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Research Article

Unknown biological mixtures evaluation using STR analytical quantification

Allelic quantification of STRs, where the presence of three or more alleles represents mixtures, provides a novel method to identify mixtures from unknown biological sources. The allelic stutters resulting in slightly different repeat containing products during fragment amplification can be mistaken for true alleles complicating a simple approach to mixture analysis. An algorithm based on the array of estimated stutters from known samples was developed and tuned to maximize the identification of true nonmixtures through the analysis of three pentanucleotide STRs. Laboratory simulated scenarios of needle sharing generated 58 mixture and 38 nonmixture samples that were blinded for determining the number of alleles. Through developing and applying an algorithm that additively estimates stuttering around the two highest peaks, mixtures and nonmixtures were characterized with sensitivity of 77.5, 82.7 and 58% while maintaining the high specificity of 100, 97.4 and 100 for the W, X, and Z STRs individually. When all three STRs were used collectively, the resulting sensitivity and specificity was 91.4 and 97.4%, respectively. The newly validated approach of using multiple STRs as highly informative biomarkers in unknown sample mixture analyses has potential applications in genetics, forensic science, and epidemiological studies.

Keywords: Mixture / Pentanucleotides / Short tandem repeats / Stutter / Stutter peaks
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1 Introduction

The discovery of polymorphisms in repetitive DNA in 1985 [1] was a major breakthrough in molecular technology. Subsequently, STR polymorphisms have been widely used in forensic and genetic applications [2–5]. STRs are highly polymorphic, abundant in the genome, and their high variability in individuals can be detected by relatively simple PCR-based assays [6, 7]. STRs can also be useful in identifying DNA from multiple individuals in a sample and the extensions of this application can be highly informative for epidemiologic studies [8].

Mixtures can be identified by the analysis of allelic patterns. However, allele calling may be complicated as a result of sample conditions, laboratory procedures, genotyping framework, and more. During the DNA-based STR amplification process, PCR products differing in size by multiple repeat units from the main allele are formed. These products are referred to in the literature as stutter, slippage, or shadow bands [9–11]. Stutters result from *Taq* DNA polymerase enzyme during DNA amplification, forming a nonbase-paired loop of usually one repeat unit causing an altered product from the parent allele which is subsequently amplified [11, 12] and in some cases can exceed 15% [13, 14]. The stutters for STR loci are inversely correlated with the length of the core repeat unit. Thus, the stutter is generally very noticeable with dinucleotides while the effect diminishes with tri-, tetra-, and pentanucleotide repeats and is almost undetectable with VNTR loci [7, 15, 16]. Pentanucleotide STRs usually have only one prevalent minor stutter [17].

In many applications of STRs (forensic, ancient DNA, and epidemiological studies), there may not be enough DNA template (especially of the minor mixture component) or

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Abbreviations: OCF, outlier capture fraction; **rfu**, relative fluorescent unit

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the DNA may be degraded such that traditional methods for tissue or blood sampling are impractical. Under such conditions, template specific products can be maximized by optimizing the PCR conditions or alternatively using touch downprotocols. However, the amount of stutter varies (even between pentanucleotide STRs) and that is especially the case when the source DNA is scarce.

While most of the optimally amplified STR markers, including pentanucleotides, show only one major stutter fragment that structurally differs by one repeat unit from the associated parent allele(s) [12, 13], other adjacent stutter ladders, both upstream and downstream from the true allele, are observed when amplifying minute or degraded DNA templates. Stutter ladders require special consideration in mixture analyses. These fragments can be troublesome in cases where the percentage of the mixture attributed to the minor contributor is of an equivalent amount and indistinguishable from the stutters of the major contributor. Thus, a quantitative analysis method is warranted to differentiate a stutter from a true allele in mixtures, especially where the sources of mixtures are not known and the templates are scarce.

If DNA templates are mixed, then this ratio will be apparently preserved throughout all the STR markers examined [18]. For instance, if the proportions in genomic mixtures are the same (*i.e.*, 50:50), then all true alleles will have essentially the same height and the allelic components can be visually interpreted. The principles of mixture interpretation were initially published in 1991 [19] and several quantitative analysis methods and interpretations have been described [20–23], but almost all are in the context of forensic science. When the individuals comprising the potential mixtures and their individual DNA profile and weights are *a priori*, a likelihood ratio approach has been previously developed to interpret mixtures [20, 24]. In most forensic cases, component DNA profiles of at least the suspect or the victim is known and a computer-based quantitative matching approach is performed with the STR data to estimate or identify the remaining contributors. However, interpretation of mixtures from unknown sources with differential proportion requires a more stringent algorithm that explicitly accounts for all possible background and artifacts for each STR used. To date, except for our own work [8, 25], genetics-based mixture analysis has only been performed in the context of forensic science and not for epidemiological evaluation. Previously, we have defined and characterized a set of pentanucleotide STRs for the specific use of determining a single genome from multiple genomes in epidemiological settings [8]. Herein, we report an analytical strategy that takes into account the peak height of all possible true alleles and pseudo-alleles for the evaluation of unknown biological mixtures.

2 Materials and methods

We followed a standard step-wise laboratory procedure including DNA extraction, amplification of STRs by PCR, fragment separation, and sizing for the development and validation of a mixture analysis method. The biological characteristics of the STRs (W, X, Y, and Z) including heterozygosity (H) and the probability of mixture discrimination (p_{MD}), which estimates the chance of detecting a third allele in a biologically mixed samples, have been previously described [8]. Upon further evaluation, we excluded STR-Y from our analyses since it had some nonspecific artifacts and its alleles overlapped with the internal size standards for CE. STRs W, X, and Z are located in different chromosomes and are in linkage equilibrium providing independent allelic profiles.

2.1 DNA samples

Initially, genomic DNA extracted from 366 cell lines from four different ethnic groups (African American = 99, European American = 95, Hispanics = 94, and Asians = 78) was used for PCR amplification and defining the stutter-based algorithm. The reference blood samples for simulating mixture and nonmixtures were obtained from 12 anonymous volunteer blood donors with informed consent for genetic studies. The blinded mixture (n = 58) and nonmixture (n = 38) samples were simulated from these reference blood samples following the laboratory-based needle sharing scenario protocol as previously described [25]. Briefly, sharing was simulated in these syringes 3–7 days following the first simulation and all syringes were left at room temperature for 14 days. The samples were washed with Tris-HCl pH 8.3 and stored at −70°C for 7 days before DNA extraction. We used DNA QIAamp blood kit (Qiagen, Hilden, Germany) to extract the DNA from the residues. A column chromatography procedure was used in a 96-well format and DNA was eluted in a volume of 30 μL. Controls of fresh blood from anonymous individuals, water, and blank samples were used and processed simultaneously with other samples throughout the laboratory protocols to assess contamination and other methodological issues.

2.2 PCR amplification

PCR was carried out separately for each STR marker since complex optimization and interpretation issues [13, 18] resulted in discordant alleles during multiplex amplification at 0.1 ng DNA as compared to the standard 1 ng DNA amplifications. However, consistent PCR products without allele drop-in or drop-out were observed at 0.1 to 10 ng while STRs performed poorly at 0.01 ng and below in single-plex reactions (data not shown). Since more

stutter at the lowest DNA amounts with reliable PCR was observed at 0.1 ng, the algorithm was developed for amplifications undertaken with these conditions. To develop the algorithm for mixture analysis, PCR was performed with 0.1 ng genomic DNA (or 5 μL eluted DNA from simulated samples) and a PCR mix consisting 0.5 mM of each of the primers (forward and reverse), 250 μM of each nucleotides (dNTP), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, and 3.5 U of Amplitaq Gold (Applied Biosystems (AB), Foster City, CA). Oligonucleotide primers for STRs W, X, and Z were obtained from Genosys (Woodlands, TX) with a gttt- tail at the 5'-end of the forward primer and a fluorescent dye attached to the 5'-end of the reverse primer. The primer sequences are identical to those previously described [8]:

W: gtttgcagggaggaggttgtgtatt and fam-actgtagatgtggctgcaaaataata,

X: gtttgagtgacaaagcaagaccctgt and vic-tggtgtgaagcagagggaa,

Z: gtttagctttggtcctgagagtcctt and fam-caacctgggcaaagcctag.

The PCR reagents and DNA were mixed in 384-well plates and amplification was performed in GeneAmp PCR system 9700 (AB). Thermal cycling conditions were as follows: 94°C for 4 min followed by ten cycles of 94°C for 30 s, 65°C for 30 s (60°C for Z), and 72°C for 30 s; 94°C for 30 s; annealing temperature step-down [26] of 0.5°C *per* cycle (65–55°C for W and X and 60–50°C for Z), 72°C for 30 s; five cycles of 94°C for 30 s, 55°C for 30 s (50°C for Z), and 72°C for 30 s; 72°C for 45 min.

2.3 Fragment analysis

Amplified products (3 μL) were combined with 9 μL of formamide and 6 fmol of an internal lane size standard (GS500, AB) and denatured at 95° for 2 min. The fluorescently tagged alleles were sized by CE (AB 3100 DNA sequencer). AB 3100 has been reported to demonstrate great precision, sizing accuracy, and produce superior data quality, including peak morphology and baseline noise [27], which are all essential for mixture analysis.

The use of automated capillary sequencer technology enables the collection of both qualitative and quantitative data (peak area and peak height) that can be explored in mixture analysis, especially to distinguish stutters from true alleles. The results of DNA fragment separation are represented as an electropherogram in relative fluorescent unit (rfu) measurements (Fig. 1). Fragment analyses were carried out with Genescan 3.6 and Genotyper 3.7 software (AB) that inferred alleles corresponding to peaks.

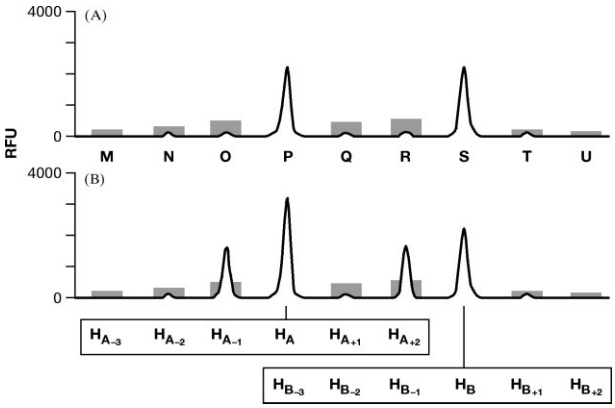

Figure 1. Electropherograms showing the conceptual basis of nonmixture (A) and mixture (B) allelic patterns (amount of stutter product shown is accentuated relative to that normally seen for clarity of presentation). Highest two peaks are located in positions P and S. To develop the algorithm for mixture analysis, the observed and expected stutter heights at (-3, -2, -1, +1, and +2) positions where stutter is observed relative to the highest two peaks are labeled. Peaks greater than the expected stutter heights at each allelic position (shown in gray boxes) are assigned as true alleles. There is no third true allele in (A), but there are third and fourth alleles at positions O and R in (B) indicating mixture.

The allele size and height of the eight highest peaks for each sample was examined to assess the characteristics of true allelic patterns, stutters in terms of peak heights, and their relative positions in the mixture analysis. Since the detection of fluorescent PCR products has thresholds in the AB 3100 genetic analyzer system, we limited the peak height of alleles to a maximum of 8000 by rerunning the samples at lower concentrations (*e.g.*, 1/10th and 1/100th) while aiming for peak heights of at least 2000. For each STR, allele peaks were labeled, sized in number of bp, and binned with an internal algorithm to represent the allele size to the nearest single base integer value.

2.4 Analysis and stutter algorithm

A matrix for three previously defined STRs (W, X, and Z) based on the height ratio of the stutter products to the true allele among the homozygotes was first developed. We developed our algorithm around the two highest peaks in the sample where the presence of a third allele indicated DNA from multiple individuals. Since rare abnormalities with three distinct alleles (0.04%) have been reported in forensic markers [28], we screened the three markers in 382 individuals from four different ethnic groups [8] and conducted additional characterization in another population [29], but did not find any trisomies. Epidemiological studies applying the STR-based mixture detection

approach are likely to be interested in detecting sharing at levels of 5% or more, far exceeding (by more than a hundred-fold) any potential confounding from trisomy. Peak heights and peak areas, which are highly correlated, have been previously used as parameters for quantifying and assigning true alleles [13, 22, 24]. Based on the peak heights, homozygotes (Table 1) were defined as those samples where the ratio of the second highest allele to the highest allele was less than 60% [22]. To develop the stutter algorithm, the evaluation thresholds of the peaks were set at a minimum height of 50 rfu to account for all possible nonallelic products. Based on peak heights (H), the array of stutter ratios (Sr_i) for all pseudo-alleles that were three positions smaller ($i = -3, -2, -1$) and two positions larger ($i = +1, +2$) relative to the highest homozygote allele ($i = 0$) were estimated as follows:

$$Sr_i = \frac{H_i}{H_0} \quad (1)$$

The largest ratio observed at each allele position in the samples was used as the basis for the threshold reference in the matrix. The algorithm was further fine-tuned by increasing the stutter ratios by a fixed fraction (0.10) that we refer to as the outlier capture fraction (OCF) (Table 1).

An algorithm of expected stutter reference height for each sample evaluated was estimated based on the matrix of stutter ratios (Eq. 1) from the homozygotes. Expected stutter reference peak heights relative to the two highest peaks were first estimated independently. (In the case of homozygotes, the highest stutter peak was considered as the second highest peak to estimate the expected stutter.) Assuming additivity, the two heights at each allelic position (i) was summed to calculate the stutter threshold height (T_i) as follows:

$$T_i = H_A\,(Sr_j) + H_B\,(Sr_k) \quad (2)$$

where Sr_j and Sr_k are the reference threshold stutter of the allele at positions j and k relative to the highest (H_A) and second highest (H_B) peaks. Each peak that exceeded the stutter threshold, T_i, at each allelic position was considered a true allele and the detection of a third allele indicated mixture. In considering multiple loci, the presence of a third allele at any one of them was taken as evidence of a mixture. In Fig. 1, alleles at positions P and S are the two highest peaks (with heights H_A and H_B, respectively). Expected stutter reference height with respect to each true allele was estimated independently and the total was calculated for each stutter position. In Fig. 1A, all observed peaks are below the stutter reference peaks (gray boxes) whereas in Fig. 1B, alleles at positions O and R are above the stutter reference indicating that they are true alleles from a mixture. Previously, we have detected mixture of known samples with as low as 95:5 ratios with these markers [8].

The stutter algorithm was first applied to the known heterozygote samples to confirm that they were not labeled as being mixtures and then to the simulated samples. The lower analytical peak height thresholds for the simulated samples were set at a minimum of 150 rfu to avoid background noise in mixtures. Based on simulated samples, the standard epidemiological measures of validity, sensitivity, and specificity were determined for each of the

Table 1. Median, range, and threshold[a)] of allelic stutter (%) for W, X, and Z STRs[b)]

Stutter position	W (N = 64)[c)]			X (N = 68)[c)]			Z (N = 78)[c)]		
	Median	Range	Thres.	Median	Range	Thres.	Median	Range	Thres.
−3	1.8	1.3–3.3	3.63	1.6	1.1–3.6	3.96	1.7	1.1–3.4	3.74
−2	3.6	2.2–6.5	7.15	4.1	2.7–7.4	8.14	3.1	1.9–5.3	5.83
−1	8.7	5.8–12.4	13.64	7.6	4.2–9.7	10.67	7.9	4.8–11.2	12.32
+1	3.5	1.6–7.6	8.36	3.8	1.9–6.9	7.59	2.9	2.0–4.6	5.06
+2	1.9	1.2–3.7	4.07	2.0	1.2–4.7	5.17	1.6	1.1–3.2	3.52

a) Threshold (Thres.) = Maximum stutter + 10% of maximum stutter determined from homozygotes. Homozygotes were defined as those samples where the second highest peak was less than 60% of the highest peak.

b) For convenience abbreviated names are used to refer to the STRs, when they are actually W (D1S71752), X (D4S18742), and Z (D2S17842) according to the GDB database (previously reported STR-Y [8] is actually D12S18547).

c) Alternatively, thresholds can be based on the mean and SD like in our previous work [8]. Considering the −1 position (which has the most data), the suggested maximum observed +10% is approximately the mean plus five SDs for these three loci (data not shown). Other positions (-3, -2, +1, and +2) had less detectable stutter peak heights (below <50 rfu). Applying a distribution-free maximum observed plus 10% threshold utilizes our best knowledge as to the range of values at all of the stutter positions. Collecting more data on homozygotes or applying a more sensitive assay methodology could alter the best method for determining the threshold of the overall sharing detection strategy.

three STRs individually and combined. Here, sensitivity measures the ability to identify correctly mixture samples and specificity measures the ability to identify correctly nonmixture samples.

3 Results

The validity of the algorithm was first tested on a known set of heterozygotes (n_w = 302, n_x = 298, n_z = 288) from STRs amplified using purified genomic DNA. The algorithm identified all heterozygote samples as containing single genomes resulting in 100% specificity. In our validation study, 96 simulated syringe washes (58 mixtures and 38 nonmixtures) were tested in blinded genotypic diagnosis. Using the initial reference algorithm of stutter threshold (T_i), we characterized mixture and nonmixture syringe samples that yielded the initial sensitivity of 77.5, 82.7, and 58% and the specificity of 100, 97.4, and 100% for W, X, and Z, respectively (Fig. 2). The combined use of W and X STRs yielded the sensitivity and specificity of 87.9 and 97.4%; W and Z combined yielded 81.0 and 100%; X and Z combined yielded 86.2 and 97.4%. When all three STRs were analyzed in a sample, a sensitivity of 91.4% and specificity of 97.4% was achieved.

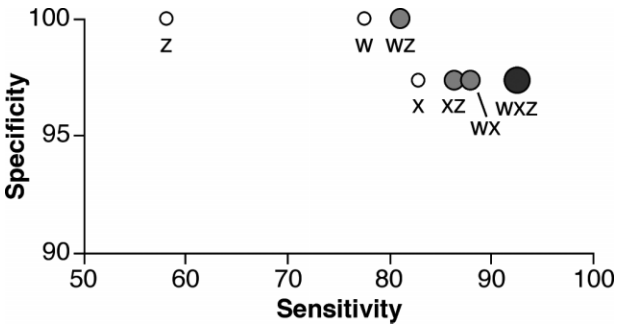

Figure 2. Sensitivity and specificity of the three pentanucleotide STRs (W, X, and Z) individually and collectively. Analyzed using the algorithm we developed and describe in the text with an OCF of 10%, the most favorable sensitivity and specificity was seen with all three loci examined together (WXZ). Some variance in the estimates is due to the inherent limited allelic diversity in examining mixtures made from just 12 individuals. Decrease in specificity for X and its combinations was due to the misidentification of 1 of the 38 nonmixed samples. Overall, sensitivity is highly dependent on the probability of mixture determination (p_{MD}) (r^2 = 0.84 using European p_{MD}, analyses not shown) which is significant if the related values were independent (p = 0.003, 5 d.f.), and in the worst case trends toward significance if analyzed as derived from just three independent loci (p = 0.12, 1 d.f.). Tuning the mixture analysis with a less stringent algorithm using just the maximal stutter peak ratios (0% OCF), sensitivities of 81.1, 87.9, and 63.8% and specificities of 92.1, 89.5, and 94.7% for W, X, and Z individually and when analyzed together 94.8% sensitivity and 89.5% specificity was observed.

4 Discussion

In forensic or epidemiologic cases where the DNA samples are scarce, increasing the number of amplification cycles or using alternate approaches such as touchdown methods can increase the amount of DNA products available for analyses [26], but can also result in the amplification of stutters even with assays to robust STRs such as pentanucleotides. The benefit of maximizing sensitivity by increasing the PCR cycles has to be balanced against the quality of analytical allelic data. However, this can cause two major stochastic variations in amplification: (i) the phenomenon of allele drop-out when one allele is not efficiently amplified or (ii) generation of artifactual PCR stutter products from true alleles. When performing STR mixture analyses, generally other problems related to STR typing such as laboratory-based contamination and nonspecific artifacts, pull-ups, chromosomal abnormalities, A-addition peaks, blobs, spikes, and other software related issues [12, 13] cannot be completely avoided. The pentanucleotide STRs used in the study were chosen from a set of 36 loci as those with the least stutter and best PCR characteristics (no drop-in or drop-out products) using 0.1 ng of DNA [8]. While issues of low copy number (LCN) amplification resulting in elevated stutter peaks cannot be ruled out, the high cutoffs chosen for −1 position (10.6–13.6%, Table 1) are meant to avoid falsely calling stutter peaks as true alleles. We have developed and assessed a quantitative algorithmic method to correct for stutter when evaluating samples in mixture analyses.

Previously, in the laboratory simulated samples, we used a single tetra-nucleotide STR, D6S502 (nomenclature since corrected to D8S1179, http://www.cstl.nist.gov/biotech/strbase/seq_info.htm) to distinguish between single-use and multi-use syringes with 68% sensitivity and 100% specificity and satisfactory agreement (κ = 0.6) [25]. We expanded upon this technique and improved it by using three pentanucleotide STRs instead of one tetranucleotide and developing an algorithm for evaluating a ladder of stutter positions. With an analysis of three loci for 58 mixtures and 38 nonmixtures from 12 individuals, the estimates of sensitivity and specificity are approximate. More precise estimates would be obtained by examining more loci, individuals, and samples. In particular, more stringent algorithmic criteria requiring evidence for mixtures from multiple loci provides a promising course for refining mixture analysis.

At individual loci, the algorithm conservatively minimizes the identification of false mixtures (maximum specificity) at some expense of identifying true mixtures (sensitivity) at low concentrations or differential proportions. By using relatively large stutter ratios at each locus, a higher specificity is achieved with the aim of identifying correctly all nonmixtures by not misclassifying stutter peaks as true alleles. Consequently, when multiple loci are collectively used, a third allele at any one locus is considered a true mixture increasing sensitivity at a cost to specificity since the chances of falsely calling a stutter peak an allele increases (Fig. 2). In alternative situations, to achieve higher sensitivity for each locus, a lower threshold (*e.g.*, without OCF) can be used at each relative stutter position, but this will adversely affect specificity (Fig. 2). We used an OCF of 10% to develop the algorithm and applied it to the subsequent analysis. The OCF should be fine-tuned as required for the purpose of the study question. In practice, we suggest a balance where the OCF is adjusted lower when the stutter ratio estimates are from a large number of known samples and increased for larger numbers of unknown mixture determinations.

Our success of identifying mixtures and nonmixtures must be considered in light of the probability of detecting a mixture (p_{MD}) [8]. While the specificity is not affected by the p_{MD}, the sensitivity observed needs to be carefully interpreted. The sensitivity depends on the p_{MD} – the chance of correctly identifying multiple genomes in a mixture [8]. Suppose, the STR algorithm is optimal (100%) where all the stutters and other artifacts are all accounted for, but the p_{MD} is only 70%, then the net sensitivity would be 70%. The algorithm that we have developed works best when the p_{MD} is maximal and multiple STRs are considered. The three STRs that we have used, W, X, and Z have the p_{MD} ranging from 74 to 80%, 71 to 79%, and 57 to 74%, respectively; combination of any two ranged from 88.8 to 96.4% and collectively with all three STRs from 97.1 to 99.1% in different ethnic populations (the lower p_{MD} of STR Z is due to its relatively lower levels of heterozygosity) [8]. The theoretical dependence of sensitivity on the probability of mixture determination is supported by the correlation seen between them with these data (Fig. 2). By analysis of three or more loci, such that $p_{MD} \approx 1$, sensitivity is not compromised by a dependence on genetic detection capabilities.

The quantitative examination of alleles relative to stutter is very useful in interpreting possible mixtures with STRs since stutter cannot be avoided with PCR amplification–especially with minute amount of or degraded DNA. The algorithm we describe accounts for a ladder of stuttering and yields high sensitivity and specificity using just three STRs. The development of this method and the application of the algorithm we present are promising advancements to the analysis of unknown biological mixtures. Beyond basic genetic and forensic studies, the application of valid mixture analysis methods can be extended to important research questions of epidemiological interest.

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