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TECHNICAL NOTE

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Accurate STR Allele Designations at the FGA and vWA Loci Despite Primer Site Polymorphisms

ABSTRACT: Polymerase chain reaction (PCR)-based STR DNA typing systems are used extensively in the field of human identification. Under optimal PCR conditions, the amplicon yield from both alleles of an STR locus is expected to be approximately equivalent. However, it is reasonable to expect that rare genomic sequence polymorphisms will co-localize with well-designed primer sets and induce allele imbalance or "dropouts". Two samples were identified in the course of genotyping thousands of individuals with AmpF/STR® Profiler Plus™ that showed strong disparity in amplitude peak height of heterozygous peaks at the loci vWA and FGA. These samples were reamplified at reduced annealing temperature in an attempt to balance the peak heights. Nucleotide sequencing documented polymorphisms at the PCR primer binding sites of the affected alleles. The results indicate that reducing the annealing temperature to improve primer-binding efficiency at the mismatch and employing an alternative multiplex enhanced the data from both samples. Reducing annealing temperatures could provide a simple general solution to improving data quality for samples where polymorphisms are suspected to cause allele imbalance. Finally, we report on additional polymorphisms surrounding the vWA locus in a genetically diverse population.

KEYWORDS: forensic science, DNA typing, single nucleotide polymorphism, FGA, vWA, short tandem repeat, allele dropout

In recent years, the development of PCR-based STR DNA typing has provided the field of human identification with highly discriminating, sensitive, and cost-effective tools (1–3). These techniques are enhanced further by the development of multiplexes that co-amplify numerous STR loci in a single tube assay, which largely stemmed from the requirement of forensic casework to derive maximum genotypic information with minimal sample consumption. The combination of STR multiplexes with laboratory automation has enabled the implementation of large-scale criminal offender genotype data banking initiatives worldwide (4). Integrating both crime scene and criminal offender genotypic data into databases of criminal identification information harnesses the full potential of these investigative tools.

An important criterion in the design of PCR systems for human identification includes avoidance of common polymorphisms at primer annealing sites. This is essential to ensure that both alleles from heterozygous individuals will be represented in the amplified fragments. However, despite extensive polymorphism searching, it is reasonable to expect that rare genomic sequence variants will co-localize with primer binding sites (5–8). The vast majority of forensic samples in the United States are processed with AmpF/STR® Profiler Plus™/AmpF/STR® COfiler™ or AmpF/STR® Identifiler™ from Applied Biosystems or the PowerPlex 16® kit from Promega. These kits employ different primers and rare instances of discordance attributable to polymorphisms have been described (9–12).

We describe single nucleotide polymorphisms (SNPs) that localized to STR primer binding sites of two loci, FGA and vWA, of AmpF/STR® Profiler Plus™ that caused variable and imbalanced allele amplitudes. Reduced annealing temperatures during thermal cycling mitigated this problem and may represent a technique with wide application to improve poor data quality suspected to result from polymorphisms. We also report on the prevalence of three other SNPs at vWA and FGA as determined by sequencing a genetically diverse set of samples.

Material and Methods

Deoxyribonucleic Acid Isolation

Genomic DNA (gDNA) was isolated from bloodstains on FTA paper or from peripheral blood mononuclear cells with the QIAamp® blood kit (Qiagen, Chatsworth, CA). Genomic DNA was quantified directly using fluorometry with a PicoGreen®-based assay (Molecular Probes, Eugene, OR).

STR Genotyping

Multiplex genotyping was performed with AmpF/STR® Profiler Plus™ (Applied Biosystems, Foster City, CA, USA) and PowerPlex 16® (a pre-production evaluation kit kindly provided by Promega, Madison, WI). All samples and controls were processed in triplicate using the manufacturer's suggested PCR thermal-cycling profile

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and with annealing temperatures reduced to 55°C and 50°C for both kits.

Sequencing

Deoxyribonucleic acid sequences were determined from M13 tailed PCR fragments (M13 tails sequences: 5′-GTTTTCCCAGTCACGACG for the forward primers and 5′-AGGAAACAGCTATGACCAT for the reverse primers) using automated instruments (BigDye™ primer chemistry and ABI Prism 377, Applied Biosystems). The following primers were used to generate fragments for sequencing (numeric base pair designations for the FGA and vWA loci correspond to GenBank #M64982, and #M25858, respectively, top strand designated forward):

FGA-F, 5′-GGACATCTTAACTGGCATTCATGGAA (bases 2765 → 2790);
FGA-R, 5′-CTCAGATCCTCTGACACTCGGTT (bases 3094 → 3072);
vWA-F, 5′-CTCCTCAGACTGATCCTATAAGGTA (bases 1547 → 1571);
vWA-R, 5′-AGAGATAGGATAGATGATAGATACAAAGGA (bases 1874 → 1845).

Nomenclature

Polymorphisms identified in these experiments were designated using the locus name (FGA or vWA), the number of base pairs upstream (−) of the first base of the STR or downstream (+) from the final base of the STR of interest (orientation based on top strand of GenBank #M64982, and #M25858, respectively), and the wildtype to variant base change (X → Y).

Results

Identification of Samples that Display Disproportionate Heterozygous Fragment Amplitudes

Initially, samples were identified that displayed strong differences in amplitudes for peaks from heterozygous markers. Furthermore, the heterozygote peak ratios would vary significantly on reprocessing of each sample. In sample 1, the FGA allele with 21 repeats showed 14% of the fluorescent amplitude generated by the allele with 24 repeats (Fig. 1*a*, 59°C). Likewise, in sample 2, the vWA allele with 19 repeats generated only 17% of the signal obtained from the allele with 17 repeats (Fig. 1*b*, 59°C). For both samples, reduced annealing temperatures enhanced the ratio of signals from

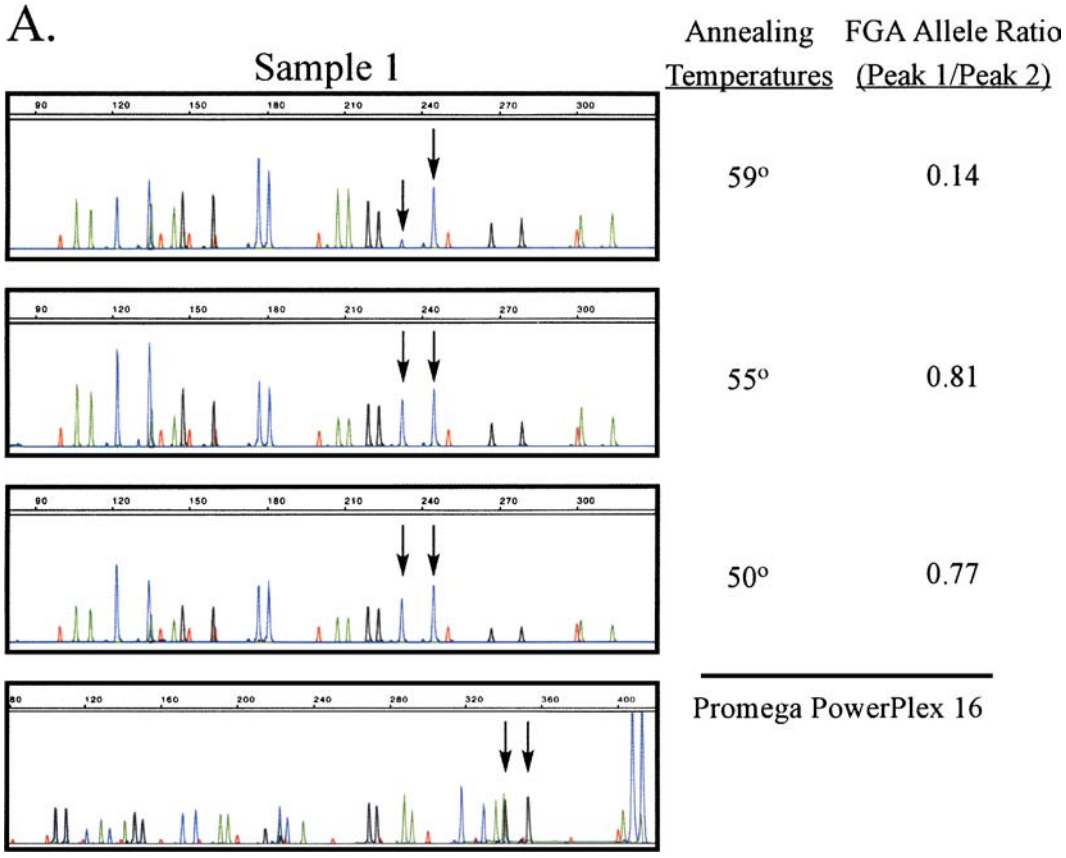

FIG. 1—*STR profiles of samples 1 and 2 processed with commercial STR kits. (A) Amplification of sample 1 with AmpFlSTR® Profiler Plus™ (top panel) resulted in unequal fragment amplitudes for the heterozygous FGA alleles of 21 and 24 repeats (fragment sizes 232 and 244, as identified with arrows). Processing this DNA using reduced annealing temperatures improved the signal from the 21 repeats allele (second and third panels). (B) Likewise, disproportionate amplitudes from fragments representing vWA alleles 17 and 19 were detected (first panel, arrows indicate fragments at 180 and 188 base pairs). The fragment amplitude of the 19 repeats allele increased to 47% of the amplitude of the 17 repeat when the sample was processed using reduced annealing temperatures (second panel). In addition, signal of the JOE labeled, heterozygous D21S11 marker (green peaks at approximately 210 bases in sample 1, and between 210 and 240 bases in sample 2) decreased as temperature was reduced for both samples (top three panels). Amplification with a second multiplex kit (PowerPlex 16®) produced heterozygous peaks for both samples when processed using the manufacturer's thermal cycling conditions (annealing temperature of 60° C). Sample 1 produced fragments with similar amplitudes for the FGA marker (Sample 1, fourth panel). As with the Profiler Plus™ system, vWA displayed unequal amplitudes of the two alleles due to some overlap of the reverse priming sites between the kits (sample 2, fourth panel). Processing the sample with reduced annealing temperature restored allele ratios in the PowerPlex 16® kit (Sample 2, fifth panel).*

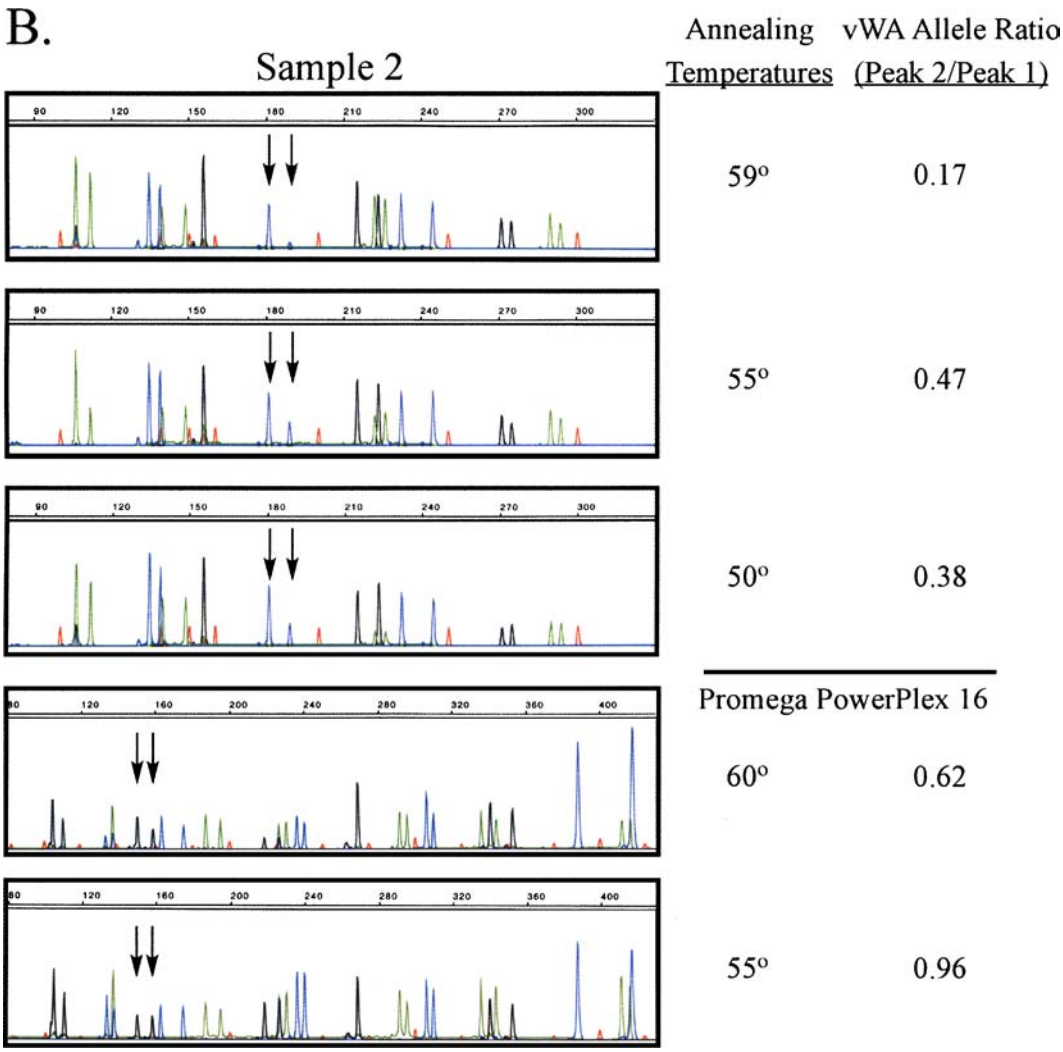

FIG. 1—*(continued)*

the alleles, with 55°C showing the greatest improvement – from 0.14 → 0.81 for sample 1 and 0.17 → 0.47 for sample 2 (Fig. 1). Additionally, no spurious fragments resulting from decreased priming specificity at the lower annealing temperatures were observed for any locus. However, D21S11 amplitudes decreased (on average 45% and 63% at annealing temperatures of 55°C and 50°C, respectively) in relation to the other peaks of the multiplex as the annealing temperature was reduced in both samples. Superficially, this may seem counterintuitive because reduced annealing temperatures can induce increased product as is the case with the mismatched primers at the polymorphic sites. However, it is known that a variety of factors including the cycling profile can influence preferential amplification in multiplexed PCR (13). Despite reduced signals, all peak amplitudes remained within interpretable levels.

Concordance Analysis

For comparison, DNA samples 1 and 2 were genotyped using the PowerPlex 16® multiplex system (Fig. 1*a* and *b*, fourth panel). These results were compared with those obtained from the AmpF*l*STR® Profiler Plus™ kit. There was no allele drop-out of FGA in sample 1 when processed with PowerPlex 16®. However, in sample 2, the 19 repeats vWA allele amplitude was 62% of the 17 repeats allele (Fig. 1*b*, fourth panel). Reducing the annealing temperature of the PowerPlex 16® reaction to 55°C (from the manufacturer recommended 60°C) caused the heterozygote vWA allele peaks to approximate equal amplitudes (Fig. 1*b*, fifth panel).

Sequence Analysis

The loss of signal from one allele in heterozygous samples that could be corrected with reduced annealing temperatures during PCR suggested that polymorphisms at priming sites might exist. This hypothesis was confirmed by nucleotide sequencing. Sample 1 contained the polymorphic nucleotide FGA-59 G → A (Fig. 2*a*) and sample 2 contained vWA + 6 C → T (Fig. 2*b*). We confirmed from the manufacturer that these polymorphisms were coincident with primer binding sites (personal communication, Applied Biosystems). Also, it was confirmed that this vWA polymorphism was located in the primer-binding site in PowerPlex 16® (personal communication, Promega).

Nucleotide Polymorphisms at the FGA and vWA Loci

Ninety-six genetically diverse DNA samples were sequenced to identify common polymorphisms at the FGA and vWA loci (96 anonymous samples selected for diverse SNP haplotype pairs at

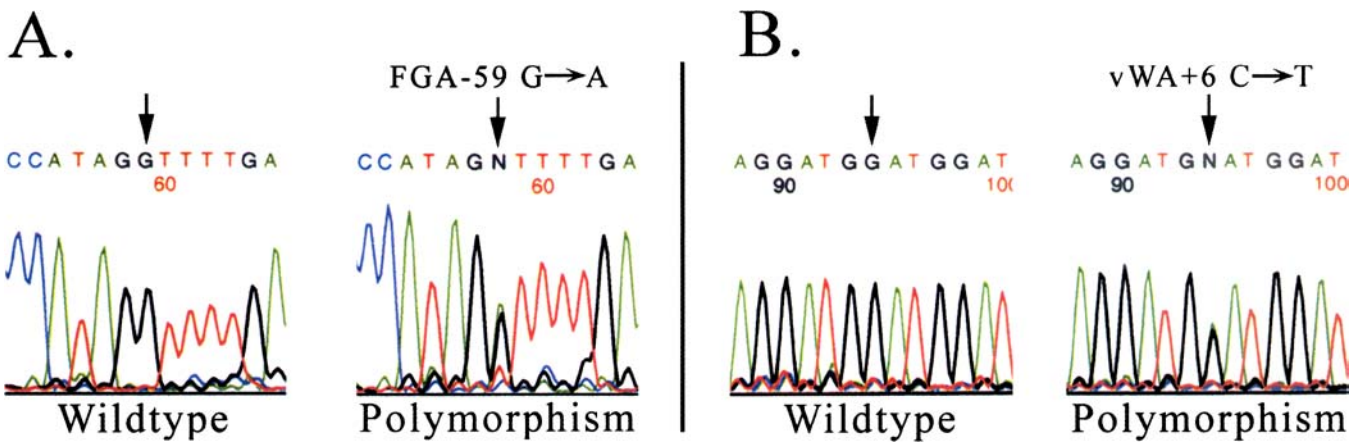

FIG. 2—*Priming site polymorphisms detected in the regions flanking the FGA and vWA loci. (A) Nucleotide sequence of DNA from sample 1 revealed a heterozygous single base change of G → A 59 nucleotides upstream of the FGA repeat. (B) DNA sequencing revealed that sample 2 was heterozygous for the polymorphism vWA + 6 C → T (reverse strand shown).*

TABLE 1—*Single Nucleotide polymorphisms identified at the vWA locus. Ninety-six genetically diverse DNAs were sequenced and screened for SNPs. Polymorphisms were designated as explained in Materials and Methods.*

Polymorphism	Allele Frequency
vWA-90 A → G	5/192
vWA-77 G → A	11/192
vWA-72 T → A	4/192

the *BRCA1* locus). Additional occurrences of the variants FGA-59 G → A and vWA + 6 C → T were not found in this sample set. Considerable sequence diversity was detected in vWA where multiple occurrences for each of three SNPs were detected (Table 1). In addition, various single nucleotide base changes were identified within the tetra-nucleotide repeats of the vWA locus (data not shown). No additional SNPs were identified at the FGA locus.

Discussion

Deoxyribonucleic acid samples that displayed markedly preferential amplification of one allele within the STR markers FGA and vWA were identified using reagents commonly employed in forensic laboratories. These differences in the signals obtained for the two alleles in these heterozygotes could result in their erroneous designation as homozygotes. In both cases, the problems resulted from polymorphisms in the regions adjacent to the STRs that reside at the annealing sites for primers.

In these samples, a thermal cycling protocol with reduced annealing temperatures diminished the competitive disadvantage during amplification at the mismatched allele and improved data quality. Although a previous attempt to restore amplification of a vWA allele by this method proved unsuccessful for Alves et al. (12), our data demonstrate that this approach may have general application to samples suspected to contain polymorphisms, regardless of the multiplex kit employed. Ultimately, the position of the mismatch within the primer is almost certainly an important determinant for success with this approach. Polymorphisms closer to the 3′ end have more impact on PCR efficiency.

Several instances of dropout have been published for different vWA alleles. In some of these reports, the presence of polymorphisms at priming sites was inferred from concordance data between multiplexes (6,9,10) while in others, polymorphisms were confirmed by DNA sequencing (7,12,14). The vWA primer binding site polymorphism identified here is novel and resides three nucleotides from the 3′ end of the downstream Profiler Plus™ primer site at position 1768 (GenBank sequence M25858). This SNP is adjacent to a more prevalent vWA SNP (vWA + 7 C → T) characterized in Lazaruk et al. (14). Additionally, two SNPs have been characterized in the region of the forward primer binding site. Alves et al. (12) reported the SNP vWA-52 A → T. Although the relationship of this SNP to the primer sequence is unclear, their failure to improve allelic balance through reduced annealing temperatures suggests that it resides near the 3′ end of the priming site. The location of the second SNP, a T → A substitution identified by Walsh (7) is not provided, but its position relative to the primer binding site is placed at the penultimate 3′ base.

The polymorphism search performed in this study confirms that the sequence surrounding the vWA repeats is highly polymorphic and provides additional results on genetic variants. These SNPs identified in the 96 diverse DNAs do not coincide with the primer binding sites for PowerPlex 16® (personal communication, Promega). Their relationship to the AmpF*l*STR® Profiler Plus™ vWA primers is unclear because the complete sequence of the primers is not published. No additional polymorphisms were identified at the FGA locus in this sample set. This result indicates that fewer SNPs exist adjacent to FGA than at vWA and concurs with reported findings (10).

The discovery of novel polymorphisms at STR loci is expected. In fact, the low incidence of allele dropouts attributable to SNPs that coincide with priming sites demonstrates that the multiplexes developed for human identification are well-designed. However, it is beneficial to understand genetic variation at these loci to support the development of new multiplex systems. Also, from a practical standpoint, this information can support the resolution of data quality issues within laboratories.

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