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ORIGINAL ARTICLE

Modeling one complete versus triplicate analyses in low template DNA typing

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Abstract There are generally two strategies for low template DNA typing: the complete strategy, which uses all available DNA in a single PCR and subsequent typing, and the consensus strategy, in which the biological sample is divided into two or more aliquots and the genotype profile is determined by consensus from these "replicates." In this study, the consensus and complete strategies are compared by a statistical approach in terms of the accuracy of obtaining the correct genotype at a single locus for single source samples. Logistic models were employed to describe the allele drop-out and drop-in events. The parameters of the models were estimated with empirical or hypothetical data. The probabilities of obtaining the true genotype and the chances to observe drop-out and drop-in alleles were estimated and compared for both strategies. Consistent with a previous experimental study, this study found that, with relatively high input DNA (e.g., ≥100 pg), the complete strategy performs better than the consensus strategy to obtain the true genotype and the complete strategy will display less dropped out alleles. The consensus strategy had less drop-in alleles for ≤100 pg DNA samples. Moreover, the limitations of the logistic models were discussed. Ideal models with better fit of empirical data approximating casework conditions were proposed for future studies.

Keywords Forensic genetics · Low template DNA · Statistical modeling · Consensus · Allele drop-out · Allele drop-in · Logistic model

Introduction

Results from typing of low quantities of DNA, known as low copy number or low template (LT) DNA typing, exhibit increased stochastic effects compared with ≥100–200 pg contributions of DNA per individual donor of a sample [1–4]. The effects present challenges regarding the reliability of interpretation of DNA profiles and validity of the significance or weight of the interpretive result. There are generally two strategies for LT DNA typing. The first strategy, i.e., consensus strategy, divides the biological sample into two or more aliquots; each aliquot is subjected to the PCR process, the amplified products of each aliquot are analyzed and alleles are identified, and a "reliable" profile is determined by consensus. Essentially, the only alleles reported as reliable are those observed in more than one of the replicates at a predefined threshold (e.g., at least observed twice in all aliquots). The premise of the consensus approach is that if drop-in occurs randomly and infrequently, then observing an allele multiple times increases the confidence that the allele is truly derived from the evidentiary sample and reduces the probability that the reported allele was due to allele drop-in or contamination. The most common choice in this strategy is "two out of three replicates," which may provide a good balance between the drop-in and drop-out rates if the amount of DNA in each aliquot is not too low [5, 6]. The second strategy, i.e., complete strategy, instead of dividing the sample into multiple aliquots, uses all available DNA in the sample for amplification in a single PCR and subsequent typing. The allele drop-in rate is expected to be lower with a potentially less sensitive assay, and higher amounts of input DNA in genotyping may or may

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not reduce the allele drop-out rate (because of its dependence on the sensitivity of the analytical method employed). Therefore, typing the sample with all available DNA can yield higher accuracy of allele calling compared with reduced template of the individual replicates by the consensus strategy.

Although LT DNA typing has been applied in casework for more than 10 years, there has been little study comparing the efficacy of these two strategies. Recently, Grisedale et al. [7] compared the two strategies by conducting empirical experiments and concluded that the consensus strategy brought a notable increase of drop-out and less allele drop-in. Benschop et al. [8] compared the performance of a consensus method (i.e., two out of four replicates) and a pooled method (i.e., four independently amplified PCR products are pooled and injected on a capillary electrophoresis instrument) with mixture samples. The pooled method attempts to approximate one complete analysis by assuming that the stochastic effects are random and the combined amplifications will average out these effects. The percentages of detecting true alleles were close for these two methods, but the pooled method had a slightly lower drop-in rate; the drop-out rates were not explicitly compared. It should be noted that most studies suggest that allele drop-in is low and random [5–7], and therefore, the reduction in allele drop-out may outweigh the impact of allele drop-in.

In this study, the consensus strategy and the complete strategy were compared by a statistical approach in terms of the accuracy of obtaining the correct genotype at a single locus for single source samples. Statistical models were proposed and employed to describe the allele drop-out and drop-in events. The parameters of the models were estimated with empirical or hypothetical data. The probabilities of obtaining the true genotype and the chances to observe drop-out and drop-in alleles were estimated and compared for both the consensus and complete strategies.

Methods

Notations

Similar statistical models were used for allele drop-in and drop-out events as described by Balding et al. [9] and Mitchell et al. [6]. In a single amplification at a single heterozygous locus, let $(1-D)$, D, and D^2 represent no drop-out, partial drop-out, and complete drop-out, respectively; let D_2 represent complete drop-out of a homozygote at a locus. The rare scenario that a locus presents more than three alleles is ignored for this study. Let C represent drop-in of one allele. Drop-in of more than two alleles at a locus is ignored because of its extremely low chance. Although a good proportion of drop-in alleles are likely exaggerated stutter from step-wise slippage [6, 7], to keep the model simple and easy for comparison purposes, step-wise slippage has not been statistically modeled in a drop-in event in other studies [6, 9]. The allele drop-in and drop-out events are assumed to be independent as suggested in [6, 9].

Transition probabilities

For a locus in a single source sample, the genotype can be heterozygous (e.g., AB) or homozygous (e.g., AA). Table 1 shows the transition probabilities of observing these genotypes given true genotypes after the PCR process. There are 16 or 8 possible observed genotypes for a heterozygous or homozygous locus, respectively.

With the complete strategy, the probabilities of obtaining the true genotypes are $(1-D)^2(1-C)^2$ and $(1-D_2)(1-C)^2$ for heterozygotes and homozygotes, respectively. For the consensus strategy, the probability of obtaining the true genotype is the sum of probabilities of all the scenarios in which the consensus genotype matches with the true genotype. Herein, only the most commonly used consensus strategy, two out of three replicates, was evaluated. With this strategy, if the true genotype is a heterozygote (i.e., AB), there are $16\times16\times16=4,096$ possible observed genotype combinations, and only 256 combinations can have the consensus genotype matching the true genotype. The probability of obtaining AB is the sum of the probabilities of these 256 combinations (Eq. 1). In Eq. 1, O_1, O_2, and O_3 are all possible observed genotypes in the triplicates as listed in Table 1; $C(O_1, O_2, O_3)$=AB presents that the consensus genotype of O_1, O_2, and O_3 is AB. The Symbolic Math Toolbox of the MATLAB version 7.0 was used to compute and simplify Eq. 1.

$$\Pr\left(\mathrm{AB}\middle|\mathrm{AB},\ \text{3 replicates}\right) = \sum_{O_1}\sum_{O_2}\sum_{O_3;\ C(O_1,O_2,O_3)\ =\ \mathrm{AB}} \Pr\left(O_1\middle|\mathrm{AB}\right)\Pr\left(O_2\middle|\mathrm{AB}\right)\Pr\left(O_3\middle|\mathrm{AB}\right) = (1\ +\ 2C)^2(1+2D)^2(1-C)^4(1-D)^4 \tag{1}$$

If the true genotype is a homozygote (i.e., AA), there are $8\times8\times8=512$ combinations, and only 64 combinations can have a consensus genotype AA. Using the same method as for heterozygote loci, the probability of obtaining AA with the consensus strategy given that the true genotype is AA can be calculated with Eq. 2.

$$\Pr\left(AA\middle|\mathrm{AA},\ \text{3 replicates}\right) = \sum_{O_1}\sum_{O_2}\sum_{O_3;C(O_1,O_2,O_3)=AA} \Pr\left(O_1\middle|\mathrm{AA}\right)\Pr\left(O_2\middle|\mathrm{AA}\right)\Pr\left(\mathrm{O}_3\middle|\mathrm{AA}\right) = (1+2C)^2(1+2D_2)(1-C)^4(1-D_2)^2 \tag{2}$$

With the same approach and the probabilities in Table 1, the probabilities of observing at least one drop-out or drop-in can be calculated given the true genotype (heterozygote or homozygote) for either one replicate or triplicates, as summarized in Table 2. The probabilities of having at least one allele drop-in are identical for heterozygote and homozygote as shown in Table 2.

Table 1 The transition probabilities from the true genotype to observed genotype as a result of the PCR process

True genotype	Observed genotype	Probability
AB	–	$D^2(1-C)^2$
	A; B	$(1-D)D(1-C)^2$
	C; D	$D^2(1-C)C$
	AB	$(1-D)^2(1-C)^2$
	AC; BC; AD; BD	$(1-D)D(1-C)C$
	CD	D^2C^2
	ABC; ABD	$(1-D)^2(1-C)C$
	ACD; BCD	$(1-D)DC^2$
	ABCD	$(1-D)^2C^2$
AA	–	$D_2(1-C)^2$
	A	$(1-D_2)(1-C)^2$
	B; C	$D_2(1-C)C$
	AB; AC	$(1-D_2)(1-C)C$
	BC	D_2C^2
	ABC	$(1-D_2)C^2$

Only one allele is presented for observed homozygotes (e.g., "A" is equivalent to "AA")

"–" represents complete drop-out

Logistic model

For a fixed setting of experiment (i.e., protocols, instruments, etc.), a logistic model has been proposed and used for potential allele drop-out events at heterozygous loci [10, 11], in which the allele drop-out rates functioned with the peak heights of the observed (i.e., not drop-out) allele. Balding and Buckleton [9] also suggested that the allele drop-out rates of homozygote loci could be estimated with linear correction of the allele drop-out rates of heterozygous loci. Since the allele drop-out rates are primarily determined by the amount of input DNA of a single source sample in the PCR process, this logistic model was adopted but instead applying the drop-out rate as a function of the amount of input DNA (Eq. 3). In Eq. 3, I is the amount of input DNA, α_D and β_D are the parameters of this model (which should be estimated from empirical data), and $\Pr(D|I)$ is the probability of drop-out given the input DNA. This model applies for both homozygotes and heterozygotes, with separate estimations of the parameters based on empirical data.

$$\Pr\left(D\middle|I\right) = \frac{1}{1+e^{\alpha_D+\beta_D I}} \tag{3}$$

For a given experimental setting, the allele drop-in rates are mainly determined by the amount of input DNA (although the sensitivity of detection of the assay will impact the drop-in rate). The same logistic model can be applied to allele drop-in events (Eq. 4), where α_C and β_C are the parameters, and $\Pr(C|I)$ is the probability of drop-in given an input DNA.

Table 2 Probabilities of observing the true genotype, at least one drop-out, or at least one drop-in for the complete strategy (one sample with all DNA) and the consensus strategy (triplicates with equal amounts of DNA in each aliquot). The D, D_2, and C in triplicates are the rates of each aliquot

Probability	One complete	Triplicates
Pr(AB|AB)	$(1-D)^2(1-C)^2$	$(1+2C)^2(1+2D)^2(1-C)^4(1-D)^4$
Pr(≥1 drop-out|AB)	$2D-D^2$	$D^2(3-2D)(2D^3-3D^2+2)$
Pr(≥1 drop-in|AB)	$2C-C^2$	$C^2(3-2C)(2C^3-3C^2+2)$
Pr(AA|AA)	$(1-D_2)(1-C)^2$	$(1+2C)^2(1+2D_2)(1-C)^4(1-D_2)^2$
Pr(≥1 drop-out |AA)	D_2	$3D^2-2D^3$
Pr(≥1 drop-in|AA)	$2C-C^2$	$C^2(3-2C)(2C^3-3C^2+2)$

Although this simple model ignores that a good proportion of dropped in alleles are actually exaggerated stutters at +1 and −1 positions, it can still apply to the primary determinant of allele drop-in events.

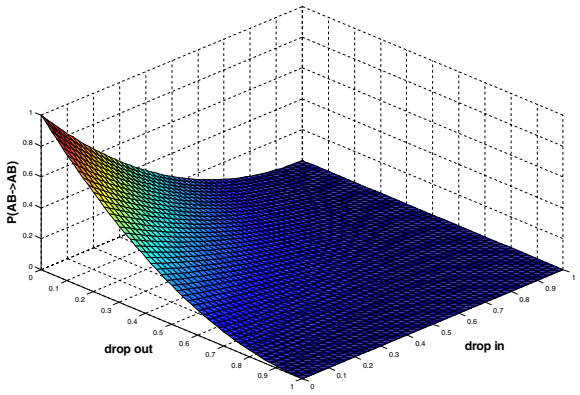
$$\Pr\left(C\middle|I\right) = \frac{1}{1+e^{\alpha_C+\beta_C I}} \tag{4}$$

Because different amounts of input DNA are used in the complete and consensus strategies, different allele drop-out and drop-in rates should be estimated and used in comparing the strategies. If a DNA sample is separated into three aliquots, for simplicity, the quantity of input DNA of each aliquot is assumed to be equal (although this assumption cannot be correct due to sampling error), and $3\times I_{\text{Consensus}}=I_{\text{Complete}}$. This assumption is an approximation for simplicity as it is unlikely that each low template replicate will contain the same amount of DNA.

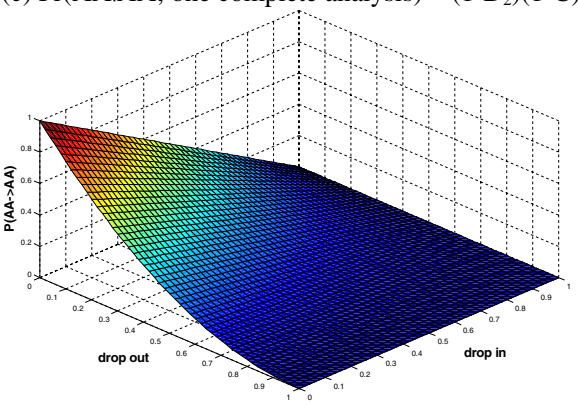
Results

The distributions of Pr(AB|AB) and Pr(AA|AA) in the complete and consensus strategies were compared (Fig. 1). The chance to obtain the true genotype decreases with higher drop-out and/or drop-in rates. Given the same allele drop-out and drop-in rates, triplicates can perform better than one aliquot of a triplicate, especially when the allele drop-out and drop-in rates are relatively low. However, in real casework applications, the rates of the single complete analysis and each analysis of the triplicates are not identical, since different quantities of DNA are used and different analytical conditions may be applied. The associations of the rates of two different strategies can be established with the statistical models, so that performance of the consensus and complete strategies can be compared for more realistic scenarios.

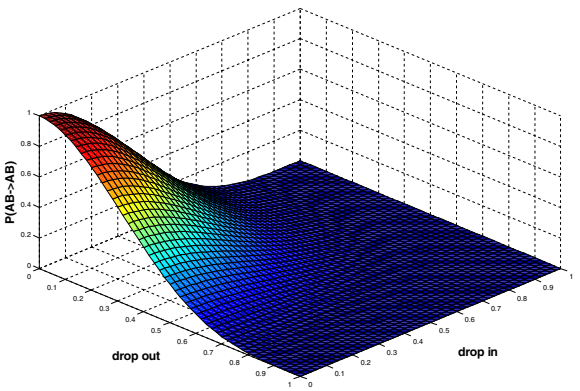
(a) Pr(AB|AB; one complete analysis) = $(1\text{-}D)^2(1\text{-}C)^2$



(c) Pr(AA|AA; one complete analysis) = $(1\text{-}D_2)(1\text{-}C)^2$



(b) Pr(AB|AB; triplicates) = $(1+2C)^2(1+2D)^2(1\text{-}C)^4(1\text{-}D)^4$



(d) Pr(AA|AA; triplicates) = $(1+2C)^2(1+2D_2)(1\text{-}C)^4(1\text{-}D_2)^2$

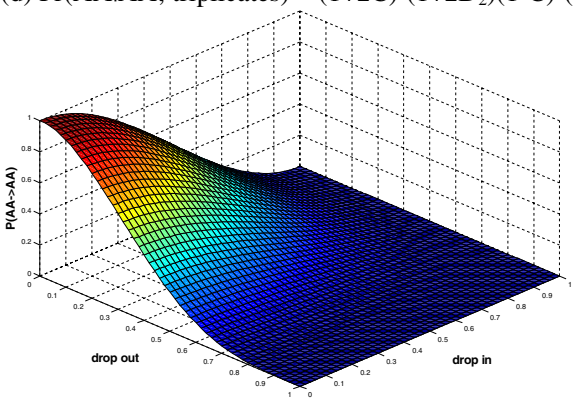


Fig. 1 Three-dimensional distributions of Pr(AB|AB) and Pr(AA|AA) for complete and consensus strategies. The *X*- and *Y*-axes are drop-out and drop-in rates, respectively. *Z*-axis is Pr(AB|AB) and Pr(AA|AA). **a** Pr(AB|AB; one complete analysis)=$(1-D)^2(1-C)^2$. **b** Pr(AB|AB; triplicates)=$(1+2C)^2(1+2D)^2(1-C)^4(1-D)^4$. **c** Pr(AA|AA; one complete analysis)=$(1-D_2)(1-C)^2$. **d** Pr(AA|AA; triplicates)=$(1+2C)^2(1+2D_2)(1-C)^4(1-D_2)^2$

To further compare these two strategies, empirical data on allele drop-out and drop-in rates were needed to estimate the parameters in the logistic models and to compare the strategies. Based on the study from Mitchell et al. [6], drop-out rates were assigned as 10 %, 15 %, 20 %, and 30 % for 100, 50, 20, and 10 pg input DNA, respectively, for a fixed analytical set of conditions (e.g., instruments, cycle numbers, injection time, locus, etc.). These drop-out rates are likely to be underestimates because they are derived from mixture studies and shared alleles will mask drop-out events. Thus, the rates are used only for illustrative purposes. A 1 % allele drop-out rate was assigned for 200 pg input DNA as an arbitrary but conservative estimation for high amounts of input DNA. For drop-in alleles, the data from Benschop et al. [5] and Mitchell et al. [6] were used as a guide and set at 0.1 %, 1 %, 5 %, and 10 % for 200, 100, 50, and 25 pg input DNA, respectively. The same caveat as above applies here as well if empirical estimates were derived from mixture studies in which the drop-in rates are likely to be underestimates as well. Based on these assigned rates, the parameters were estimated with Eq. 5.

$$\Pr\left(D\middle|I\right)=\frac{1}{1+e^{1.040+0.0169\times I}}\quad\text{and}\quad\Pr\left(C\middle|I\right)=\frac{1}{1+e^{1.733+0.0263\times I}} \tag{5}$$

With these models and the estimated parameters, the allele drop-out and drop-in rates given the quantity of input DNA, namely, the distributions of Pr($D|I$) and Pr($C|I$), are shown in Fig. 2a. To compare the different rates in the single complete analysis and the 1/3 aliquot analysis (i.e., one of the triplicates in the consensus strategy), the difference of Pr($D|I$) and Pr($D|$I/3), as well as Pr($C|I$) and Pr($C|$I/3), is shown in Fig. 2b. For example, for a total of 150 pg input DNA, the drop-out and drop-in rates were 2.73 % and 0.34 %, respectively, with the complete strategy; the drop-out and drop-in rates were 13.18 % and 4.53 %, respectively, for each aliquot with the consensus strategy. As expected, when the input DNA is high (e.g., ≥500 pg), Pr($D|I$) is low and thus Pr($D|I$) and Pr($D|$I/3) are close to each other (e.g., Pr($D|I$)−Pr($D|$I/3)= 1.4 % for 500 pg total input DNA). Neither of these quantities

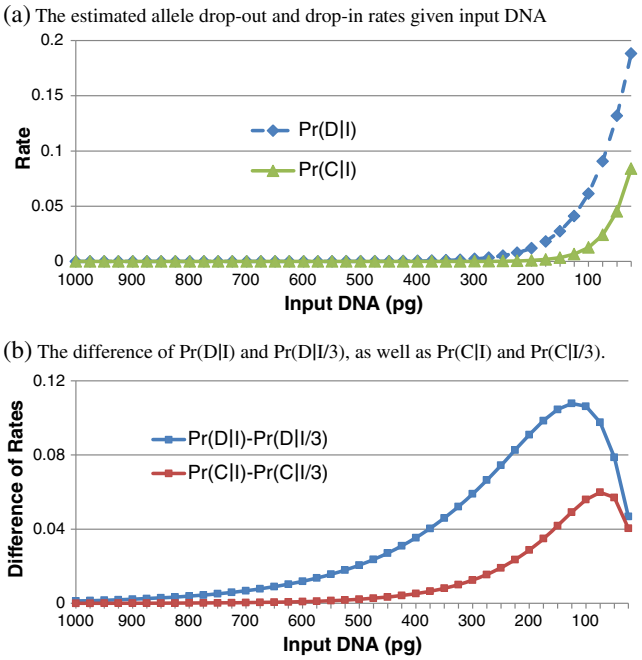


Fig. 2 The estimated allele drop-out and drop-in rates with the logistic model. Pr($D|I$) is the allele drop-out rate with the complete strategy; Pr(D|I/3) is the allele drop-out rate for one of the triplicates with the consensus strategy; Pr($C|I$) is the allele drop-in rate with the complete strategy; and Pr(C|I/3) is the allele drop-in rate for one of the triplicates with the consensus strategy. **a** The estimated allele drop-out and drop-in rates given input DNA. **b** The difference of Pr($D|I$) and Pr(D|I/3), as well as Pr($C|I$) and Pr(C|I/3)

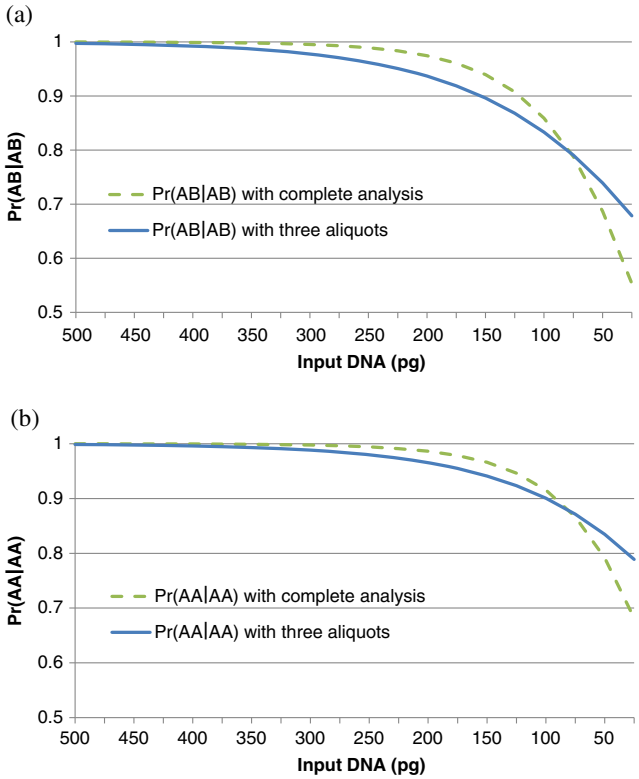


Fig. 3 The distributions of **a** Pr(AB|AB) and **b** Pr(AA|AA) for the consensus and complete strategies

demonstrated substantial effects of drop-in and drop-out. Large differences of the rates can be found when the amount of DNA is much lower (e.g., 50~500 pg). But, when the input DNA is extremely low (e.g., ≤50 pg), Pr($D|I$) and Pr(D|I/3) begin to become similar (i.e., both approach 100 %) and the difference eventually is nominal. Similar curves are observed for the allele drop-in rates (Fig. 2b). The largest difference of the drop-out and drop-in rates between the complete strategy and the 1/3 aliquot method is reached at about 125 and 75 pg total input DNA, respectively, using the empirical data from Mitchell et al. [6].

The distributions of Pr(AB|AB) and Pr(AA|AA), or the capabilities of the strategies to obtain the true genotype (Fig. 3), were estimated. With 500 pg total input DNA, both consensus and complete strategies are able to derive the correct genotype with almost 100 % accuracy. The accuracies start to decrease quickly when the input DNA is about 250~200 pg, which is close to the threshold defining LT-DNA typing for single source samples [12, 13], although the threshold can vary with the technologies and protocols. For both Pr(AB|AB) and Pr(AA|AA), the complete strategy is more likely to obtain the true genotype than would the consensus strategy when total input DNA is more than 75 pg, especially in the range from 300 to 100 pg, where the largest differences of the rates are reached. This observation is due to the greater difference in the allele drop-out and drop-in rates for the complete analysis and the 1/3 aliquot analysis with more than 75 pg input DNA. When the input DNA is lower than 75 pg because of the drop-out rates and drop-in rates used herein, the complete and the 1/3 aliquot methods approach similar values, three aliquots together apparently perform better than a single complete sample. Thus, the consensus strategy is more likely to obtain the true genotype than the complete strategy in this scenario.

The probabilities of observing at least one drop-out allele by the consensus and complete strategies were compared (Fig. 4). With relatively high input DNA (i.e., 75~500 pg), Pr(≥1 drop-out|AA) with one complete is lower than Pr(≥1 drop-out|AA) with triplicates, namely, the consensus strategy is more likely to observe allele drop-out compared with the complete strategy. Similar to the distributions of Pr(AB|AB) and Pr(AA|AA) in Fig. 3, the greatest differences of Pr(≥1 drop-out) of both strategies were found in the range of 300 to 100 pg input DNA. When the input DNA is lower than 75 pg, more allele drop-out will be observed with the complete strategy compared with the consensus strategy, since the drop-out rates are becoming more similar with extremely low input DNA, each aliquot of the consensus strategy can perform comparably with the complete strategy, and consensus from three aliquots provided a greater chance to reduce allele drop-out.

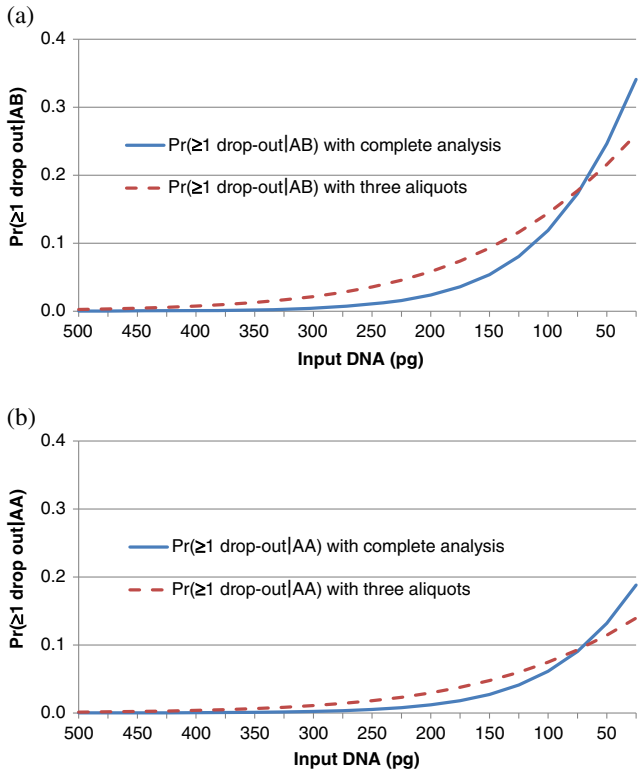


Fig. 4 The distributions of **a** Pr(≥1 drop-out|AB) and **b** Pr(≥1 drop-out|AA) for the consensus and complete strategies

For the probabilities of observing at least one drop-in event, Pr(≥1 drop-in) is the same for both AB and AA according to the equations in Table 2. Figure 5 shows the distributions of Pr(≥1 drop-in) for both consensus and complete strategies. When the input DNA is greater than 100 pg, the Pr(≥1 drop-in) with the consensus strategy is slightly higher than that of the complete strategy, but the difference is small. With input DNA lower than 100 pg, the chance of observing a drop-in allele is much higher with the complete strategy than the consensus strategy (i.e., observing a drop-in allele after consensus from triplicates). Same as Pr(≥1 drop-out), with similar drop-in rates, consensus from three aliquots has a higher chance to reduce the rate of allele drop-in.

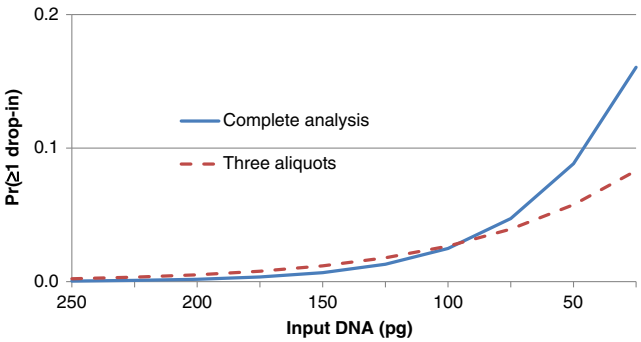


Fig. 5 The distributions of Pr(≥1 drop-in) for the consensus and complete strategies

Although one set of empirical data was used in the above analysis, similar results were observed for a different set of empirical data (Supplementary material), which suggests that the general trend is relatively consistent with the logistic model.

Discussion

This study compared the consensus (three aliquots) and complete strategies in terms of their capabilities of obtaining the true genotype and detecting at least one drop-out or drop-in allele. The same statistical models as [6, 8] were used for the allele drop-out and allele drop-in events. Logistic models [9, 10] were used to estimate the allele drop-out and drop-in rates functioning with the amount of input DNA. The parameters of the logistic models were estimated using reported empirical data for illustration purposes only. For real applications, the parameters should be estimated by each laboratory based on its protocol (such as loci, instruments, kits, PCR cycle numbers, injection times, etc.).

Generally, the complete strategy performed better than the consensus strategy for relatively high amounts of input DNA (e.g., ≥75 pg). If the amount of input DNA is low, the consensus strategy performed better because the drop-out and drop-in rates of the complete sample and 1/3 aliquot become closer with low input DNA. Consensus from three aliquot can have a higher chance of obtaining the true genotype if drop-out and drop-in rates are similar between the two strategies. However, the logistic model may not reflect the true conditions in the PCR process. When the input DNA is extremely low (e.g., ≤50 pg), the drop-in rate cannot increase indefinitely, since if there is no DNA, the drop-in rate should be low as it is in a large part dependant on stutter generation.

The results of this study are generally consistent with the conclusions of the empirical study by Grisedale et al. [7]. With relatively high input DNA, the complete strategy performs better than the consensus strategy to obtain the true genotype, and the complete strategy will display less dropped out alleles. Grisedale et al. [7] found that the consensus strategy had less drop-in alleles for ≤100 pg DNA samples. This study also found consistent results, namely, Pr(≥1 drop-in) of the complete strategy is much higher with low amount of DNA (e.g., ≤100 pg), although Pr(≥1 drop-in) with the consensus strategy is slightly higher than that of the complete strategy for relatively high amounts of DNA (e.g., ≥100 pg).

The logistic model implemented in this study is continuous in terms of the input DNA and each replicate was assumed to contain equal amounts of DNA. This model and its assumptions may perform well for high amounts of DNA, but likely is not a good fit for extremely low amounts of DNA (e.g.,

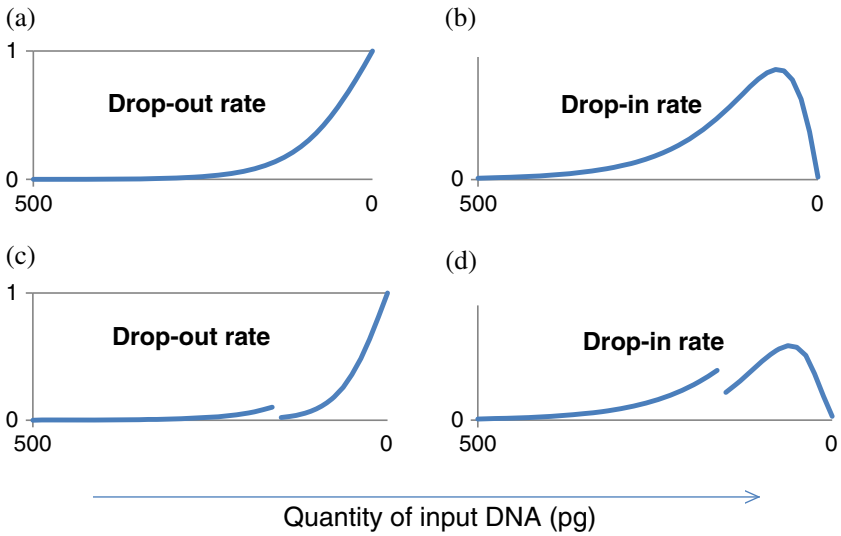


Fig. 6 More applicable distributions of the drop-out and drop-in rates for two different protocols segmented by the quantity of input DNA. *X*-axis is the quantity of input DNA and *Y*-axis is drop-in or drop-out rate. **a** The distribution for drop-out rate for a fixed protocol for any quantities of DNA. **b** The distribution for drop-in rate for a fixed protocol for any quantities of DNA. **c** The distribution for drop-out rate for two protocols for varying quantities of DNA. **d** The distribution for drop-in rate for two protocols for varying quantities of DNA

equivalent to a few cells). When the amount of input DNA is high, the probability that each replicate has equal or close to equal amounts of DNA is high, and the chance to obtain the true genotype is relatively equal for each replicate. However, when there are only a few cells worth of DNA, the distributions of the probabilities of obtaining true genotype and drop-in and drop-out events are more discrete (i.e., multinomial distribution), instead of continuous, and the amount of DNA in each replicate may not (and in fact are more likely not to) be equal. For example, suppose there are three cells equivalent of DNA (6 pg for each cell) in a sample. For the consensus strategy with triplicates, the chance that that there is 6 pg in each replicate is not 100 %, but 22.2 % according to multinomial distribution. The allocation of the aliquots also can be that (1) one aliquot has 18 pg and two other replicates have no DNA, or (2) one replicate has 12 pg, one replicate has 6 pg, and the other replicate has no DNA (12 and 6 are used here for illustrative purposes; the amounts need not vary solely by the discrete quantity of a cell and it may also depend on the volume in which the sample is dispersed). Therefore, stochastic events will be further exaggerated with the greater chances of unbalanced allocation and higher drop-out and drop-in rates brought by lower amounts of input DNA in a particular aliquot. Better statistical models (e.g., incorporation of discrete events) are needed for interpreting extremely low copy DNA typing and the observations presented in the study herein will likely be a lower amount of DNA where the two methods perform similarly.

On the other hand, when the quantity of DNA is lower than a certain threshold (e.g., 100 pg), an augmented PCR protocol, different from the standard protocol, is applied in real LT DNA typing casework (e.g., increased PCR cycles, increased injection time, post-PCR purification, etc.). Thus, a simple logistic model for any quantity of input DNA may not be the best fit for this scenario. The drop-out and drop-in rates between the complete and consensus methods are not one continuum as the sensitivity of detection of the separate methods is quite different. For allele drop-out, two separate logistic models with different parameters may be implemented for input DNA greater or less than the threshold, as shown in Eq. 6, where I_0 is the DNA quantity threshold for different protocols (e.g., 100 pg), α_{D1}, β_{D1}, α_{D2}, and β_{D2} are the parameters of the logistic models. A model with more than two segments may be applied if more than two protocols are implemented for the different quantities of DNA in casework analyses.

$$\Pr\left(D\middle|I\right) = \begin{cases} \dfrac{1}{1+e^{\alpha_{D1}+\beta_{D1}I}} & I \geq I_0 \\ \dfrac{1}{1+e^{\alpha_{D2}+\beta_{D2}I}} & I < I_0 \end{cases} \tag{6}$$

Allele drop-in events may arise from slippage events during PCR amplification (i.e., exaggerated stutter) or by contamination before PCR amplification. As analytical conditions increase the sensitivity of detection, the contamination rate and stutter artifacts are further exacerbated. Thus, the drop-in rate should be the sum of contamination rate and the chance of other stochastic artifacts. The contamination rate is relatively stable for a protocol and less affected by the quantity of DNA. Thus, the contamination rate can be two uniform distributions for the different PCR protocols, respectively. The stutter rate is relatively low and stable for relatively high quantity of DNA; the rate may increase significantly with low quantity of DNA and more sensitive protocols. Therefore, the drop-in rate of the standard protocol for high quantity of DNA is the sum of the stutter rate (S_1) and the contamination rate (T_1) with an augmented protocol; the drop-in rate of the augmented protocol for extremely low quantity of DNA is the sum of the

contamination rate with augmented protocol (T_2) and the stutter rate estimated by a logistic model with parameters (α_{C2} and β_{C2}), as shown in the Eq. 7.

$$\Pr\left(C\middle|I\right) = \begin{cases} S_1 + T_1 & I \geq I_0 \\ \dfrac{1}{1 + e^{\alpha_{C2} + \beta_{C2} I}} + T_2 & I < I_0 \end{cases} \quad (7)$$

Figure 6 shows the ideal distributions of the drop-out and drop-in rates given the quantity of input DNA, if two protocols (i.e., standard and augmented) are used. The drop-out and drop-in rates at the protocol switch point are apparently different given different protocols. With a more sensitive protocol, the drop-in rate increases and drop-out rate initially decreases for the same quantity of DNA. However, the proportion of exaggerated stutters might significantly reduce with only a few cells worth of DNA, since lower amounts of DNA are available to generate stutters and eventually no exaggerated stutter will be generated if there is no DNA (note: there will be an empirically defined chance that if stutter occurs in one of the early cycles of PCR than stutter can still be high with a few cells worth of DNA). Therefore, a simple logistic drop-in model may not be a good fit for real situations with extremely low quantities of DNA (e.g., ≤50 pg). But the logistic model should fit higher quantities of DNA (e.g., ≥50 pg) well.

Since there are no empirical data available to estimate the rates of drop-out, drop-in, contamination, and stutter for extremely low quantities of DNA, the multiple segment models were not implemented and used to compare different strategies in this study. However, these models are apparently more sophisticated and may be a good base for future study.

The study was conducted only for single source genotypes (i.e., homozygote or heterozygote) at a single locus. The same models and conclusions can be easily applied to multiple loci assuming that the drop-out and drop-in events are independent across the loci. Of course, this assumption may not hold and could be dependent on the degree of degradation or inhibition which may correlate with amplicon size.

Since mixture samples are composed of single source samples, in theory, the same principle and general trends from the single source samples can form a basis to apply in the interpretation of mixture. However, such an endeavor will be far more complicated to compare the consensus and complete strategies for mixture profiles, since the probabilities would be affected by more factors, such as overlap of the alleles from different contributors, the mixture ratio of the contributors, the ability to infer mixture ratios, etc. For mixture analysis, one approach is to conduct replicate analyses on a sample where a major component of the profile comes from a known person (e.g., the victim), accounting for 90 % of the DNA in the sample, and the perpetrator's profile only accounts for the remaining 10 % and this lower quantity DNA is subject to stochastic events. In this case, multiple replicates may perform better than a complete analysis to confirm the presence of alleles of the minor contributor. Mitchell et al. [6] applied a total input DNA for their LT DNA typing method to estimate the parameters of drop-in and drop-out. This approach is not sustainable for mixtures because these rates are dependent on the amount of DNA of each contributor. The rates should be different for each contributing component of a mixture when the contributions are not equal. Specific genotypes of the contributors within a mixture, the number of the contributors, and the mixture ratio can change the probabilities of detecting the true genotypes, as well as drop-out and drop-in alleles. Lastly, we assumed that drop-out and drop-in events were independent across the loci as this assumption is used in other studies [6, 9]. This assumption is not likely valid and future models will consider adding a dependency factor.

In conclusion, this study has provided a simplified model to assess the performance of the complete strategy and the consensus strategy for low template DNA typing of single source samples. Clearly for relatively high input DNA, the complete strategy is preferred. The simulations show that with this model, there are points of DNA quantities for single source samples where further definition is needed. These points are where the two methods cross-over. For extremely low quantity of DNA, unbalanced allocation of the DNA within the triplicates will exacerbate the performance of the consensus approach, and the current continuous statistical model may not be the best fit for this situation. Multiple segment models are proposed to better fit the practical situation of casework analyses, but more studies are needed to estimate the parameters and test these models. Lastly, the estimate of drop-out rates can be quite variable for a given amount of low-level DNA. Therefore, it may be better to provide a range of likelihood ratios to accommodate this variation.

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