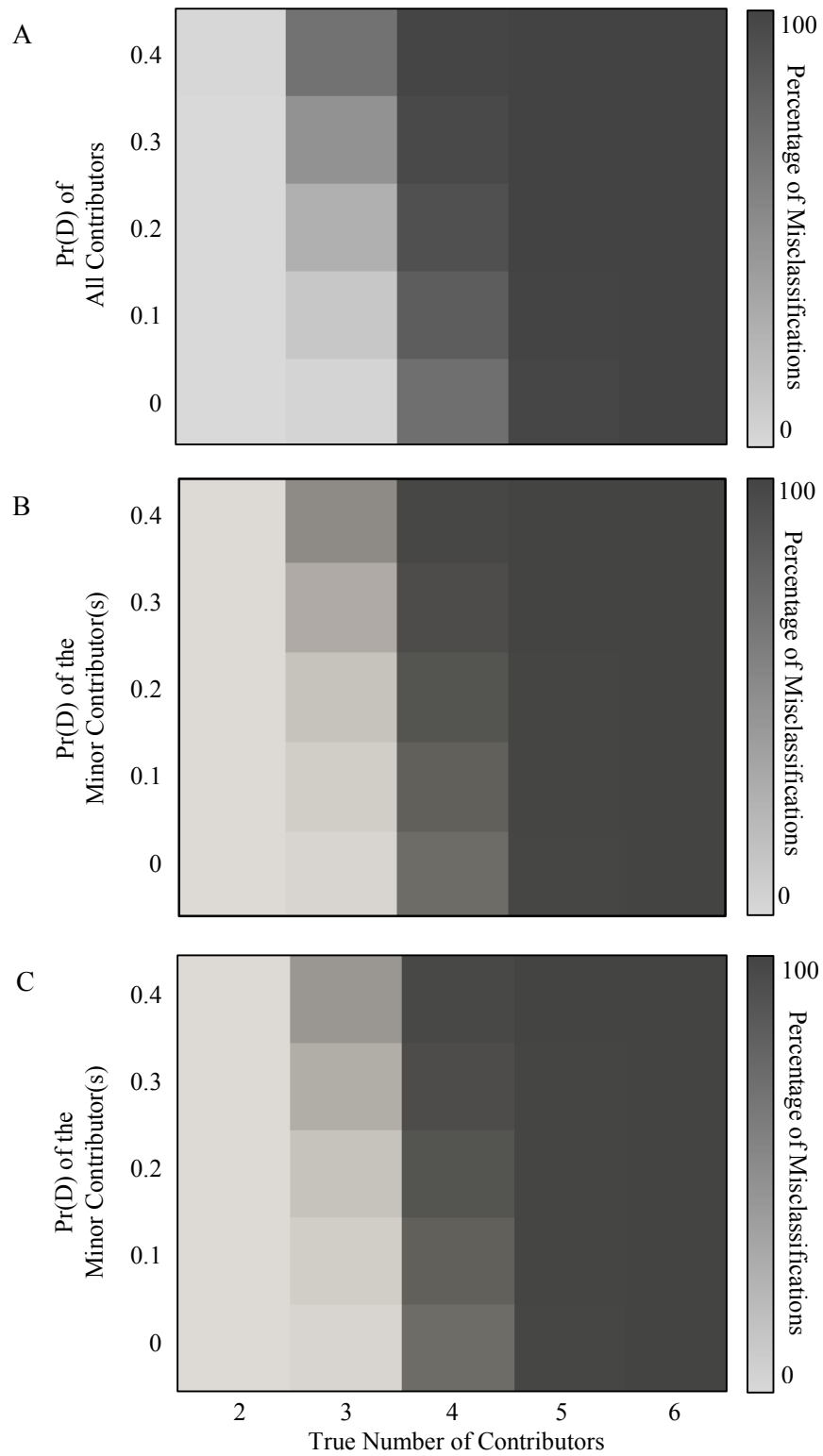


Figure 23. A summary of the percentage of misclassifications for Identifiler® Plus simulated mixtures with (A) no major, (B) one moderate major, and (C) one substantial major contributor.

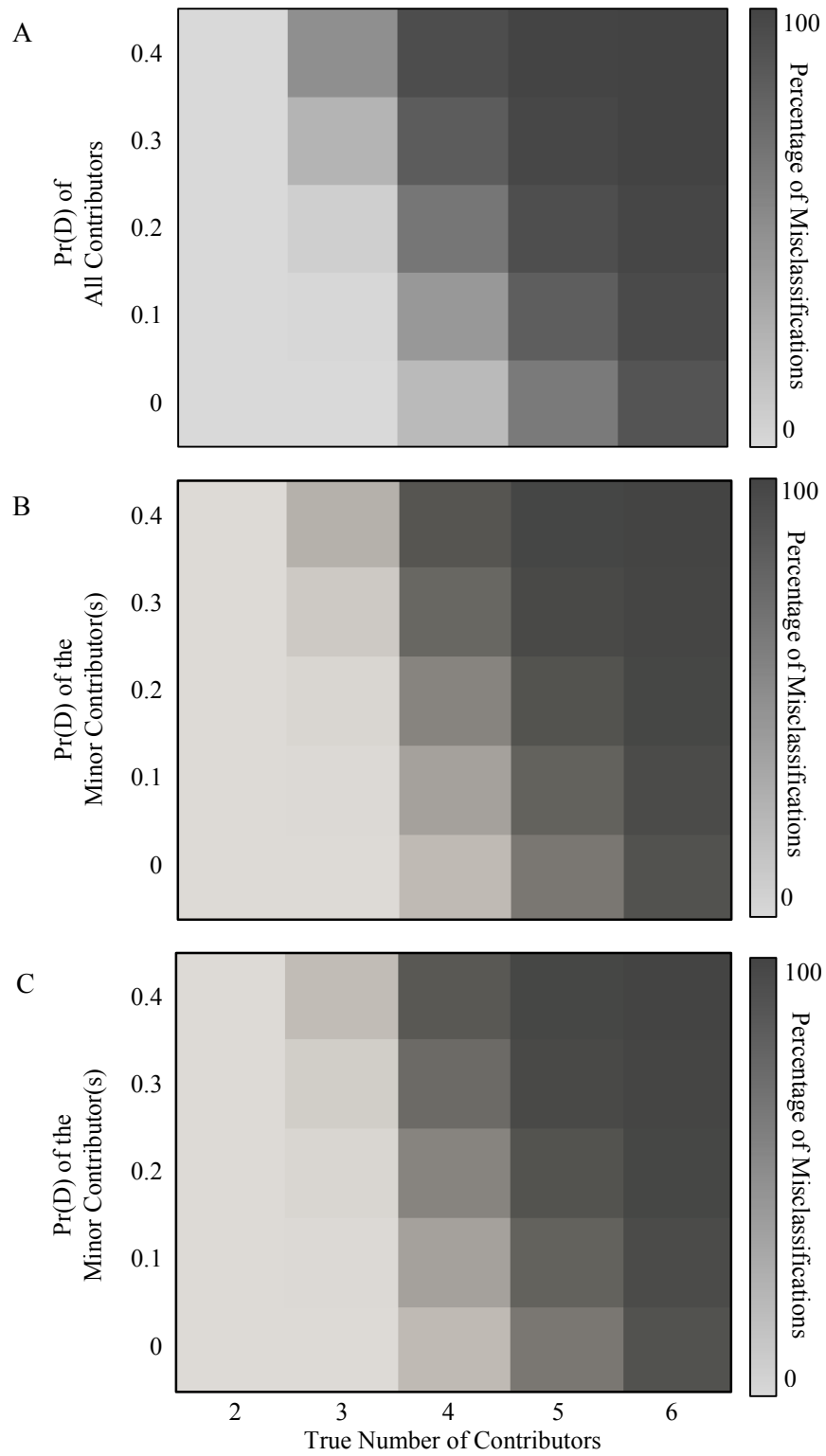
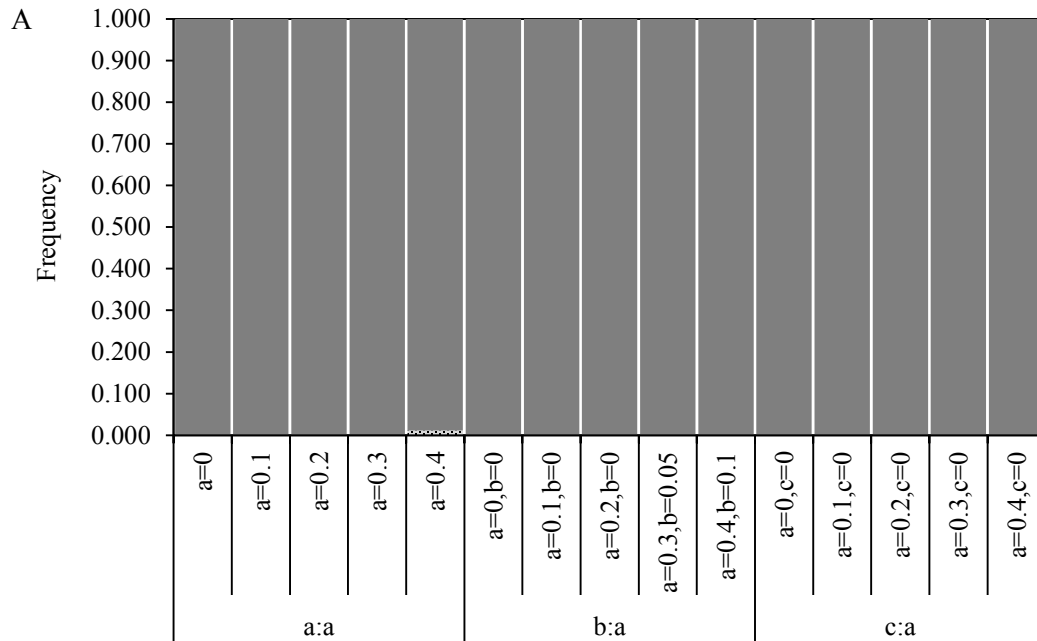


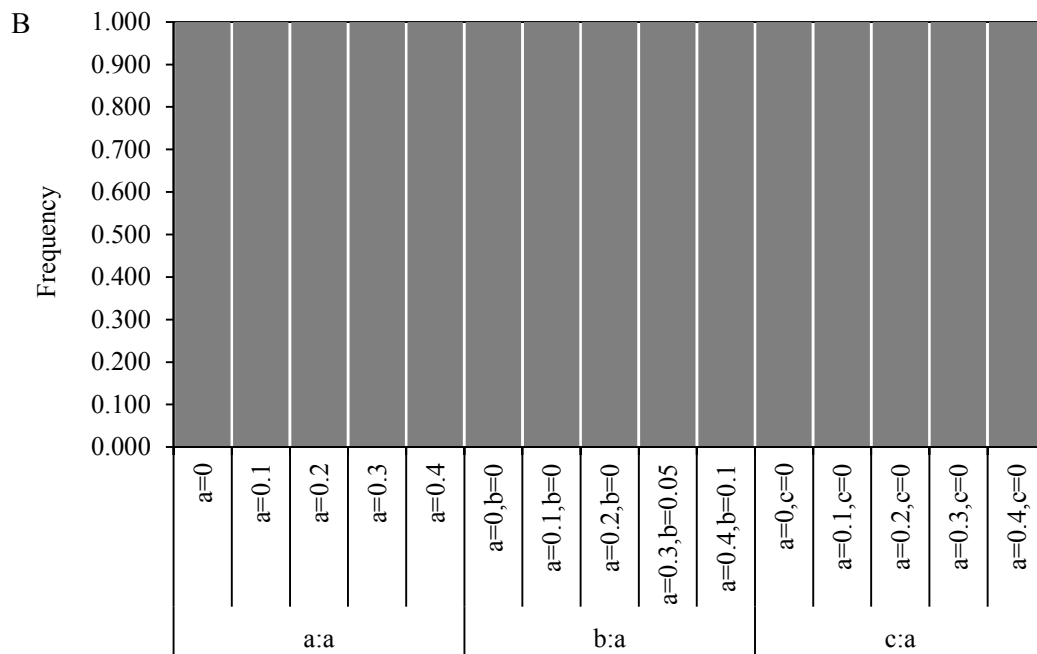
Figure 24. A summary of the percentage of misclassifications for GlobalFiler™ simulated mixtures with (A) no major, (B) one moderate major, and (C) one substantial major contributor.

2.3.1 Two-Person Mixtures

The likelihood of classifying a two-person mixture as originating from a single contributor was assessed for both the GlobalFiler™ and Identifiler® Plus kits. Figure 25 displays the frequency of classifying simulated Identifiler® Plus and GlobalFiler™ two-person mixtures, respectively, as a single-source profile and a two-person mixture using MAC. Nearly 100% of true two-person Identifiler® Plus and GlobalFiler™ mixtures were correctly recognized as originating from two contributors even with a 0.4 dropout probability for both contributors, regardless of mixture ratio. This analysis indicates there is a very small chance of misclassifying a two-person mixture as a single contributor stain.



Contributor Combination and Pr(D) Scenario



Contributor Combination and Pr(D) Scenario

Figure 25. The frequency of observing 1 (☐) and 2 (■) contributors using MAC for simulated Identifiler® Plus (A) and GlobalFiler™ (B) two-person mixtures with no major contributor (a:a), one moderate major contributor (b:a), and one substantial major contributor (c:a) for all Pr(D) scenarios described in Figure 22.

2.3.2 Three-Person Mixtures

The results of these simulations appear in Figure 26. Without any dropout, 3.6% of three-person Identifiler® Plus mixtures were misidentified as two-person mixtures for all mixture ratios. This value increased to 68.9% with the maximum dropout rate for mixtures with no major contributor and to 50.0% and 42.6% for $b:a:a$ and $c:a:a$ mixtures, respectively. The number of misclassifications decreased for GlobalFiler™ mixtures with only 0.1% of GlobalFiler™ mixtures without dropout appearing to have originated from two contributors. Again, there was an increase in misidentifications as the dropout probability increased. For the highest Pr(D) for all contributors in an $a:a:a$ mixture, 49.1% presented as two-person mixtures. This value decreased by half for $b:a:a$ mixtures and to 19.3% for $c:a:a$ mixtures. No three-person mixture was ever misclassified as originating from a single contributor for either kit.

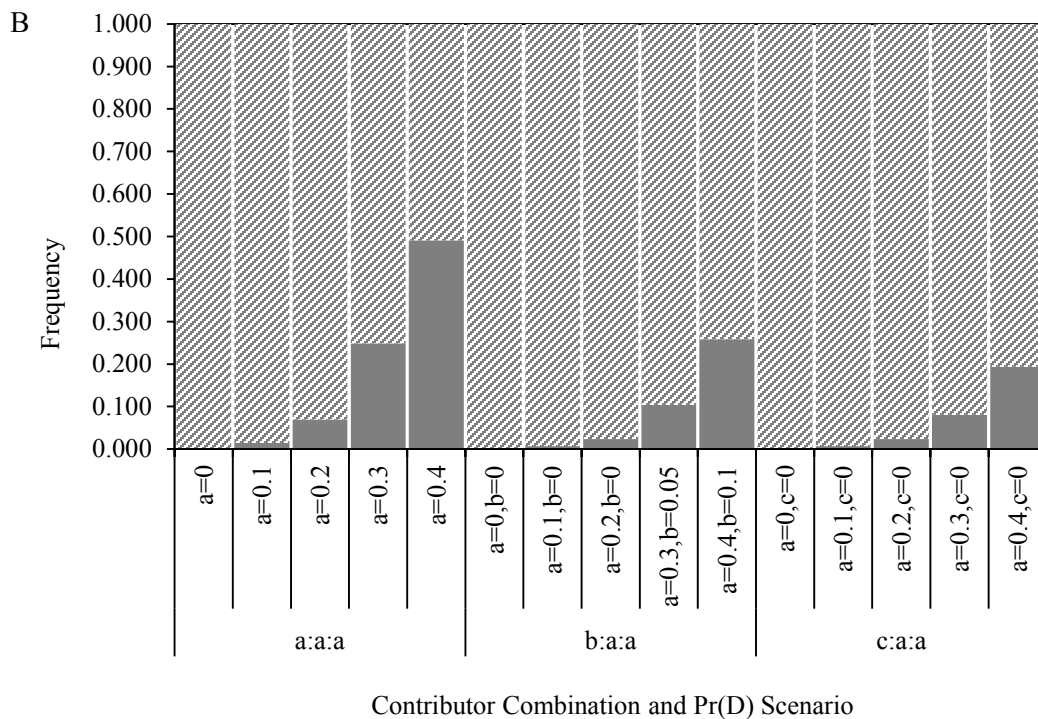
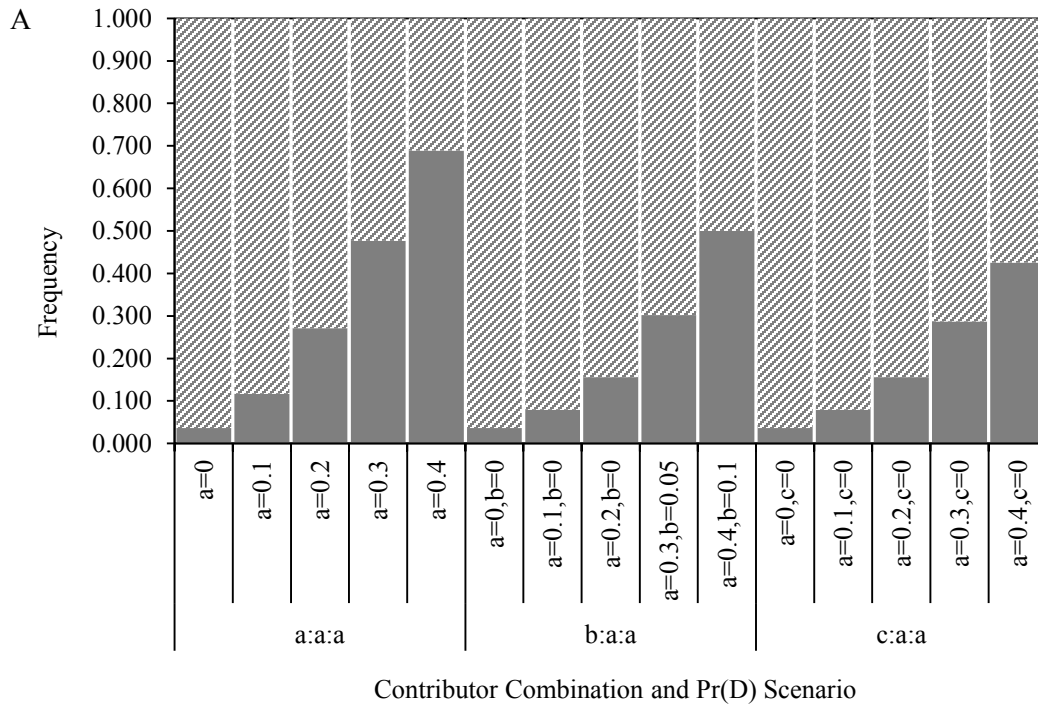


Figure 26. The frequency of observing 1 (▨), 2 (■), and 3 (▩) contributors using MAC for simulated Identifiler® Plus (A) and GlobalFiler™ (B) three-person mixtures with no major contributor (a:a:a), one moderate major contributor (b:a:a), and one substantial major contributor (c:a:a) for all Pr(D) scenarios described in Figure 22.

2.3.3 Four-Person Mixtures

The majority of simulated four-person Identifiler® Plus mixtures (70-90%) were misclassified as three-person mixtures using MAC. At the maximum dropout rate for all contributors in an *a:a:a:a* mixture, 20.8% presented as two-person mixtures. This percentage decreased for mixtures with one major contributor (10.4% for the *b:a:a:a* mixture ratio and 6.5% for the *c:a:a:a* mixture ratio). For the GlobalFiler™ simulations, the NOC was correctly identified for 56.7-79.6% of profiles at low dropout probabilities, 0 to 0.1. At higher dropout probabilities, the majority of profiles were identified as three-person mixtures (54.8-87.7%). The number of profiles presenting as two-person mixtures was lower for the GlobalFiler™ simulations (5.4%) compared to the Identifiler® Plus simulations (20.8%) when examining the *a:a:a:a* mixture ratio with the maximum dropout probability. No mixtures were misclassified as originating from a single source for either kit. The results of these simulations are summarized in Figure 27.

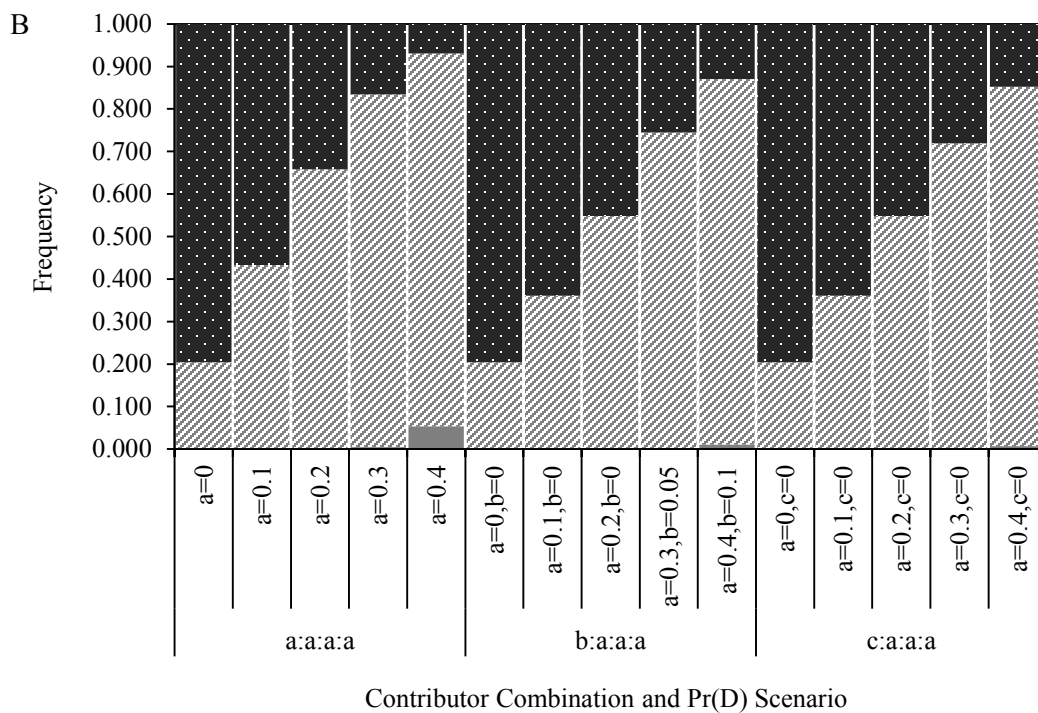
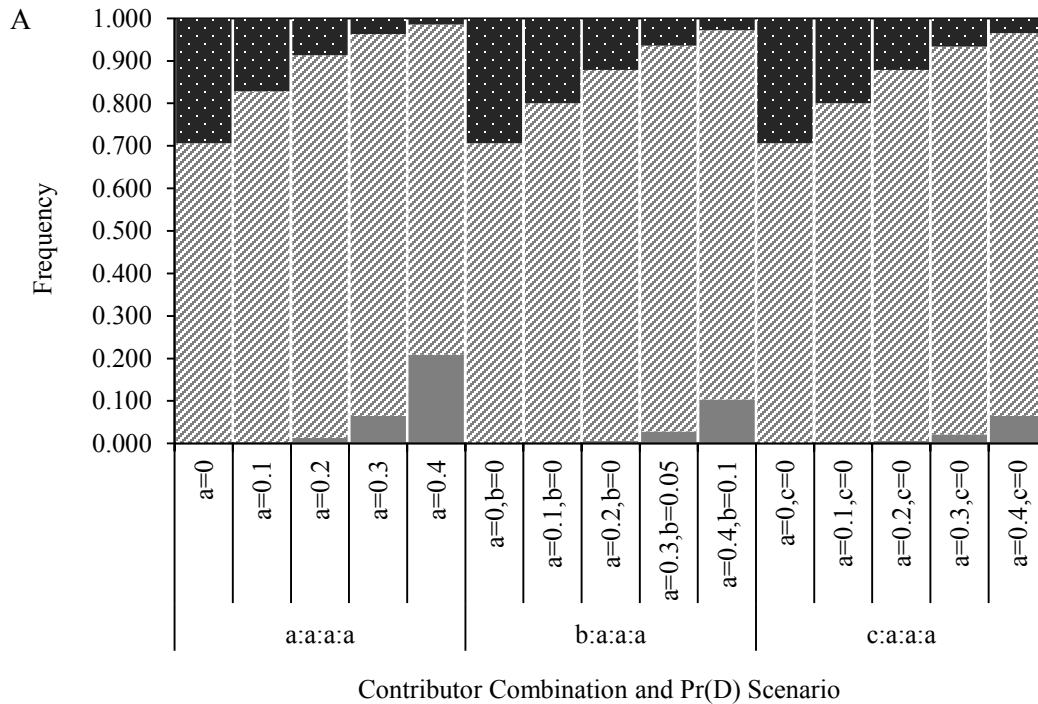


Figure 27. The frequency of observing 1 (□), 2 (▨), 3 (▩), and 4 (■) contributors using MAC for Identifiler® Plus (A) and GlobalFiler™ (B) four-person mixtures with no major contributor (a:a:a:a), one moderate major contributor (b:a:a:a), and one substantial major contributor (c:a:a:a) for all Pr(D) scenarios described in Figure 22.

2.3.4 Five-Person Mixtures

A very small number (1.7%) of five-person Identifiler® Plus mixtures were correctly classified using MAC even when the Pr(D) for all contributors was zero. The majority of profiles were classified as four-person mixtures (53.0-70.3%) when the dropout probability was low (0 or 0.1) and three-person mixtures for the higher rates of dropout (56.3-87.3%). Up to 0.3% of profiles presented as two-person mixtures for a dropout probability of 0.3 and up to 3% for a dropout probability of 0.4. These numbers varied between the different mixture ratios. The percentage of profiles correctly classified improved with the GlobalFiler™ simulations (36.8% compared to 1.7% for Identifiler® Plus mixtures). However, the majority of profiles were classified as four-person mixtures for dropout probabilities up to 0.3 and three-person mixtures at a dropout probability of 0.4. Fewer profiles (0.2%) were misclassified as two-person mixtures compared to Identifiler® Plus (3%) for the *a:a:a:a:a* mixture ratio with the highest dropout probability (0.4). Again no profiles were misclassified as originating from a single contributor for either kit. The results of these simulations appear in Figure 28.

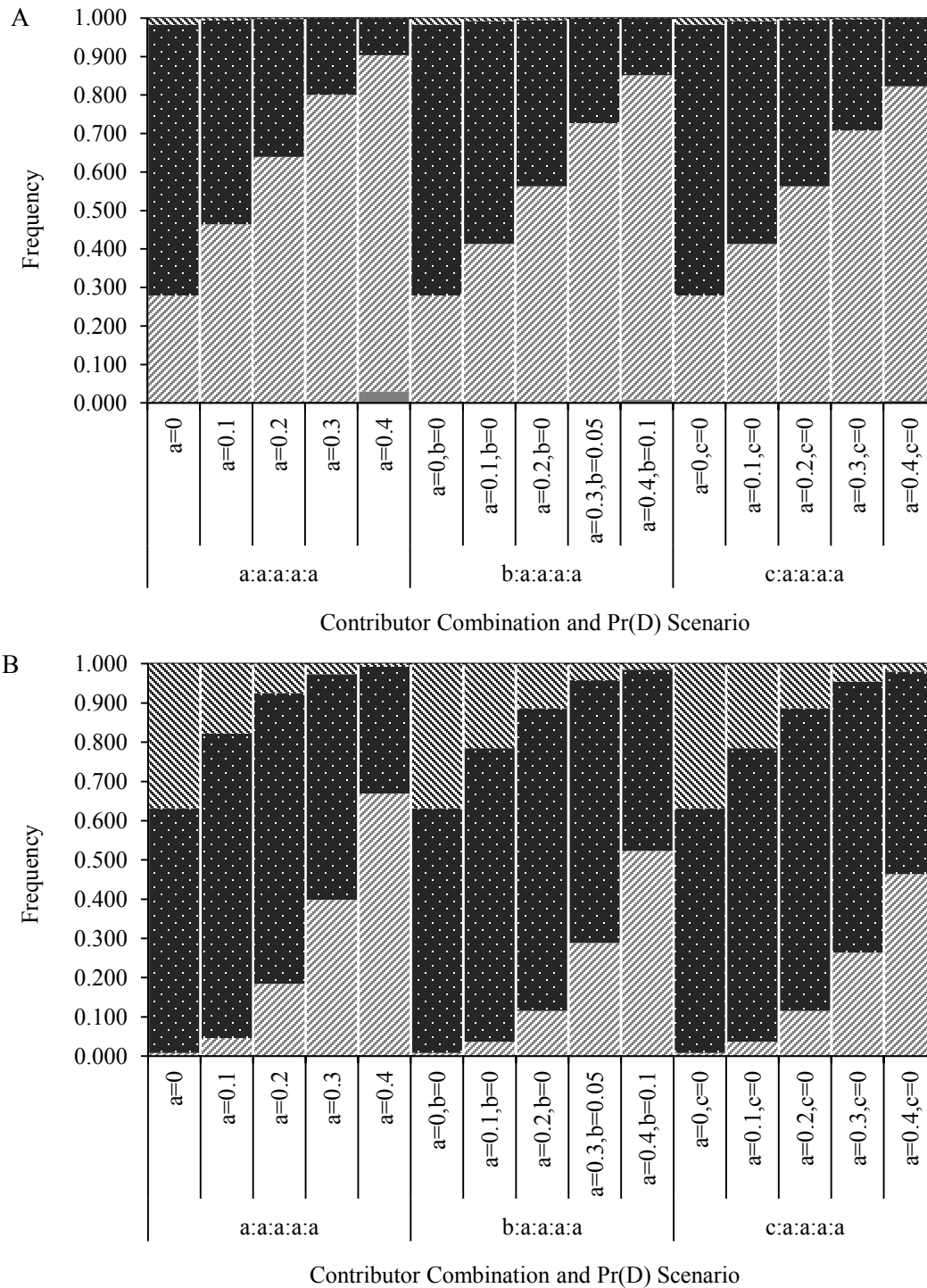


Figure 28. The frequency of observing 1 (▣), 2 (■), 3 (▧), 4 (▨), and 5 (▩) contributors using MAC for simulated Identifiler® Plus (A) and GlobalFiler™ (B) five-person mixtures with no major contributor (a:a:a:a), one moderate major contributor (b:a:a:a), and one substantial major contributor (c:a:a:a) for all Pr(D) scenarios described in Figure 22.

2.3.5 Six-Person Mixtures

The results of these simulations are summarized in Figure 29. True six-person Identifiler® Plus mixtures were never classified correctly using MAC and across all mixture ratios, most profiles presented as four-person mixtures (up to 83.6%) at low dropout probabilities (0 to 0.2) and three-person mixtures (up to 73.3%) at high dropout probabilities (0.3 to 0.4). These results improved for GlobalFiler™ mixtures with a small number correctly classified as six-person mixtures (maximum 11.2%) and the majority characterized as five-person mixtures for dropout probabilities between 0-0.1 (52.0-67.2%) or four-person mixtures for dropout probabilities between 0.2-0.4 (57.0-72.6%). For both kits, few or no profiles were classified as two-person mixtures, and no profiles were misclassified as originating from a single contributor.

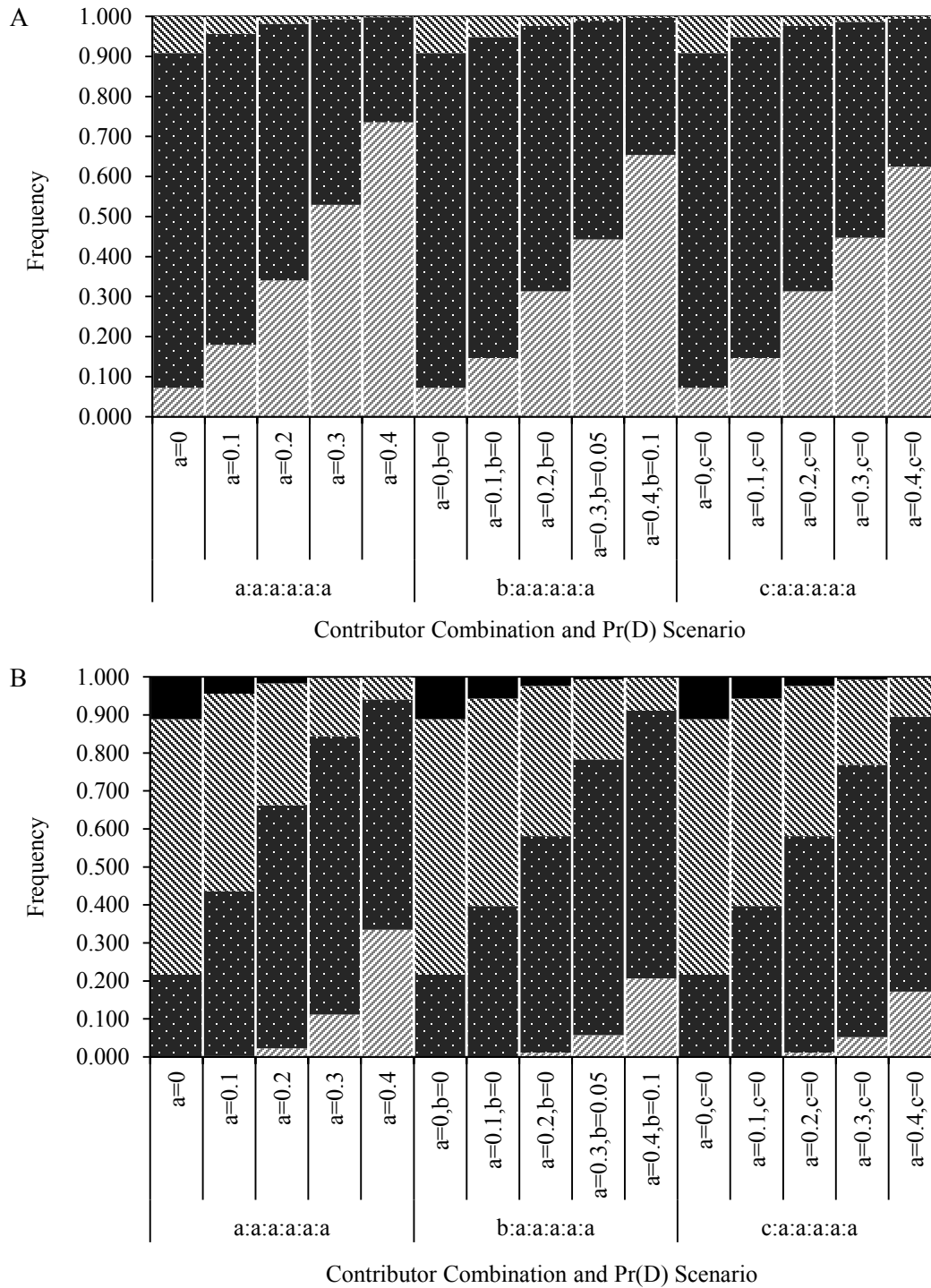


Figure 29. The frequency of observing 1 (▣), 2 (■), 3 (▤), 4 (▥), 5 (▦), and 6 (■) contributors using MAC for simulated Identifiler® Plus (A) and GlobalFiler™ (B) six-person mixtures with no major contributor (a:a:a:a:a), one moderate major contributor (b:a:a:a:a), and one substantial major contributor (c:a:a:a:a) for all Pr(D) scenarios described in Figure 22.

2.4 Discussion

The impact that allelic dropout has on estimating the NOC to a forensic DNA sample was evaluated by varying the $\text{Pr}(D)$ in mixtures with up to six contributors. The MAC method performed efficiently for two- and three-person mixtures with a $\text{Pr}(D)$ equal to zero for all contributors but underestimated the NOC for more complex mixtures with greater than three contributors. Therefore, increasing the NOC increases the degree of allele sharing which in turn reduces the number of unique alleles at each locus. Increasing the $\text{Pr}(D)$ increased the degree of underestimation, since the number of observed alleles decreased. Though the MAC method seems to work relatively well for pristine three-person mixtures with no dropout, moderate levels of allelic dropout (e.g. approximately 0.1) start to impact the ability to infer three contributors correctly using MAC. The absence of a major contributor in a mixture increased the percentage of profiles for which the NOC was underestimated. The additional loci, especially the highly polymorphic locus SE33, in the GlobalFiler™ amplification kit, reduced the frequency of misidentifying the NOC compared to the Identifiler® Plus kit. Though SE33 is helpful in evaluating the NOC, it is to be noted that it is one of the longer molecular weight loci and prone to degradation effects. It is expected that degradation would further complicate this process.

Results from these simulations, when dropout did not occur, were consistent with the results of previous studies. For example, Buckleton et al. investigated the probability of observing x alleles for simulated mixtures of two, three, and four contributors in order to assess the risk associated with making an assumption of the NOC to a mixed DNA

sample [39]. Mixtures were simulated by drawing alleles at their relative frequencies independently for the ten SGM Plus™ loci; allelic dropout was not factored into these simulations. Their analysis suggested there is a small (4.4×10^{-8}) chance that a two-person mixture would exhibit two or fewer alleles at all loci and about 3.3% of three-person mixtures exhibited four or fewer alleles at all loci studied. When they considered four-person mixtures, about 66% exhibited six or fewer alleles. Comparable results were obtained by Paoletti et al. [20], who concluded that MAC is not reliable in terms of predicting the NOC to mixed DNA samples. These are both consistent with the data presented herein, where 100% of two-person mixtures were correctly identified, 3.6% of three-person mixtures presented as two-person mixtures, and 70.6% of four-person mixtures were misclassified as three-person mixtures.

Coble et al. extended the study of Buckleton et al. by exploring the uncertainty in the NOC in the existing CODIS set, the proposed new CODIS set, and two amplification kits that include the new CODIS set, GlobalFiler™ and PowerPlex® Fusion (Promega Corp., Madison, WI) through the simulation of mixtures [40]. Again dropout was not incorporated into the simulations. They reported results similar to Buckleton et al. for two- and three-person mixtures for the new CODIS and GlobalFiler™ datasets. However, with the additional loci, the percentage of four-person mixtures appearing to have originated from three or fewer contributors decreased to 43.2%. This number of misclassifications dropped even lower to 16.5% when the highly polymorphic locus SE33 was added to the dataset. Five-person mixtures were incorrectly identified as four-person mixtures 61% of the time, and 67% and 19% of six-person mixtures characterized

respectively as five- and four-person mixtures. Results for GlobalFiler™ simulations of the present study are similar, with 20.4% of four-person mixtures identified originating from three contributors, 62.4% of five-person mixtures characterized as four-person mixtures, and 67.2 and 21.4% of six-person mixtures characterized, respectively, as five- and four-person mixtures.

In other work, a comparison of the MAC and maximum likelihood estimator (MLE) method, which takes into account the allele frequencies of the target population, was assessed by Haned et al. [41]. This study concluded that the accuracy of estimations of the NOC to mixed DNA samples decreased as the NOC increased for both the MAC and the MLE. While the probability of correct estimations was greater than 90% for mixtures with two to three contributors with the MAC, the efficiency dramatically decreased for mixtures with more than three contributors, 34% for four-person and 2% for five-person mixtures. Using MLE improved these probabilities to 77% and 64%, respectively. This inference was found to be true from the simulations of the present study – as the NOC and Pr(D) increased the efficiency of the MAC decreased, particularly for mixtures with greater than three contributors.

Taken together, the present and previous studies indicate that while MAC may perform well for two- and three-person mixtures with no instances of allelic dropout, it is not a reliable estimator of the actual NOC to complex high-order, low-template DNA mixtures. Increasing the Pr(D) to 0.4, the maximum observed Fr(D) for approximately one cell of DNA, decreased the percentage of correctly identified three-person Identifiler® Plus mixtures to 31.1%. Despite the benefit of the additional loci in the

GlobalFiler™ kit, 49.1% were still misclassified. Even in the best circumstances, i.e. no dropout, 20.4% of four-person, 63.2% of five-person, and 88.8% of six-person GlobalFiler™ mixtures were underestimated. The percentage of misidentifications increased to 93.0%, 99.2%, and 99.9%, respectively, when the Pr(D) was 0.4 for all contributors. The presence of a major contributor did not greatly improve the results. Further, a substantial number of gross underestimations on the NOC were observed for MAC designations greater than two.

Forensic laboratories using MAC to determine the NOC to mixed samples should be aware of the shortcomings of the method and accompany all inclusions with statistics that illustrate their strength. Furthermore, it is essential to understand the impact that allelic dropout has on correctly estimating the NOC. The probability that dropout may have occurred in a sample should be evaluated when attempting to determine the NOC that could reasonably explain the signal obtained from an item of evidence.

2.5. Conclusions

This research aimed to determine the impact allelic dropout has on estimating the NOC to a mixed DNA sample. Additionally, it was of interest to explore if the presence of a major contributor aids or hinders this assessment. Basing the NOC on the MAC of a profile was found to be unreliable for complex mixtures with greater than two contributors or with one or more minor contributors present at low levels. While a high level of dropout did not affect correctly identifying two-person mixtures, it greatly increased the number of misclassifications of the NOC of mixtures with three or more contributors. This number of misclassifications was reduced for mixtures with the STR loci consistent with the GlobalFiler™ kit due to the additional polymorphic loci SE33, D1S1656 and D12S391, which are not included in the Identifiler® Plus kit. The presence or absence of a major contributor did not appear to substantially affect the results. To conclude, these data suggest that estimating the NOC in a high-order mixed, low-template DNA profile will be prone to underestimation if based solely on MAC. Instead, a combination of factors that, at a minimum, include the allele frequencies in the population, the probability of allelic dropout, and evaluation of additional genetic data, may aid in evaluating the number of contributors that gave rise to the evidence.

LIST OF JOURNAL ABBREVIATIONS

Biometrika	Biometrika
Croat Med J	Croatian Medical Journal
Forensic Sci Int	Forensic Science International
Forensic Sci Int Genet	Forensic Science International: Genetics
Int J Legal Med	International Journal of Legal Medicine
J Forensic Sci	Journal of Forensic Sciences
Nat Biotechnol	Nature Biotechnology
Nucleic Acids Res	Nucleic Acids Research

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EDUCATION

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PRESENTATIONS

Sarah Norsworthy, Desmond S. Lun, Harish Swaminathan, Muriel Medard, and Catherine M. Grgicak. Characterizing rates of allelic dropout and its impact on estimating the number of contributors. 66th Annual Meeting of the American Academy of Forensic Sciences; Feb 2014; Seattle, WA.