CEESIt: A Computational Tool for the Interpretation of STR Mixtures

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ABSTRACT

In forensic DNA interpretation, the Likelihood Ratio (LR) is often used to convey the strength of a match. Expanding on binary and semi-continuous methods that do not use all of the quantitative data contained in an electropherogram, fully continuous methods to calculate the LR have been created. These fully continuous methods utilize all of the information captured in the electropherogram, including the peak heights. Recently, methods that calculate the distribution of the LR using semi-continuous methods have also been developed. The LR distribution has been proposed as a way of studying the robustness of the LR, which varies depending on the probabilistic model used for its calculation. For example, the LR distribution can be used to calculate the p -value, which is the probability that a randomly chosen individual results in a LR greater than the LR obtained from the person-of-interest (POI). Hence, the p -value is a statistic that is different from, but related to, the LR; and it may be interpreted as the false positive rate resulting from a binary hypothesis test between the prosecution and defense hypotheses. Here, we present CEESIt, a method that combines the twin features of a fully continuous model to calculate the LR and its distribution, conditioned on the defense hypothesis, along with an associated p -value. CEESIt incorporates dropout, noise and stutter (reverse and forward) in its calculation. As calibration data, CEESIt uses single source samples with known genotypes and calculates a LR for a specified POI on a question sample, along with the LR distribution and a p -value. The method was tested on 303 files representing 1-, 2- and 3- person samples injected using three injection times containing between 0.016 and 1 ng of template DNA. Our data allows us to evaluate changes in the LR and p -value with respect to the complexity of the sample and to facilitate discussions regarding complex DNA mixture interpretation. We observed that the amount of template DNA from the contributor impacted the LR – small LRs resulted from contributors with low template masses. Moreover, as expected, we observed a decrease of p -values as the LR increased. A p -value of 10^{-9} or lower was achieved in all the cases where the LR was greater than 10^8 . We tested the repeatability of CEESIt by running all samples in duplicate and found the results to be repeatable.

KEYWORDS

Mixture interpretation, Likelihood ratio, p -value, DNA analysis, LR distribution

INTRODUCTION

Until recently, statements of inclusion or exclusion were exclusively used when reporting or presenting DNA comparisons to the trier-of-fact. If a suspect, or other known, is 'included' as a potential contributor to the item of evidence, then the inclusion statement must be accompanied

by the calculation of a statistic that conveys the strength of the match [1]. Alternatives to inclusion/exclusion statements have, of late, been adopted, where a verbal scale is used to describe the number obtained [2]. Two protocols for calculating a match statistic are the Random Man Not Excluded (RMNE) approach, based on the Combined Probability of Inclusion (CPI) statistic, and the Likelihood Ratio (LR) approach. The RMNE method seeks to determine the fraction of the population that would not be excluded as a contributor to the profile. During the calculation of the CPI statistic, some information like the genotype of the suspect, the peak heights and the number of contributors to the profile is not utilized [3]. Though RMNE is still employed in practice, this method of evaluation is being replaced with the LR approach [4]. The Likelihood Ratio is defined as:

$$LR = \frac{\Pr(E|H_p, n_p)}{\Pr(E|H_d, n_d)},$$

where E is the evidence in the form of the electropherogram (epg); H_p and H_d are the hypotheses specified by the prosecution and the defense, respectively; and n_p and n_d are the number of contributors specified by the prosecution and the defense, respectively. The Likelihood Ratio can be expressed either as the ratio of probabilities or as the ratio of probability densities, depending on whether the evidence is treated as a discrete or as a continuous random variable. The numerator is the probability of observing the evidence given the prosecution's hypothesis and the denominator is the probability of observing the evidence given the defense's hypothesis. The evidence shows support for the prosecution's hypotheses if $LR > 1$; if $LR < 1$ the defense's hypothesis is supported by the evidence. Unlike the RMNE method, the LR can use information like the number of contributors to the sample, the heights of the peaks observed and the genotype of the suspect [3].

The LR framework can be applied using a binary model that uses the set of alleles observed in a DNA profile [5]. This method assigns a probability of 0 or 1 to genotypes based on the presence or absence of alleles. Alternatives to the binary model have been proposed that allow for drop-in and/or dropout of alleles [6,7]. These 'semi-continuous' methods use the peak heights to establish probabilities of dropout and drop-in. Unlike binary methods, they can be used to interpret profiles in which one or more of the suspect's alleles are not observed, or when there are incidences of drop-in. Fully continuous methods that employ probabilistic genotyping by modeling the peak heights have also been created, resulting in the ability to incorporate stutter and noise, or drop-in, into the calculation of the statistic [8-12]. Fully continuous methods make use of the entire data obtained in the epg, including the qualitative (alleles observed) and the quantitative (peak heights) data. The TrueAllele system [8-9] uses an MCMC sampler to compute a probability for every possible genotype combination based on how well it explains the observed data. The peak heights are linearly modeled with respect to the mixture weights using a

multivariate normal distribution. Degradation is modeled as an exponential decay with respect to the allele product length and stutter as a linear function of the allele. Cowell et al [10] model the peak heights using a Gamma distribution and employ a Bayesian network for analyzing mixtures that incorporate dropout and a stutter model that is independent of DNA mass and the marker. Puch-Solis et al [11] also use a gamma distribution for stutter and allele heights but differ from [10] by using the total peak height at a locus as a proxy for the DNA mass, estimating parameters conditional on peak heights and jointly modeling stutter and allelic peaks. Both these methods do not take into account drop-in. Taylor et al [12] implement MCMC using the Metropolis-Hastings algorithm to compute the genotype probabilities. Allele peak heights are modeled using an exponential decay with respect to the molecular weight of the allele and stutter peak heights are modeled as a linear function of the allele height. Drop-in is modeled as an exponential decay with respect to the peak height.

In addition to the methods and models that evaluate LR_s, computational methods that compute the LR distribution have recently garnered attention [7,13-16]. The LR distribution can be used to evaluate the robustness of the model by performing Tippett tests – “*what is the probability that a non-contributor will give rise to an LR greater than 1 (Type I error)?*” [13]. Another statistic that can be obtained from the LR distribution is the *p*-value [7,14-16]. The *p*-value is a summary statistic that provides the probability that a randomly picked person from the population would give rise to an LR at least as large as the one observed for the person of interest. It can be interpreted as the false positive rate and may be useful when the analyst wants to compute the probability of a random non-contributor giving rise to an LR greater than the one observed for the suspect. While there is controversy surrounding the use of *p*-values [17], several authors have shown that it is a useful statistic that assists in the interpretation of LR_s and has other applications like database searching [18] and kinship analysis [19].

In this work, we seek to combine the twin features of a fully continuous method to calculate the LR and the calculation of the LR distribution and a *p*-value. To this end, we developed a computational method called ‘CEESIt’ to calculate the LR for a person of interest, given an STR profile. CEESIt (CEES: Computational Evaluation of Evidentiary Signal) is a fully continuous method that works by modeling the peak heights observed in a calibration data set consisting of single source samples with known genotypes. CEESIt accounts for dropout, noise and stutter (both reverse and forward), artifacts observed regularly in low template samples [19]. Additionally, CEESIt also computes a *p*-value for the LR by sampling a large number of random genotypes from the population. The method was tested on 303, 1-, 2- and 3-person experimental sample files with template masses ranging from 0.016 to 1 ng, and represents the largest empirical study to evaluate the *p*-value and LR that we know of. We found that the amount of template DNA from the contributor had an impact on the LR – small LR_s were associated with low template masses. Since we used 10^9 samples to calculate the *p*-value, the lowest *p*-value that CEESIt reports is 10^{-9} .

⁹, and this was obtained in all the cases where the LR was greater than 10^8 . We also tested the system's repeatability and found that the results were repeatable.

MATERIALS AND METHODS

Calibration Set

CEESIt employs a continuous method to calculate the LR and hence uses the peak heights in the signal to calculate probabilities. Characterization of the peak heights is accomplished by using single source calibration profiles with known genotypes obtained from samples amplified from a wide range of input DNA masses. For a detailed description of how the calibration samples (Calibration Set – Supplementary Table 1) were created, refer to [20]. Briefly, DNA was extracted from 28 individuals. Absolute DNA quantification was performed using real-time PCR and the Quantifiler[®] Duo[™] Quantification kit according to the manufacturer's recommended protocol and one external calibration curve [21, 22]. The extracted DNA was amplified using the manufacturer's recommended protocol for AmpF ℓ STR[®] Identifiler[®] Plus Amplification Kit (Life Technologies, Inc.) [23]. Separation of the STR fragments was accomplished with a 3130 Genetic Analyzer using an injection voltage of 3 kV and injection times of 5, 10 and 20 seconds. Analysis was performed using GeneMapper IDX v1.1.1 (Life Technologies, Inc.) and an RFU threshold of 1. A threshold of 1 RFU was used in order to capture all peak height information, i.e. the allelic peaks, baseline noise and stutter peaks, in the signal. Known artifacts such as pull-up, spikes, -A, and artifacts due to dye dissociation were manually removed, as previously detailed in [20].

Testing Set

A total of 303 1-, 2- and 3-person sample files were used to test CEESIt (Testing Set – Supplementary Tables 2-4). These 1-person test samples were created using the same protocol described for the single source samples in the calibration set. The mixtures were created by mixing appropriate volumes of the single source DNA extracts to attain the various ratios specified in Supplementary Table 5. Once mixed, these samples were re-quantified and then amplified using the targets from Supplementary Tables 3 and 4. In the case of mixtures, the samples were created using various kinds of mixture ratios in such a way that each individual contributed at least two cells' worth of DNA, which corresponds to approximately 0.013 ng of DNA [24]. The 1-person samples contained DNA from 32 different individuals, the 2-person samples contained DNA from 6 different individuals (3 combinations) and the 3-person samples contained DNA from 6 different individuals (2 combinations). When testing the samples injected for 5 sec, the calibration file also consisted of data from 5 sec injections. The same was true for the 10 and 20 sec injection files. None of the contributors to the calibration set were present in the testing set and none of the contributors to the testing set were present in the calibration set.

Modeling of variables

For a detailed description of the model used for the various variables, see [20]. In short, the allelic and noise peak heights were modeled using a Gaussian distribution. For stutter, since the height of the stutter peak depends upon the height of the parent allele, the stutter ratio was modeled using a Gaussian distribution. The allele frequencies used in this study were those of the US Caucasian population published in [23], though any population database can be used. The functions used to model the different variables with respect to the DNA mass were chosen by fitting a curve to the calibration data in MATLAB® (R2015b, The Mathworks, Natick, Massachusetts) and are shown in Table 1.

CEESIt's Algorithm

The Likelihood Ratio (LR) is defined as:

$$LR = \frac{\Pr(E|H_p, n_p)}{\Pr(E|H_d, n_d)}.$$

The evidence is in the form of a discrete random variable that takes non-negative integer values and CEESIt uses a continuous distribution, specifically the normal distribution, as the underlying distribution from which a discrete distribution to model the peak heights is derived. Hence we define the Likelihood Ratio to be the ratio of the two probabilities, rather than probability densities. In practice, n_p and n_d can be chosen by the prosecution and the defense to maximize their respective probabilities and there is no necessity for n_p to be equal to n_d . However, we have developed CEESIt to use the same number of contributors in both the numerator and denominator to calculate the LR. It should be noted that the method could be extended to work on different assumptions on the number of contributors. We note that for purposes of this work, $n_p = n_d$ in all cases presented herein and we use the known, and thus the true n to test the capabilities of CEESIt, unless otherwise mentioned.

For this study, we use the following hypotheses for H_p and H_d :

H_p : The evidence is a mixture of the genotype profile of a suspect and the profiles of $n - 1$ other unknown, unrelated contributors, whom we term the 'interference' contributors.

H_d : The evidence is from n unknown individuals unrelated to the suspect.

It should be noted that the algorithm can be expanded to incorporate a victim or other known profile in the prosecution and/or the defense hypothesis, but the current design of CEESIt allows only for the genotype of a suspect to be included in the algorithm.

The LR for the suspect with genotype s is:

$$LR(s) = \frac{\Pr(E|R = s, N = n)}{\Pr(E|N = n)}.$$

CEESIt's model incorporates a population substructure correction factor θ . In this work, a value of 0.01 was used for θ .

LR numerator calculation:

Our algorithm assumes a constant mixture ratio at all the loci. The mixture ratio specifies the proportion of the total template mass contributed by each contributor to the sample. The underlying mixture ratio of an evidence sample is unknown and needs to be described by a model in order to compute a continuous LR. A constant mixture ratio model assumes that the mixture ratio is the same at all the markers, whereas a variable mixture model accounts for the possibility of the mixture ratio being different at the various markers. Both models are reasonable and are used in existing continuous methods to compute the LR. Perlin et al [9] assign a uniform prior probability for the template mixture weight and construct its probability distribution by drawing individual locus weights using a multivariate normal distribution. Cowell et al [10] and Puch-Solis et al [11] use a constant mixture ratio model and implement a discrete approximation over the interval (0,1) by assigning a uniform prior. Taylor et al [12] use the variable model and assume the mixture weights to be independent across the loci. Since we adopt the constant mixture ratio approach, we integrate over all possible mixture ratios:

$$\Pr(E|R = s, N = n) = \int_{\theta \in \Delta^{n-1}} \Pr(E|\theta = \theta, R = s, N = n) f_{\theta}(\theta),$$

where θ is the vector with components θ_i , the mixture proportion of each contributor $i \in \{1, \dots, n_{\max}\}$; $\Delta^{n-1} = \{(\theta_1, \dots, \theta_n) \in \mathbb{R}^n | \sum_{i=1}^n \theta_i = 1, \theta_i > 0 \forall i\}$ is the unit $n - 1$ simplex; and f_{θ} is the probability density function of θ , which we assume to be uniform over Δ^{n-1} . For $n = 1$, Δ^{n-1} consists of the single element $\{1\}$. For mixtures, we implement the integration over Δ^{n-1} by dividing it into equal-sized subsets and representing each subset with its centroid, resulting in a discrete sum. This was done by performing k-means clustering in Python (Python Software Foundation, Beaverton, Oregon). k-means clustering is an algorithm used to partition observations into a set of clusters by repeated minimization of the distance from an observation to the centroid of its cluster [25]. For $n = 2$, the space was divided into 9 equally sized clusters, while for $n = 3$, 12 clusters were used. See Supplementary Table 5 for a list of the mixture ratios used in CEESIt's algorithm. For the k-means algorithm, we used 10,000 randomly generated mixture ratios for $n = 2$ and 100,000 randomly generated mixture ratios for $n = 3$. The algorithm was stopped when there was no further possible minimization of the distance from a point to a centroid, i.e. when each point was assigned to the cluster with the closest centroid. Since the number of clusters 'k' is an input to the k-means clustering algorithm, we need to check if the choice made for the number of clusters is reasonable. To do this, we analyzed the effect of increasing the number of clusters on the probability that is conditional upon the mixture ratios. For this sensitivity test on the number of clusters, we used the forty-one 2-person samples and the thirty 3-person samples at the 10s injection time that were part of our testing set. In these seventy-one samples, we computed $\Pr(E|R = s, N = n)$ for all the true contributors. We observed that increasing the number of clusters beyond our chosen values (9 for $n = 2$ and 12 for $n = 3$)

had little effect on the probability (Supplementary Figures 1 and 2), indicating that the values chosen are appropriate.

Let L be the set of all loci in the evidence sample, E_l be the evidence at locus l and s_l be the genotype of the suspect at locus l . The STR loci used for forensic DNA analysis are assumed to be in linkage equilibrium and independent of each other [24]. Hence we obtain:

$$\Pr(E|\Theta = \theta, R = s, N = n) = \prod_{l \in L} \Pr(E_l|\Theta = \theta, R_l = s_l, N = n).$$

The prosecution's hypothesis states that the profile is made of the suspect's contribution plus the contribution from $n - 1$ other random, unrelated contributors. Therefore we get:

$$\Pr(E_l|\Theta = \theta, R_l = s_l, N = n) = \sum_{\mathbf{u}^{n-1}} \Pr(E_l|\mathbf{U}^{n-1} = \mathbf{u}^{n-1}, \Theta = \theta, R_l = s_l, N = n) \Pr(\mathbf{U}^{n-1} = \mathbf{u}^{n-1}),$$

where $\mathbf{u}^i = \{u_1, \dots, u_i\}$ is a vector of the random genotypes of i contributors. Since there are many possibilities for the genotype of these interference contributors at each locus and summing over each possibility would take a large amount of time, we approximate $\Pr(E_l|\Theta = \theta, R_l = s_l, N = n)$ using importance sampling. Importance sampling is a Monte Carlo sampling algorithm in which, instead of sampling directly from the target distribution, samples are generated from a different distribution that is easier to sample from [26]. To take into account the fact that the samples have come from the 'wrong' distribution, weights are introduced to adjust the 'importance' of each sample. For the problem at hand, instead of sampling using the allele frequency distribution, we generate samples of the interference genotypes using the peak height distribution observed at the locus. The reason for sampling from the peak height distribution is that this method is faster and requires fewer samples for convergence than the method that samples from the allele frequency distribution.

Let J be the number of interference samples used. Now we obtain:

$$\Pr(E_l|\Theta = \theta, R_l = s_l, N = n) \approx \frac{\sum_{i=1}^J \Pr(E_l|\mathbf{U}_i^{n-1} = \mathbf{u}_i^{n-1}, \Theta = \theta, R_l = s_l) w_i}{J},$$

where $w_i = P(\mathbf{u}_i^{n-1})/Q(\mathbf{u}_i^{n-1})$ is the weight of sample i ; $P(\mathbf{u}_i^{n-1})$ is the probability of the interference genotypes under the allele frequency distribution; and $Q(\mathbf{u}_i^{n-1})$ is the probability of the interference genotypes under the peak height distribution.

Importance sampling is done for autosomal loci, which are based on STRs. The locus AMEL, the sex-determining locus, is not based on STRs and has only 2 possible genotypes: $\{X, X\}$ for females and $\{X, Y\}$ for males. At this locus, we assume that both genotypes are equally likely to occur and generate genotype samples for the n contributors and calculate the probability of observing the evidence as:

$$\Pr(E_l|\Theta = \theta, R_l = s_l, N = n) \approx \frac{\sum_{i=1}^J \Pr(E_l|\mathbf{U}_i^{n-1} = \mathbf{u}_i^{n-1}, \Theta = \theta, R_l = s_l)}{J}.$$

239 Calculation of $\Pr(E_l | \mathbf{U}_i^{n-1} = \mathbf{u}_i^{n-1}, \boldsymbol{\theta} = \boldsymbol{\theta}, R_l = s_l)$ is explained in the Appendix as part of the
 240 Supplementary Material.

241

242 *LR distribution and p-value calculation:*

243

244 The p -value for the suspect is defined as the probability that a randomly picked person from
 245 the population would give rise to an LR at least as large as the one observed for the suspect.

$$p\text{-value}(s) = \Pr(LR(R) \geq LR(s)),$$

246 where R is a random variable denoting the genotype of an individual. Since the denominator of
 247 the LR is the same for all genotypes, it is sufficient if we compare the numerator of the LR for R
 248 and s .

$$p\text{-value}(s) = \Pr(\Pr(E|R, N = n) \geq \Pr(E|R = s, N = n)).$$

249 During testing of CEESIt, we observed that because of floating-point precision, $\Pr(E|R, N = n)$
 250 evaluated to 0 for many of the random genotypes R that fit the data poorly. As a result, we were
 251 able to eliminate those genotypes from the p -value calculation as a preliminary step. Formally, let
 252 $\mathbf{R}$ be the set of all genotypes. We define $\mathbf{R}_1 = \{r \in \mathbf{R} \mid \Pr(E_l | R_l = r_l) \neq 0 \text{ for all loci } l\}$ and
 253 $\mathbf{R}_2 = \{r \in \mathbf{R} \mid \exists \text{ locus } l \text{ s.t. } \Pr(E_l | R_l = r_l) \simeq 0\}$, where $\simeq 0$ means “evaluates to 0 using double-
 254 precision 64-bit floating-point arithmetic”. Thus, we have $\mathbf{R} = \mathbf{R}_1 \cup \mathbf{R}_2$ and $\mathbf{R}_1 \cap \mathbf{R}_2 = \emptyset$. We see
 255 that for all $r \in \mathbf{R}_2$, $\Pr(E|R = r) \simeq 0$. We omit the notation on N for the sake of brevity. We have:

$$p\text{-value}(s) = \left\{ \Pr(R \in \mathbf{R}_1) \sum_{r \in \mathbf{R}_1} \Pr(\Pr(E|R = r) \geq \Pr(E|R = s)) \Pr(R = r | R \in \mathbf{R}_1) \right. \\ \left. + \Pr(R \in \mathbf{R}_2) \sum_{r \in \mathbf{R}_2} \Pr(\Pr(E|R = r) \geq \Pr(E|R = s)) \Pr(R = r | R \in \mathbf{R}_2) \right\}$$

256 We see that the second term is 0, provided $\Pr(E|R = s)$ is greater than 0. Hence we get:

$$p\text{-value}(s) = \Pr(R \in \mathbf{R}_1) \sum_{r \in \mathbf{R}_1} \mathbf{1}(\Pr(E|R = r) \geq \Pr(E|R = s)) \Pr(R = r | R \in \mathbf{R}_1),$$

257 where

$$258 \quad \mathbf{1}(\Pr(E|R = r) \geq \Pr(E|R = s)) = \begin{cases} 1, & \text{if } \Pr(E|R = r) \geq \Pr(E|R = s), \\ 0, & \text{otherwise.} \end{cases}$$

259 We have:

$$\Pr(R \in \mathbf{R}_1) = \prod_{l \in L} \sum_{r_l \in \{r \mid \Pr(E_l | R_l = r) \neq 0\}} \Pr(R_l = r_l).$$

260 We compute the p -value using Monte Carlo simulation. We generate M random genotypes

261 $r^1, \dots, r^M$ according to the distribution $\Pr(R | R \in \mathbf{R}_1)$ and calculate the p -value as:

$$p\text{-value}(s) = \Pr(R \in \mathbf{R}_1) \frac{\sum_{i=1}^M \mathbf{1}(\Pr(E|R = r^i) \geq \Pr(E|R = s))}{M}.$$

262 Increasing the value of M increases the accuracy of the p -value computed, but this also increases

the run time and a hence a tradeoff has to be achieved between the two. In this study, we have used 1 billion or 10^9 random genotypes to compute the p -value.

In some cases, $\sum_{i=1}^M \mathbf{1} \left((\Pr(E|R = r^i) \geq \Pr(E|R = s)) \right)$ evaluated to 0 when no simulated genotype had an LR at least as large as the POI's. This means that the p -value for the POI is less than $\Pr(R \in \mathbf{R}_1) / M$ and more samples are required for estimation. As a result, we could only arrive at an upper bound on the p -value for such samples since we calculate the p -value by sampling and do not perform an exact computation. Since $\Pr(R \in \mathbf{R}_1)$ in general varies with the sample, the upper bound also in general varies from one sample to another. Hence, to maintain consistency in our representation of the CEESIt results, whenever the p -value evaluated to less than $1/M$ for a contributor, we report $1/M$ or 10^{-9} as an upper bound on the p -value.

In order to facilitate the computation of the p -value, as an initial step $\Pr(E_l | \boldsymbol{\theta} = \boldsymbol{\theta}, R_l = g_l)$ is computed for all possible genotypes g_l at all loci l for all values of $\boldsymbol{\theta}$. Once this is done, for the p -value computation, 10^9 genotypes r^i are generated based on the allele frequencies. Since we know $\Pr(E_l | \boldsymbol{\theta} = \boldsymbol{\theta}, R_l = r_l^i)$ at all loci l , we can compute $\Pr(E|R = r^i)$ as:

$$\Pr(E|R = r^i) = \int \prod_{\boldsymbol{\theta} \in \Delta^{n-1}} \prod_l \Pr(E_l | \boldsymbol{\theta} = \boldsymbol{\theta}, R_l = r_l^i) f_{\boldsymbol{\theta}}(\boldsymbol{\theta}).$$

LR denominator calculation:

Let $\bar{R}$ be the genotype of an unknown contributor in the defense's hypothesis. The denominator of the LR can be written as:

$$\Pr(E|N = n) = \sum_{\bar{r}} \Pr(E|\bar{R} = \bar{r}, N = n) \Pr(\bar{R} = \bar{r})$$

Since the number of possible values that $\bar{r}$ can take is large and summing over all of them is computationally intensive, we utilize the calculations done for the random genotypes r^i that are sampled for the p -value estimation to compute the denominator of the LR as:

$$\Pr(E|N = n) = \Pr(R \in \mathbf{R}_1) \frac{\sum_{i=1}^M \Pr(E|R = r^i)}{M}.$$

RESULTS

Contributors with LR less than 1 have low template DNA masses

Figure 1 shows LR vs contributor template mass for all the contributors for 101, 1-, 2- and 3-person samples at the 10s injection in the testing set. The LRs for the 5s and the 20s samples were similar to the 10s samples and are shown in Supplementary Figures 3 and 4. All the log values shown in this study are to base 10. For each sample, the LR was computed for all the contributors. Hence, a single source sample would have 1 LR; a 2-person sample would have 2 LRs, etc. We observed that the amount of template DNA from the contributor impacted the LR

from CEESIt, i.e. high LR's corresponded to high template DNA amounts. This effect was previously reported by Taylor et al [12]. While most log(LR) values in Figure 1 are greater than 0 (the highest was 100, with a mean of 15), CEESIt computed the log(LR) as less than 0 (or LR < 1) for a contributor in 7 cases (two 1-person samples and five 3-person samples). These samples had a low template DNA from the contributor and a high level of dropout and stutter. For example, a 3-person sample with 0.59 ng of template DNA and a mixture ratio of 1:9:9 resulted in a log(LR) of -7 for the minor contributor (~0.03 ng template mass per qPCR quantification). Figure 2 shows the profile of the sample and Table 2 shows the genotype of the 3 contributors. Supplementary Table 6 contains the heights of the peaks in Figure 2. One of Contributor 1's alleles completely dropped out at 3 loci: allele 8 at D13S317, allele 20 at D2S1338 and allele 18 at vWA. Moreover, at locus D18S51 the forward stutter (height of 10 RFU) from allele 17 has a larger height than the parent peak (height of 9 RFU). These factors caused CEESIt to compute a low probability for the minor contributor. On the other hand, the alleles of major contributors 2 and 3 (~0.28 ng template mass each) are well represented in the epg resulting in a LR of 10^{12} and 10^{11} , respectively. The distribution of the LR's for the 1 billion random genotypes simulated for this sample is seen in Figure 3. A vast majority of the 1 billion samples had a log(LR) less than 0, with a peak around -30. The LR's for the 3 contributors were near the right end of the distribution; the resulting p -values were 7×10^{-4} for the minor contributor and 10^{-9} for the two major contributors.

When dropout levels were not high and the alleles were well represented in the epg, even minor contributors had high LR's. Figure 4 shows the profile of a 2-person mixture, with 0.25 ng of template DNA and a 1:2 mixture ratio. Supplementary Table 7 contains the heights of the peaks in Figure 4. The genotypes of the 2 contributors can be seen in Table 3. All the alleles of the contributors can be seen in the profile and the LR was 10^{19} for both contributors. Figure 5 shows the LR distribution for the 1 billion random genotypes simulated for this sample. Most of the samples had a log(LR) less than 0. The LR's for the contributors to the sample were in the right tail of the distribution; the p -value was 10^{-9} for the major and the minor contributor.

The Random Match Probability (RMP) is an inclusion statistic that stems from a binary approach, i.e. alleles are either present or absent [19]. The statistic is calculated using only the frequencies of the alleles observed. We computed the RMP for the single source samples using an analytical threshold of 50 RFU and the stutter filter and the US Caucasian allele frequencies published in [23]. In our testing set, 34 out of the 91 single source files resulted in a 'match' and hence a RMP was computed for the 34 samples. For all those 34 samples, the LR from CEESIt was similar to 1 / RMP [1] – the absolute value of the difference between log(LR) and log(1/RMP) was less than 1 in 26 out of the 33 samples, with the lowest value being 0.109. The largest difference observed between log(LR) and log(1/RMP) was 16, with log(LR) being smaller.

Addition of 'interference' contributors reduces the LR for a POI

There were two contributors to the 1-person samples who were also minor contributors in 2-person and 3-person samples in the testing set. We observed that for a given template DNA mass, the LR was highest for the 1-person sample and decreased in the presence of an interference contributor in a 2-person sample with a 1:1 mixture ratio. The LR further decreased with 2 interference contributors in a 3-person sample with a 1:2:1 mixture ratio (Figure 6). We did observe that at all three injection times, for some 2-person samples the $\log(\text{LR})$ for the minor and major contributors was large, and even larger than the $\log(\text{LR})$ for some 1-person samples. We found that the $\log(\text{LR})$'s were large in these instances because the two contributors gave the best, or close to the best, fit to the signal and the denominator of the LR had a very low probability.

Large LRs correspond to small p-values

Figure 7 (a) shows how the p -values from CEESIt vary with LR for the samples at the 10s injection. In general, as the LR increased in value, the p -value decreased, indicating a greater confidence in including the individual as a contributor. All the 1-person samples had the lowest possible p -value of 10^{-9} . Even the 1-person samples that had a $\text{LR} < 1$ had a p -value of 10^{-9} – though their probability was low because of high levels of dropout and stutter, it was still high relative to the population. For the 2-person and 3-person samples, in all cases where the $\log(\text{LR})$ was greater than 8, the $\log(p\text{-value})$ from CEESIt was -9, indicating that CEESIt was able to identify the individual as a contributor with great certainty. Figure 7 (b) shows the same plot zoomed in on the $\log(\text{LR})$ values between 0 and 9. All the $\log(p\text{-value})$ points represent 2- and 3-person samples and they lie below the line representing $-\log(\text{LR})$. This relationship between the p -value and the LR is expected because the $p\text{-value} \leq 1 / \text{LR}$ according to Markov's inequality [27]. The 5s and the 20s samples showed a trend similar to the 10s samples (Supplementary Figures 5 and 6).

LRs for non-contributors are low

As described in Materials and Methods, for each of the 303 sample files in our testing set, $\mathbf{R}_1$ contains all the genotypes with a non-0 probability. CEESIt produced the LR distribution for a sample by sampling the genotypes of 10^9 random contributors from the set $\mathbf{R}_1$. This resulted in a total of $303 \times 10^9 \approx 3 \times 10^{11}$ random genotypes for which we obtained a LR from CEESIt. The LR distribution for every sample was similar to the ones shown in Figures 3 and 5, with a majority of the LRs falling below 1. For each sample, we calculated the fraction of the 10^9 genotypes that resulted in a $\text{LR} > 1$ and multiplied it by $\Pr(R \in \mathbf{R}_1)$ to calculate the Tippett statistic $\Pr(\text{LR} > 1 | H_d)$. We observed that the average of the Tippett statistic increased as the number of contributors to the sample increased, indicating that the probability of a random non-contributor having a $\text{LR} > 1$ is low for small n and increases as n increases. The average $\Pr(\text{LR} > 1 | H_d)$ for the 1-person samples was 2.34×10^{-12} and it increased to 0.0004 and 0.0008 for the 2- and 3-person samples.

The smallest LR observed for the random contributors was 0, while the maximum was 10^{31} (for a 1-person sample in which the true contributor to the sample had a LR of 10^{31}).

The results from CEESIt are repeatable

One of the consequences of using a sampling algorithm (like importance sampling or Monte Carlo simulation) is that the results of two analyses on the same sample are generally not identical. While increasing the number of samples reduces the run-to-run variation, doing so also increases the running time. Hence a trade-off between the two has to be achieved. We ran CEESIt on all the samples in the testing set twice to check if the results were repeatable, i.e. if the LRs from the two runs were similar to each other. Figure 8 shows the results on the 101, 1-, 2- and 3-person samples at the 10s injection. There is very little variation from run to run; the points lie close to the $x=y$ diagonal line with very few outliers (Correlation coefficient = 0.9905, slope = 1.04, intercept = -0.1). Additionally we tested repeatability of CEESIt on one 2-person sample and one 3-person sample after 5 runs and found that the LRs and p -values obtained were repeatable (Supplementary Figures 7 and 8). Tests for repeatability on the 5s and the 20s samples also produced similar results (Supplementary Figures 9 and 10).

Sensitivity to the number of contributors

Since the true number of contributors to an evidence sample is never known with certainty, we examined the performance of CEESIt when conditioned upon different assumptions on the number of contributors. For this sensitivity study on the number of contributors, we focus on the samples at the 10s injection time that were incorrectly called by the maximum allele count (MAC) method. MAC is a binary method to estimate the number of contributors to a sample [28]. It works by counting the number of alleles observed at every locus, taking the maximum value over all the loci and dividing it by 2 to get the minimum number of contributors needed to explain the profile. MAC works by the application of an analytical threshold and a stutter threshold. Peaks below the analytical and the stutter thresholds are labeled as noise and stutter, respectively, and peaks above the thresholds are considered as signal and used in the calculation. In this study, we used an analytical threshold of 50 RFU, which is commonly used in forensic laboratories, and the stutter threshold for each locus as specified in [23].

MAC had 1 underestimate among the 2-person samples and 2 underestimates among the 3-person samples. In all 3 samples, we observed that using an incorrect number of contributors tended to decrease the LR to very small values, even resulting in a shift from $LR > 1$ to $LR < 1$. This effect was more pronounced for the 2-person sample than for the 3-person samples. Except for one contributor in one of the 3-person samples, all the other LRs using an incorrect number of contributors were less than 1 (Figure 9).

There were 3 overestimates among the 1-person samples and 2 overestimates among the 2-person samples, all of which were called as one more than actual number by the MAC method. Although MAC typically gives the minimum number of contributors needed to explain the profile, these overestimates occurred due to stutter peaks that exceeded the stutter threshold and hence were wrongly labeled as signal peaks. Using an overestimate on the number of contributors, unlike underestimates, did not result in a large decrease in the LR. For the 1-person samples, using an overestimating the number of contributors resulted in a similar LR and the LR even slightly increased for one of the samples. For the 2-person samples which were analyzed as 3-person mixtures, the LR did decrease, though it was greater than 1 (Figure 10). These results suggest that the output from CEESIt is indeed sensitive to the number of contributors specified and a good estimate on the number is crucial for interpretation of the sample.

Runtime

The running time of CEESIt increases as the number of contributors increases. For single source samples, the average running time was 7 minutes, and it increased to 50 and 140 minutes for 2- and 3-person samples, respectively. CEESIt has been implemented to employ multi-threaded processing to take advantage of multiple cores when available. The calculations were done using 8 cores on an Intel E3 3.4GHz processor.

DISCUSSION

We have developed a computational algorithm - CEESIt - to compute the LR for a POI and the distribution of the LR given an evidence profile. CEESIt also provides a summary statistic of the distribution in the form of the p -value of the LR. There is no 'correct' value for the LR to compare the results with – the LR computed is dependent upon the model used. Hence, we need to perform tests to check if the results from the model are reasonable, i.e. if CEESIt computes a 'large' LR in the case of a true contributor to the mixture and a 'small' LR for a non-contributor. CEESIt was tested on 303 sample files containing between 1 and 3 contributors with total template mass ranging from 0.016ng to 1ng, injected using 3 injection times. Across all samples tested, the mean of the LRs was 10^{14} ; the lowest and highest LRs were 10^{-74} and 10^{100} respectively for minor (or single) contributors ranging in mass from ~0.013 to 0.33 ng. We observed that the large LRs were obtained in instances where the LR denominator was small and the true contributor to the sample gave the best (or close to the best) fit for the evidence. For example, a LR of 10^{100} was obtained for a 1-person sample at the 10s injection with 0.0156ng of template DNA. The numerator of the LR evaluated to 10^{-135} . The contributor's genotype gave the best, or close to the best, explanation for the evidence and the denominator of the LR was extremely small (10^{-235}), resulting in the large LR. Such large LRs are typically not obtained from

binary interpretation methods such as [5], which take into account only the alleles observed and ignore other information like the heights of the peaks in the signal. In instances where CEESIt produced a small LR, the template DNA mass from the contributor was low, resulting in extreme dropout and stutter.

We observed that the presence of additional contributors impacted the LR for a POI. Specifically, we observed that the addition of interference contributors decreased the LR for a POI that contributed the same template mass to a 1-, 2- and 3-person sample, where, the LR was highest for the 1-person sample, followed by the 2-person sample and then the 3-person sample.

For comparison, we calculated a binary inclusion statistic for the single source samples in the form of the RMP. We observed that for samples for which we could calculate a RMP, the LR from CEESIt was close to $1 / \text{RMP}$, as expected. The smallest difference observed between $\log(\text{LR})$ and $\log(1/\text{RMP})$ was 0.109, while the largest difference was 16, with $\log(\text{LR})$ being smaller.

In order to compare CEESIt with other published results we focus on 2-person mixture cases. We note that in order to test CEESIt, the template masses used for the 2-person samples set in this studied varied from 0.2 to 1 ng and the mixture ratios varied from 1:1 to 1:49, where the lowest DNA mass of the minor contributed ~ 0.013 ng of DNA. For all of the 122 2-person mixture files tested in this work, the LR ranged from 10^{-63} to 10^{79} . Taylor et al [12] performed a similar study using an alternative continuous interpretation scheme and observed LRs in the range 10^8 to 10^{23} on 127 artificially constructed 2-person mixtures where the total template mass varied from 0.1 to 0.5 ng and the mixture ratios from 1:1 to 1:5, resulting in a minor contributor mass of at least 0.017 ng. TrueAllele [9] was tested on 101 casework profiles containing between 2 and 4 contributors. The mixture weight for the matched genotype varied from 0.05 to 0.95. The LRs ranged from 10 to 10^{23} , with a mean of 10^{11} .

In addition to calculating the LR, CEESIt also makes available to the user a distribution on the LR. The LR distribution can be useful while performing error rate calculations such as $\Pr(\text{LR} > 1 | H_d)$ [13]. CEESIt also provides a summary statistic of the LR distribution in the form of the p -value. The p -value, which can be thought of as the false positive rate, is the probability that a randomly picked person results in a LR greater than the LR observed for the POI. In this study, CEESIt calculated the p -value by generating 10^9 random genotypes according to the allele frequency distribution and computing the fraction of those genotypes that have a LR greater than the POI's LR. All the 1-person samples in the testing set had a p -value of 10^{-9} or lower, indicating that CEESIt was able to identify the individual as a contributor with great certainty. With regard to the 2- and 3-person samples, the p -value was the lowest possible value of 10^{-9} when the LR was greater than 10^8 .

One desirable quality of a mixture interpretation method is specificity: How often would a method misidentify a non-contributor? In our testing, CEESIt produced a LR distribution for a test sample by sampling 10^9 genotypes, giving $\sim 3 \times 10^{11}$ random genotypes with a LR from CEESIt. For each

experimental sample, we calculated the Tippett test statistic $\Pr(LR > 1|H_d)$ [13]. We observed that the probability of a random non-contributor resulting in a $LR > 1$ is low for small n and increases as n increases. The average $\Pr(LR > 1|H_d)$ for the 1-person samples was 2.34×10^{-12} and it increased to 0.0004 and 0.0008 for the 2- and 3-person samples, respectively. The minimum LR observed for the random contributors (sampled over the set R_1 , see Materials and Methods) was 0, while the maximum was 10^{31} . Perlin et al [9] tested TrueAllele by computing the LR for 10,000 random contributors on each of the 101 samples in their testing set and found 133 instances in which the LR was greater than 1. The highest LR was 10^4 and lowest was 10^{-30} . Similarly, Taylor et al observed a maximum LR of 10^4 and 1168 LRs greater than 1 after testing 57, 609 non-contributors on a 3-person mixture [12].

Another desirable aspect of a sampling algorithm is reproducibility from one run to the next on the same sample, i.e. obtaining similar results. CEESIt was run twice on all the samples in the testing set and the results were repeatable (The correlation coefficient was greater than 0.9 for the samples at all 3 injection times). Additionally, testing on one 2-person and one 3-person sample confirmed the repeatability of CEESIt after 5 runs. TrueAllele variability from run to run was tested on duplicate runs and on average the LR varied by a factor of 2 ($10^{0.305}$) standard deviations [9]. Taylor et al [12] showed their model to give reproducible results over 10 runs on a single 2-person mixture, with a standard deviation of ~3% of the mean.

CONCLUSION

Interpretation of low template samples is a challenging problem. Lately there has been a push towards the LR as the method of choice for calculating a match statistic. Fully continuous methods to calculate the LR have the benefit of making use of all the information contained in the signal.

One question that forensic analysts are interested in is “How many times would a non-contributor come up with a statistic that is as large or larger?” or in other words, “What is the false positive rate?” In addition to the LR, calculation of the LR distribution and a resulting summary statistic - the p -value - has been reported, with the p -value serving as a false positive rate. To date, the only systems available to compute both the LR and p -value used semi-continuous models.

In this work, we have brought together a continuous method to compute the LR based on modeling of the peak heights in known data and to calculate a p -value from the LR distribution by simulation of genotypes based on allele frequencies in order to demonstrate, using a large set of data, that systems which determine both a p -value and an LR are computationally viable. We do not propose to prescribe the use of one statistic over the other for purposes of reporting or testimony. Rather, we describe a study that examines the impact of the complexity of the sample on both statistics using a fully continuous interpretation scheme. The method was tested on 303 1-, 2- and 3-person sample files containing between 0.016 and 1 ng of DNA, injected for 5, 10

and 20 sec. We found that the amount of template DNA from the contributor had an impact on the LR – small LRs arose from contributors with low template masses, indicating that high levels of dropout and stutter could decrease the probability of the evidence under the prosecution's hypothesis even for true contributors. Since we used 10^9 samples to calculate the p -value, the lowest p -value that we report is 10^{-9} , and this was obtained in all the cases where the LR was greater than 10^8 . The results from CEESIt were found to be repeatable after duplicate runs on all the samples in the testing set. Even though we used low-medium template masses in our study, CEESIt can be tested on unknown samples with any template mass, provided the mass falls within the range of the masses of the calibration samples. While we have compared the results from CEESIt with the published results of other continuous interpretation software, comparison of the results from CEESIt with other continuous interpretation methods on the same set of data is important and would facilitate standardization of validation approaches. Future work involves developing CEESIt to handle different numbers of contributors in the prosecution and the defense hypotheses and accounting for known (and possibly related) contributors. In a follow up paper, we plan on testing the sensitivity of the LR to the changes in the model. Information on downloading and using CEESIt can be found at www.bu.edu/dnamixtures.

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FIGURES AND TABLES CAPTIONS

Table 1. The variables used in the study and the distribution used to model them as a function of DNA mass. At each time of injection, different parameters were used for different loci.

Table 2. Genotypes of the contributors to the 3-person sample in Fig 2.

Table 3. Genotypes of the contributors to the 2-person sample in Fig 4.

Figure 1. LR from CEESIt for the contributors to the 1-, 2- and 3-person samples at the 10s injection in the testing set. High LRs corresponded to high template DNA amounts. Contributors with low template masses had high levels of dropout and stutter, resulting in small LRs. Similar results were obtained at the other injection times.

Figure 2. Profile of a 3-person sample, amplified from 0.59ng of template DNA and a mixture ratio of 1:9:9. Arrows represent alleles of minor contributor that had a height < 1 RFU. The minor contributor's alleles dropped out at 3 loci, leading to a low LR (10^{-7}) and a high p -value (7×10^{-4}). The major contributors had LRs of 10^{12} and 10^{11} and p -values of 10^{-9} and 10^{-9} .

Figure 3. LR distribution from CEESIt for the sample in Fig 2. Most of the 1 billion samples had a log(LR) less than 0, with a peak at -30.

Figure 4. Profile of a 2-person sample, amplified from 0.25ng of template DNA and a mixture ratio of 1:2. The alleles of the minor contributor are well represented in the signal resulting in a LR of 10^{19} and a p -value of 10^{-9} . The major contributor also had a LR of 10^{19} and a p -value of 10^{-9} .

Figure 5. LR distribution from CEESIt for the sample in Fig 4. Many genotype samples had a log(LR) less than 0. 481 million samples had a LR of 0 (and a Log(LR) of $-\infty$). The plot shows the distribution of the remaining 519 million samples..

Figure 6. The LR for a contributor with 0.0625ng template mass in a 1-, 2- and 3-person sample at the 10s injection. The contributor was the major in the 3-person sample (0.125ng total template

mass, 1:2:1 mixture ratio), while the 2-person sample had a total template mass of 0.125ng and a 1:1 mixture ratio. The LR decreased as the number of interference contributors increased.

Figure 7 (a-b). p -values from CEESIt as a function of LR for all the samples at the 10s injection in the testing set. All the 1-person samples had a p -value of 10^{-9} . With regard to the 2- and 3-person samples, the p -value was the lowest possible value of 10^{-9} when the LR was greater than 10^8 . Similar results were obtained at the other injection times.

Figure 8. The results of 2 different runs on the 101, 1-, 2- and 3-person samples at the 10s injection. There is very little variation from run to run; the points lie close to the $x=y$ diagonal line with very few outliers (Pearson correlation coefficient = 0.9781, slope = 1.01, intercept = 0.03). Similar results were obtained at the other injection times.

Figure 9. LR for one 2-person sample (a) and two 3-person samples (b) using the true number of contributors and an underestimate on the number of contributors.

Figure 10. LR for three 1-person samples (a) and two 2-person samples (b) using the true number of contributors and an overestimate on the number of contributors.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

a

b

Figure 8

Figure 9

a

b

Figure 10

a

b

Table 1

Variable	Model description	Model function
Dropout rate of alleles (α)	Exponentially decreasing curve	$\alpha = ae^{bx}$, where x is the DNA mass from the contributor with the allele
Rate of non-detection of stutter (β)	Exponentially decreasing curve	$\beta = ae^{bx}$, where x is the DNA mass in the parent allele that gives rise to stutter
Rate of non- detection of noise (γ)	Constant	Estimated from calibration data
Mean of true peak heights (μ_t)	Line with a positive slope	$\mu_t = ax + b$, where x is the DNA mass in the true peak
Standard deviation of true peak heights (σ_t)	Line with a positive slope	$\sigma_t = ax + b$, where x is the DNA mass in the true peak
Mean of noise peak heights (μ_n)	Line with a positive slope	$\mu_n = ax + b$, where x is the DNA mass that the sample was amplified with
Standard deviation of noise peak heights (σ_n)	Line with a positive slope	$\sigma_n = ax + b$, where x is the DNA mass that the sample was amplified with
Mean of stutter ratios (μ_s)	Exponentially decreasing curve	$\mu_s = ae^{bx} + c$, where x is the DNA mass in the parent allele that gives rise to stutter
Standard deviation of stutter ratios (σ_s)	Exponentially decreasing curve	$\sigma_s = ae^{bx} + c$, where x is the DNA mass in the parent allele that gives rise to stutter

Table 2

Locus	Contributor 1	Contributor 2	Contributor 3
D8S1179	12,13	10,10	8,14
D21S11	28,30	30,30	28,29
D7S820	8,10	10,12	8,12
CSF1PO	12,12	11,12	10,12
D3S1358	16,17	14,18	15,16
TH01	8,9	7,9.3	8,9
D13S317	8,13	10,11	10,11
D16S539	11,12	9,12	8,13
D2S1338	19,20	23,24	23,24
D19S433	12,13	13,15	13,15
vWA	15,18	15,17	14,16
TPOX	8,11	8,8	8,11
D18S51	17,20	15,20	13,15
AMEL	X,X	X,X	X,X
D5S818	9,13	11,11	10,12
FGA	19,24	22,23	21,23

Table 3

Locus	Contributor 1	Contributor 2
D8S1179	13,14	14,14
D21S11	27,29	30,30.2
D7S820	8,12	8,9
CSF1PO	10,12	12,12
D3S1358	16,18	15,18.2
TH01	7,8	7,9.3
D13S317	12,14	11,13
D16S539	8,9	9,11
D2S1338	17,17	22,24
D19S433	13,13	11,13
vWA	17,19	15,17
TPOX	9,10	8,8
D18S51	15,16	14,17
AM	X,Y	X,Y
D5S818	12,12	11,11
FGA	22,24	22,23