TECHNICAL NOTE

Jeanette M. Wallin,[1] M.P.H.; Martin R. Buoncristiani,[2] M.P.H.; Katherine D. Lazaruk,[1] Ph.D.; Nicola Fildes,[1] B.S.; Cydne L. Holt,[1] Ph.D.; and P. Sean Walsh,[1] M.P.H.

TWGDAM Validation of the AmpFISTR™ Blue PCR Amplification Kit for Forensic Casework Analysis*

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ABSTRACT: Studies were performed as recommended by the Technical Working Group on DNA Analysis Methods (TWGDAM) committee to validate the AmpFISTR Blue PCR Amplification Kit for forensic casework applications. The kit coamplifies the tetranucleotide short tandem repeat (STR) loci D3S1358, vWA, and FGA. The dye-labeled amplification products were electrophoresed and detected directly using the ABI PRISM™ 377 DNA Sequencer or the 310 Genetic Analyzer. CEPH family studies demonstrated Mendelian inheritance of these loci and probability of identity values from population studies were 1/4,830 (African-American), 1/5,479 (U.S. Caucasian), and 1/3,443 (U.S. West Coast Hispanic). In all studies examining different body tissues and fluids, the expected genotypes were observed. Studies to determine and test the PCR reagent components and thermal cycling parameters demonstrated specificity, sensitivity, and balance over a wide range of conditions. Reliable results were obtained from DNA quantities as low as 0.25 ng. A variety of environmental studies were performed, as forensic samples are often exposed to different environmental conditions and substances which may degrade DNA or inhibit the amplification process. Highly degraded samples demonstrated that FGA was the first locus to become undetectable, followed by vWA, and then D3S1358; this is the expected pattern according to locus size. In studies of PCR inhibition, the pattern in which the loci became undetectable was different; FGA was the first locus to become undetectable, followed by D3S1358, and then vWA. Single versus multiple locus amplifications revealed no benefit to single locus analysis, even in cases of degradation or inhibition. The occurrence of preferential amplification was very rare, particularly in noncompromised, unmixed samples. Artifact peaks were not observed in any instance. Mixture studies confirmed the ability to detect mixed DNA samples and included the characterization of stutter and peak height ratios; the limit of detection was 1:10 for 1 ng total genomic DNA and 1:30 for 5 ng. DNA extracted from nonprobative case evidence was successfully amplified and genotyped. All such studies indicate that the AmpFISTR Blue PCR Amplification Kit will reproducibly yield specific and sensitive results.

KEYWORDS: forensic science, DNA typing, AmpFISTR Blue, short tandem repeat, D3S1358, vWA, FGA, validation, polymerase chain reaction

[1]PE Applied Biosystems, Foster City, CA.
[2]California Department of Justice DNA Laboratory, Berkeley, CA.

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The forensic community has been applying techniques of molecular biology to the analysis of DNA extracted from casework samples since the mid 1980's. Initially, restriction fragment length polymorphism (RFLP) analysis (1–3) was implemented. While providing excellent discrimination and mixture interpretation, RFLP analysis is time consuming, requires a relatively large amount (at least 20-50 nanograms) of high molecular weight DNA, and is unable to provide discrete alleles. Polymerase chain reaction (PCR) based assays were subsequently developed (4,5), which address these disadvantages. Currently, the most popular PCR-based systems used for forensic applications are the AmpliType® reverse dot-blot typing kits (6–11) and the AmpliFLP™ D1S80 fragment sizing kit (12–14). While these assays address the shortcomings of RFLP analysis, there are limitations to their use. Mainly, the discrimination potential of the reverse dot-blot typing kits is greatly reduced relative to that with RFLP, making it more difficult to exculpate suspects and resolve mixtures. With regard to the D1S80 kit, the discrimination potential of this locus can be very informative yet the allele size range is rather large, resulting in potential for preferential amplification.

Ideally, forensic assays should include high discrimination power, maximal mixture interpretation capability, high sensitivity, ability to obtain results from degraded DNA, minimal risk of preferential amplification, discrete alleles, and high throughout potential. In an attempt to better achieve these desirable features, the forensic community has recently been exploring the utility of short tandem repeat (STR) polymorphic loci (15–20). STR loci contain reiterated nucleotide sequences 2 to 7 bases in length, are present in great abundance throughout the human genome, and exhibit a high degree of polymorphism in humans. STR loci are similar to RFLP loci in that the alleles vary in repeat number, and therefore length, among individuals. The STR size ranges, however, are smaller and the alleles are discrete. Because the overall size range of STR repeat regions is relatively small, primers that delimit these regions may be designed to produce short PCR products (100 to 350 base pairs (bp)). This makes it possible to obtain results from degraded DNA samples (21,22) and minimizes the chance for preferential amplification.

As with other PCR-based tests, the analysis of STR loci requires only small amounts of DNA. By choosing STR loci with at least five common alleles, mixture detection is improved relative to that with loci having only two or three alleles, as with several of the reverse dot-blot loci. While STR loci contain fewer alleles than RFLP loci, it is possible to approach discrimination levels similar to those of RFLP analysis by simultaneously amplifying (multiplexing) several STR loci. The coamplification of several STR loci in one tube provides the additional benefits of conserving sample, minimizing chance of contamination, and facilitating minimal manual processing time.

When combined with automated laser-induced fluorescence technology, STR locus amplification systems provide a simple, rapid, and reliable high throughput approach for processing forensic samples. PCR products of STR loci can be easily resolved by size using electrophoresis with high resolution gels (18,23) or capillaries (24–26). By directly detecting the products, further steps, such as hybridizations, are eliminated. In utilizing fluorescent dye-labeling technology, STR loci with overlapping size ranges can be coamplified in a single tube and yet detected individually due to the different characteristic emission spectrum of each dye (27). Dye-labeling technology also facilitates the use of an in-lane size standard which can greatly improve the sizing precision of alleles (26,27).

The AmpFISTR Blue PCR Amplification Kit was developed to provide the forensic community with technology and methodology that combines many of the desirable features of both RFLP analysis and the PCR-based assays. The AmpFISTR Blue PCR Amplification Kit coamplifies three STR loci: D3S1358 (114 to 142 bp; chromosome 3) (28), vWA (157 to 197 bp; chromosome 12) (29,30), and FGA (219 to 267 bp; chromosome 4) (31). Locus specific primers are labeled with the NHS-ester dye 5-FAM, designated as blue on PE Applied Biosystem's instrumentation. The kit contains preformulated PCR reaction mix, AmpliTaq Gold™ DNA Polymerase (32), blended AmpFISTR Blue Primer Set, positive control DNA 9947A (33), allelic ladder, and mineral oil.

To systematically evaluate the reliability of this STR kit for forensic applications, studies as suggested by the Technical Working Group on DNA Analysis Methods (TWGDAM) committee (34) were performed. The TWGDAM committee has published recommended studies as guidelines to evaluate, and thus to ''validate'' the performance of a new DNA assay under consideration for forensic applications. Forensic samples of biological origin are unique because they may be exposed to an infinite variety of environmental elements. Validation is particularly important to empower the forensic analyst to understand how to reliably interpret results from such unique samples.

Materials and Methods

DNA Extraction and Quantitation

Two methods for DNA extraction were used to isolate DNA: organic phenol/chloroform (35,36) and Chelex® (PE Applied Biosystems; 36,37). The organic procedure was used to extract DNA from liquid and stain specimens of blood, semen, and saliva, which were concentrated by Centricon-100 ultrafiltration devices (Amicon, Beverly, MA). The organic procedure was additionally used for DNA extractions from cadaver tissues and recovered bones. DNA from nonprobative sexual assault case vaginal swabs was extracted according to an organic differential extraction protocol (36) and concentrated by Centricon-100 devices. The Chelex extraction method was used in DNA extractions from bloodstains, hair, and buccal cell swabbings for the 4.1.5.1 Standard Specimens Study.

Population database DNA samples were extracted from liquid blood of 195 African Americans, 200 U.S. Caucasians, and 113 U.S. West Coast Hispanics. The African American and Caucasian genomic DNA samples were provided by Laboratory Corporation of America (Research Triangle Park, NC), who collected the liquid blood from unrelated individuals throughout the United States without geographical preference. DNA was extracted by the phenol/chloroform method and ethanol precipitated (35). The liquid blood samples from unrelated Hispanic individuals were collected by the California (CA) Department of Justice DNA Laboratory for the CA convicted offender DNA database program. DNA from these samples was also extracted by the phenol/chloroform method (35,36) and concentrated by Centricon-100 devices. All DNA extracts were quantitated using the QuantiBlot® Human DNA Quantitation Kit (Perkin Elmer, Norwalk, CT) (38).

Nonhuman and Centre d'Etude du Polymorphisme Humain (CEPH) family DNA samples were obtained from and quantitated by BIOS Laboratories, Inc. (New Haven, CT). Degraded DNA was prepared using 0.005 units/microliter (μL) of Deoxyribonuclease I (DNase I; Gibco BRL, Gaithersberg, MD) as previously described (14).

AmpFISTR Blue PCR Amplification

Using the AmpFISTR Blue PCR Amplification Kit reagents and suggested protocol (39), the target amount of 1.0 to 2.5 nanograms (ng) of DNA was amplified in 50 μL reaction volumes. For the inhibition study, hematin (Sigma Chemical Co., St. Louis, MO) was diluted to 1 mM in 0.1 N NaOH and added to the reaction mix at each concentration examined. Samples were amplified in either GeneAmp® Thin-Walled Reaction Tubes in the DNA Thermal Cycler 480 (Perkin Elmer) or MicroAmp® Reaction Tubes with Caps in the GeneAmp PCR Systems 9600 or 2400 (Perkin Elmer). The amplification parameters were those recommended in the AmpFISTR Blue User's Manual (39) and are the same for each thermal cycler: initial denaturation at 95°C for 11 min, followed by 28 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 1 min. A final extension was performed at 60°C for 30 min.

Sample Electrophoresis and Data Analysis

AmpFISTR Blue amplification products were electrophoresed on either the ABI PRISM 377 DNA Sequencer or the ABI PRISM 310 Genetic Analyzer; the 377 DNA Sequencer was the instrument used in each study except where otherwise stated. The 377 DNA Sequencer protocol is outlined in the AmpFISTR Blue User's Manual (39). Briefly, 2 μL of amplified product and 0.5 μL of GeneScan™-350 [ROX] Internal Lane Size Standard (PE Applied Biosystems) were added to a 4.5 μL mixture of dextran blue dye and deionized formamide; the samples were denatured at 95°C for 2 min and 1.5 μL of each sample was loaded on each gel. Gels were poured on 36 cm well-to-read plates, composed of 5% Long Ranger (Pharmacia Biotech Inc., Piscataway, NJ), and run for 2.25 h at 3000 volts. Data was collected using the ABI PRISM 377 Collection software application, version 1.1, with the GeneScan® run module GS 36A-2400 (virtual filter set A). The results were analyzed using the GeneScan Analysis software application, version 2.0.2.

The ABI PRISM 310 Genetic Analyzer protocol used in this study is also outlined in the AmpFISTR Blue User's Manual (39). One μL of amplified product and 1 μL of GeneScan-350 [ROX] Internal Lane Size Standard were added to 24 μL of deionized

formamide and denatured at 95°C for 3 min. The PCR products were injected (5 s) and electrophoresed at 15 kV in Performance Optimized Polymer 4 (POP4™; 2.5 mL syringe). Data were collected using the ABI PRISM 310 Collection software application, version 1.0.2, with the GeneScan run module GS POP4 A (virtual filter set A). The results were analyzed using the 310 GeneScan Analysis software application, version 2.0.2.

Results and Discussion

The following studies are presented according to the format of the TWGDAM guidelines and were performed to validate the AmpFISTR Blue PCR Amplification Kit for forensic applications.

4.1.5.1 Standard Specimens and 4.1.5.4 Reproducibility

In the validation of an amplification system, it is important to evaluate the performance of the system on forensic-like samples, including DNA samples from a variety of body tissues and fluids. The TWGDAM Standard Specimen guideline states that fresh body tissues and fluids, obtained and stored in a controlled manner, should be examined to confirm that DNA isolated from different tissues from the same donor will produce the same genotype result. Similarly, the Reproducibility guideline states that prepared dried stains from donors of known types should accurately and reproducibly produce the same genotype results as those obtained from liquid specimens from the same donors.

To address both the Standard Specimen and Reproducibility guidelines, blood, semen, and saliva were collected from two donors. DNA was immediately extracted from liquid and stain specimens and then stored for approximately one year at −15 to −25°C. For each individual, the genotype results obtained from the liquid and stain specimens of each body fluid and tissue were the same (data not shown).

Additionally, DNA was extracted from bloodstains, buccal cell swabbings, and hair roots of two other donors using the Chelex method. For each individual, the results showed the same genotype from each of the different tissues (data not shown).

Lastly, DNA from different organ tissues of a fresh cadaver was tested since forensic samples often originate from autopsies. DNA was extracted from bone, brain, kidney, liver, and muscle. Once again, the results showed the same genotype for the different tissues (data not shown). Collectively, these studies demonstrate somatic stability of each of the AmpFISTR Blue loci.

4.1.5.2 Consistency

The TWGDAM Consistency guideline suggests the examination of samples from donors of known types to evaluate reproducibility of genetic typing results both within a particular laboratory and among different laboratories. Thus two laboratories, the CA Department of Justice DNA Laboratory and the Florida Department of Law Enforcement Orlando Regional Crime Laboratory, extracted and exchanged ten genomic DNA samples. The instrumentation used included the DNA Thermal Cycler 480 and GeneAmp PCR System 9600 along with 377 DNA Sequencers. The genotype results of all 20 samples were forwarded to PE Applied Biosystems where they were evaluated. Both laboratories obtained the same genotype results for each sample (data not shown).

Reproducibility within the PE Applied Biosystems laboratories has been tested with several control and database samples many times. Each time, the same genotype results have been obtained (data not shown).

4.1.5.3 Population Studies

The TWGDAM Population Studies guideline recommends establishing ''population distribution data in different racial and/or ethnic groups.'' To address this guideline, three populations were examined: African-American, U.S. Caucasian, and U.S. West Coast Hispanic. From the database genotypes, allele frequencies (39,40) and Probability of Identity (P_I) values (Table 1; 41,42) were calculated. The P_I values, the probability that two individuals selected at random will have an identical genotype, for all three loci were 1/4,830 (African-American), 1/5,479 (U.S. Caucasian), and 1/3,443 (U.S. West Coast Hispanic). Thus, the STR loci chosen for the AmpFISTR Blue Kit are extremely informative. Additionally, statistical studies were performed on these population databases and confirmed Hardy-Weinberg equilibrium (manuscript submitted).

4.1.5.5 Mixed Specimen Studies

Forensic DNA samples frequently contain DNA from more than a single source. Thus, the Mixed Specimen Studies are suggested by TWGDAM for the purpose of investigating ''the ability of the system to detect the components of mixed specimens and define the limitations of the system.'' Through such studies, guidelines may be established to assist in both the recognition and interpretation of such samples. To address these issues, the following mixture studies were performed: mixtures of purified DNA samples in defined ratios, examination of peak height ratios of heterozygous alleles within a locus, and determination of the range of stutter percentage for each allele of each locus (the stutter peak is four base pairs shorter than the allele peak; 43).

Mixtures of purified DNA samples in defined ratios were examined for the purpose of qualitatively and quantitatively characterizing major and minor components of such samples. Pairs of DNA samples were mixed together at the following ratios while holding the total amount of input genomic DNA (either 1 ng or 5 ng) constant: 1:1, 1:3, 1:5, 1:10, 1:20, 1:30, 1:40, and 1:50. Following electrophoresis and data analysis, the resulting peak heights were used to calculate ratios of major:minor component alleles at each locus. At a 1:1 and generally a 1:3 mixture, the ratio of the resulting peak heights of the major component alleles to the minor component alleles of each mixture quantitatively reflected the ratio of input genomic DNA. For example, a 1:1 mixture of two genotypes which are both heterozygous at a particular locus and for which none of the alleles are of the same size yielded four peaks of approximately equal height (see the FGA locus of Fig. 1B, panel 1). For mixtures in which the ratio between the two genomic DNA samples was greater than 1:3, such quantitative analysis revealed that relative peak heights for the minor components were often greater than expected. Nonetheless, qualitative interpretations

TABLE 1—*Probability of identity values for the individual and combined AmpFISTR Blue loci.*

Locus	African-American $n = 195$	U.S. Caucasian $n = 200$	U.S. West Coast Hispanic $n = 113$
D3S1358	0.102	0.078	0.103
vWA	0.058	0.065	0.094
FGA	0.035	0.036	0.030
Combined	0.00021	0.00018	0.00029

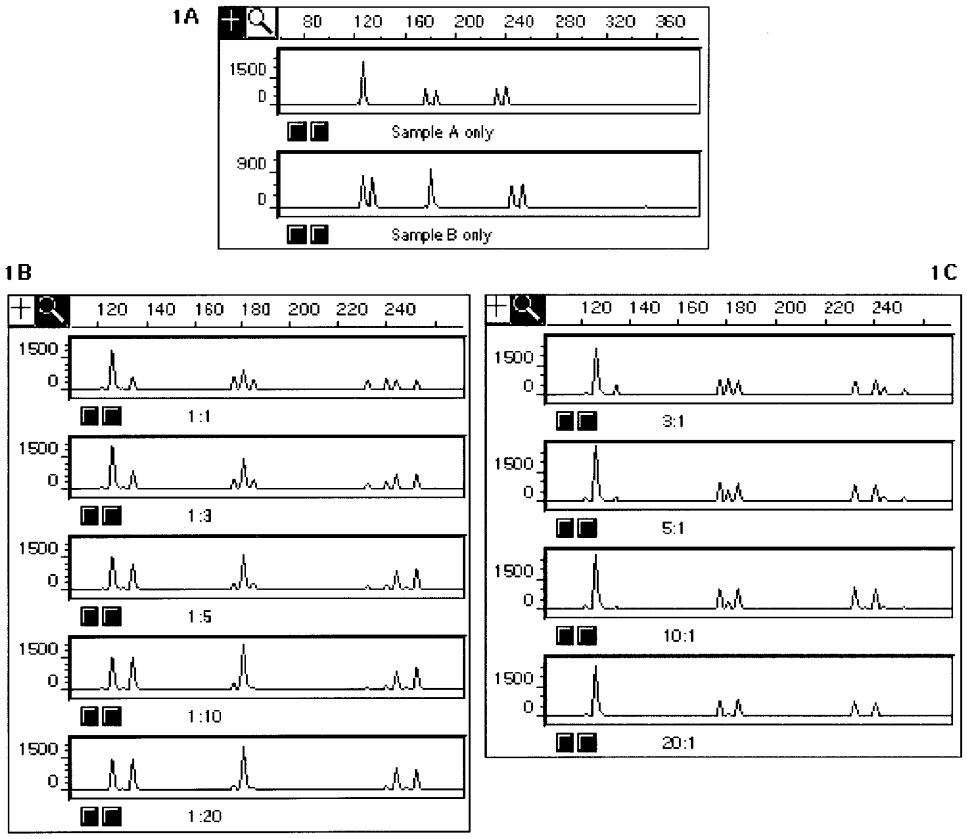

FIG. 1—*DNA samples mixed in defined ratios, run on the 377 DNA Sequencer. Two DNA samples, A and B (see 1A), were mixed together at different ratios prior to amplification. The first panel of 1B (1:1) shows the results of a mixture of equal proportions of samples A and B. In panels 2 to 5 of 1B, sample A is the minor component of the mixtures, and in Fig. 1C, sample B is the minor component of the mixtures. The total amount of DNA amplified in each case was 1 ng. The x-axes indicate base pair size of the PCR products and the y-axes indicate signal intensity (RFU).*

revealed that the relative peak heights for the minor components steadily decreased as the relative proportion of the minor components decreased in each mixture (see Figs. 1B and 1C).

From the ratios examined, the limit of detection of the minor component was determined. This limit is the point before which a mixture is no longer recognized as a mixture; in other words, it is the point before the mixture result appears to be DNA template from only a single source. The limit of detection for mixtures in which a total of 1 ng was amplified was 1:10. Thus, when the minor component was present at 10% the quantity of the major component, the minor component was still detectable, depending on the particular allele combination. The limit of detection for mixtures in which a total of 5 ng was amplified was 1:30 (data not shown).

To characterize a potential quantitative tool for use in the recognition and interpretation of mixed samples, the ratio of peak heights of heterozygous genotypes (within a locus) was examined. The peak height ratio was calculated as the peak height of the lower intensity allele (in relative fluorescence units (RFU)) divided by the peak height of the higher intensity allele and is expressed as a percentage. Data were compiled from unmixed, population database samples amplified with the AmpFISTR Blue Kit to characterize the expected peak height ratio in single source samples. The average peak height ratio for each locus was 92%. Thus, the average difference in peak heights between heterozygous alleles at either D3S1358, vWA, or FGA was approximately 8%. The most extreme peak height ratio observed was 77% for D3S1358 (n = 80; 5% Standard Deviation (S.D.)), 74% for vWA (n = 80; 7% S.D.), and 76% for FGA (n = 87; 6% S.D.).

While these data indicate that the two alleles of a heterozygous type are normally very similar, there was one sample for which the peak height ratio was 66% at vWA. Upon duplicate reamplification, this sample gave peak height ratios of 86% and 96%. Since 99.7% of all observations are expected to fall within three standard deviations of the mean (92%), 99.7% of all peak height ratio observations at any of the AmpFISTR loci should be ≥71% (using 7% S.D., which was the largest standard deviation observed). With that in mind, it is not surprising that 1 ratio out of 247 total observations (0.4%) was less than 71%. Thus, ratios less than approximately 70% will be rare in unmixed samples. Instances which can potentially result in peak height ratios <70% are cases of extremely low amounts of genomic DNA, inhibition, degradation, or an allele containing a mutation at the priming site that effects the efficiency of amplification of that allele.

As another potential quantitative tool for use in the recognition and interpretation of mixed samples, relative stutter peak heights (stutter percentages) were examined. Stutter percentages were calculated by dividing the peak height of a stutter peak by the peak height of its associated allele, expressed as a percentage. Stutter percentages determined from AmpFISTR Blue results of 164 unmixed, population database samples are shown in Fig. 2. The stutter percentages were very reproducible, with an average standard deviation of 0.6%. Again, since 99.7% of all observations should fall within three standard deviations of the mean, a threshold

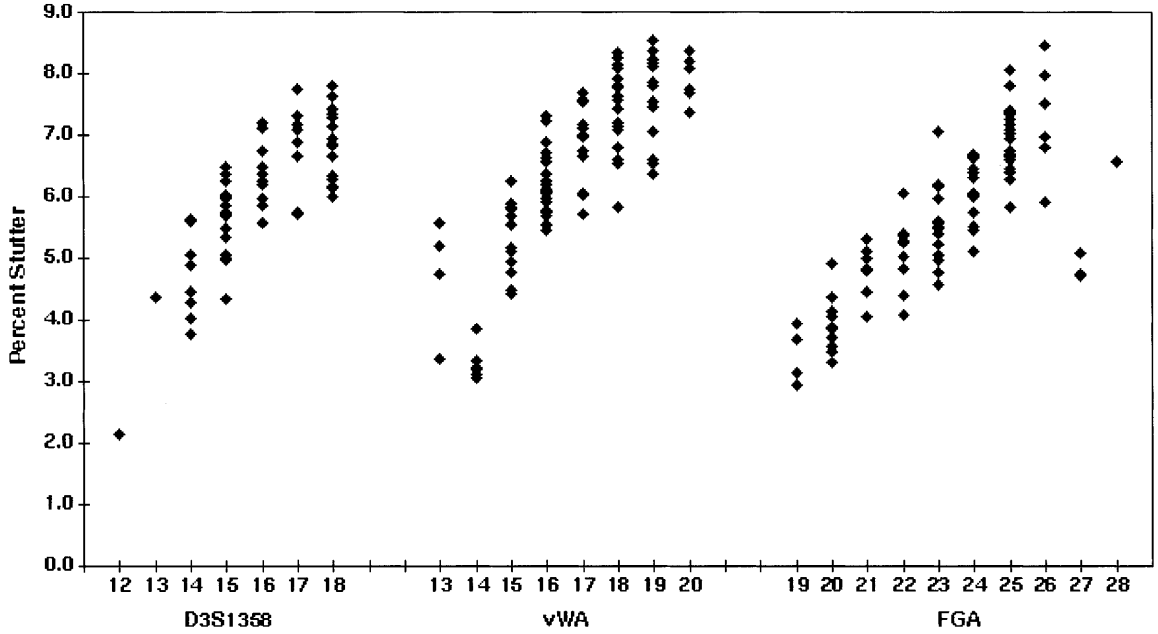

FIG. 2—*Stutter percentages determined from 164 population database samples, run on the 377 DNA Sequencer. The highest stutter percentage observed at any locus was 8.56% (vWA). The X-axis represents the alleles at each locus from which results were determined; the Y-axis represents the percentage of stutter.*

can be applied to facilitate quantitative interpretations of stutter and minor mixture components. In using the greatest stutter percentage observed (8.56%), rather than the mean value observed for all alleles, a conservative threshold of 11% can be applied. Thus, all stutter percentages for any of the AmpFISTR Blue alleles should be $\leq$11% at least 99.7% of the time, and hence, stutter percentages $>$11% may indicate that the peak at the stutter position is due not only to stutter but also to a minor component mixture allele. Furthermore, as has been previously observed (43), the stutter percentages increased with allele length, attributed to the increase in number of repeat units. Consequently, it may be possible to interpret stutter percentages more rigorously, considering the particular allele of the specific locus in question.

Stutter percentages were not included in Fig. 2 for stutter peaks residing between alleles differing by eight base pairs. In this situation, the stutter peak can reside on the shoulder of the shorter allele peak, depending on the resolution of the gel or capillary system used, and cause the stutter peak height to be artificially high (43). Additionally, stutter percentages for allele peaks which were greater than 4500 RFU were not included as these peaks were likely to be off-scale (not within the linear dynamic range of the instrument detector). Off-scale peaks similarly cause stutter percentages to be artificially high since the actual height of the allele peak cannot be determined and is consequently underestimated. A third instance that was not included in Fig. 2 was stutter percentages for alleles which differed by four base pairs since the stutter of the longer allele comigrates with the shorter allele. Taken together, these mixture studies indicate that it is possible to detect a mixture and sometimes resolve the genotypes of each contributor, depending on the genomic DNA ratios, number of contributors, and particular combination of alleles present.

4.1.5.6 Environmental Studies

Because of the environmental conditions to which forensic specimens may be exposed, casework samples are often compromised, exhibiting degradation of the DNA or inhibition of PCR, or a combination of both. The TWGDAM Environmental Studies guideline suggests the examination of samples exposed to a variety of environmental conditions which closely resemble those conditions to which actual evidence may be exposed. Such samples should be compared to reference samples to verify that the resulting genetic types are correct and to define limitations of the DNA assay. The following studies were designed to test the effects of enzymatic degradation, temperature, and sunlight over time.

To determine the effects of degradation on DNA amplified with the AmpFISTR Blue Kit, 8 μg high molecular weight genomic DNA was incubated with the enzyme DNase I for several time periods. Agarose gel analysis was performed to assess degradation at each time point (see Fig. 3A). One and a half ng of undegraded DNA (time 0) and 4 ng of degraded DNA from each time point were then amplified using the AmpFISTR Blue Kit.

In general, the PCR product yield for a locus was reduced as the DNA became more degraded. At the one minute time point (see Fig. 3B), there was a significant loss of signal relative to the undegraded sample. At 4 min, FGA (the longest locus) was no longer detectable, and at the next time point, 8 min, only D3S1358 was detectable. Such amplifications in which one or more loci amplify to a lesser extent relative to the other loci are referred to, in this manuscript, as differential amplifications. No signal from any of the loci was detectable at the time points longer than 8 min. These results paralleled the extent of degradation observed in the agarose gel. For example, at the 4 min time point, the agarose gel showed that most of the genomic DNA was less than 222 bp and, similarly, results of amplification showed that most of the PCR products were less than 222 bp, corresponding to D3S1358 and vWA alleles. This indicates that the amplification efficiency is directly related to the size of the genomic DNA, as expected in an optimized system.

To examine DNA samples exposed to conditions similar to those which evidence may be exposed, environmentally stressed samples

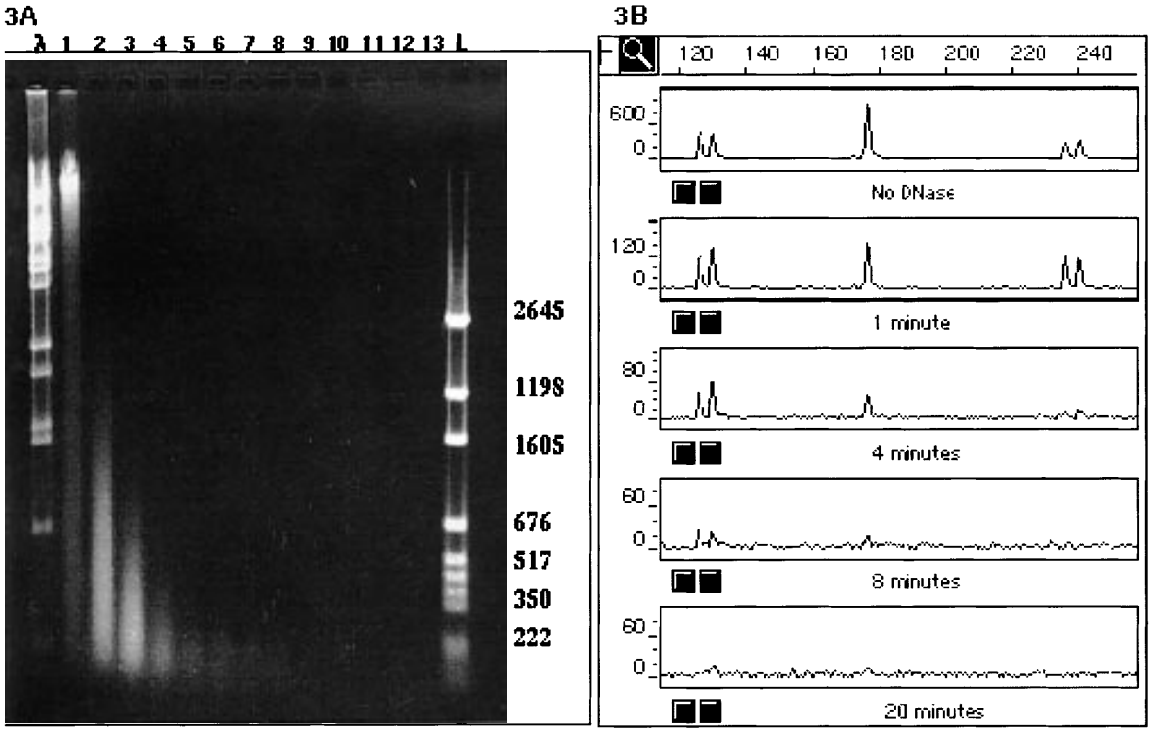

FIG. 3—*DNA degraded with DNase I over increasing lengths of time. Degradation at each time point was evaluated by agarose gel analysis (3A), using 1% NuSieve agarose (FMC Bioproducts, ME) for separation and ethidium bromide for detection. Lanes 1 to 13 were loaded with DNA from each of the following time points: 0, 0.5, 1, 4, 8, 12, 20, 30, 60, 120, 240, 480, and 1440 min, respectively. Two molecular weight markers were included: lamda DNA digested with the restriction enzyme Bste I (λ) and pGEM DNA Marker (L) (Promega Corporation, Madison, WI). 3B shows some of the results of amplification of the degraded DNA samples, run on the 377 DNA Sequencer.*

were analyzed using the AmpFISTR Blue Kit. Dried blood and semen stains (from a single donor) were exposed to sunlight (and shade, as a control) or variations in storage temperature (4°C, room temperature, 37°C, and 65°C). The time points collected and analyzed were 0 time, 1 week, 1 month, 2 months, and for the storage temperature study only, 6 and 12 months. Once again, the PCR product yield for a locus was reduced as the DNA became more degraded. The reduced peak heights seen in the amplified product paralleled the extent of genomic DNA degradation observed by agarose gel analysis (data not shown). As observed in the DNase I study, the first locus to show reduced yield was FGA, followed by vWA, and then D3S1358, which is the expected pattern of differential amplification based on locus size; this is illustrated in the 65°C semen stain results shown in Fig. 4B. Furthermore, the AmpFISTR Blue results of these environmentally compromised samples reflected results obtained previously with the AmpliType PM PCR Amplification and Typing Kit (6): FGA failed to amplify at the same time point as HLA DQA1 (242 bp), which is of similar base pair size.

Results were obtained for all three loci at all the conditions tested with the exception of the few instances described below. No PCR product was detected from the bloodstains stored at 65°C for one year (see Fig. 4A), and very little PCR product was detected for FGA at 65°C for six months (data not shown). From the semen stains, no results were obtained for FGA at 65°C for six months and one year, and very little PCR product was detected for D3S1358 and vWA at 65°C for one year (see Fig. 4B). PCR product at the vWA and FGA loci from semen stains exposed to sunlight was minimally detected for vWA at two months and for FGA at one month (see Fig. 5).; no signal for FGA was detectable at two months. Because the AmpFISTR Blue Kit amplifies degraded DNA very efficiently, these studies demonstrate that the chance of obtaining results from a degraded DNA sample is very good when the PCR target region is still intact.

4.1.5.7 Matrix Studies

While forensic samples may be degraded, they may also contain PCR inhibitors originating from the substance in contact with the biological material (e.g., dyes in denim). In this instance, the inhibitors are coextracted with the DNA and consequently influence the success of PCR by preventing efficient amplification. Therefore, the TWGDAM Matrix Studies guideline suggests evaluating the DNA assay with body fluids that have been mixed with or deposited on a variety of commonly encountered substances.

To address this guideline, DNA was extracted from blood and semen (from a single donor) that had been mixed with or deposited on the following substrates: nylon panties, wool, blue denim, black denim, newspaper, leaves, leather (not tested with semen), cotton swab, carpet, cotton crotch of panties, and soil. Denim and leather were included in this study because they can be particularly challenging due to frequently coextracted inhibiting dyes and pigments. Soil was chosen as its components often additionally degrade DNA.

Bloodstains were dried overnight at room temperature, extracted, and then stored for approximately two years at −15 to −25°C. Semen stains were dried overnight at room temperature, stored −15 to −25°C for approximately two years, and then extracted. Genotype results were obtained from all samples including those from denim, leather, and soil. No spurious peaks or artifacts were observed in any of these amplification results. Figure

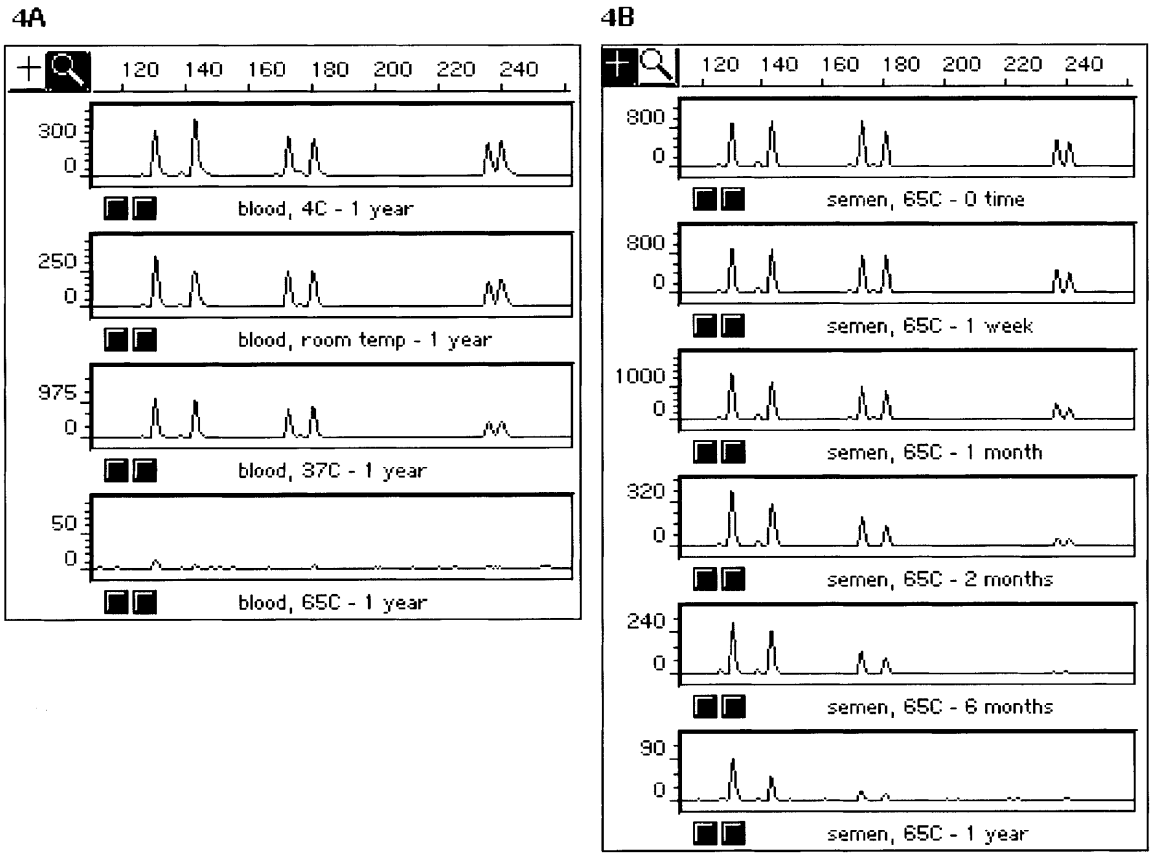

FIG. 4—*Blood stored at different temperatures and semen samples stored for varying lengths of time, run on the 377 DNA Sequencer. Shown in 4A are the AmpFISTR Blue results from blood samples stored for one year at the temperatures 4°C, room temperature, 37°C, and 65°C. Shown in 4B are the AmpFISTR Blue results from semen samples stored at 65°C for 0 time, 1 week, 1 month, 2 months, 6 months, and 1 year.*

6 shows the results obtained for blood deposited on blue denim, black denim, leather, and mixed with soil.

Degradation and inhibition were not observed, as inferred by the balance between the loci. This result is somewhat surprising in regard to the more challenging substrates examined. Several possible parameters may account for the ability to achieve results with such difficult samples, including: optimized extraction protocols, types of dyes and pigments present, types and abundance of bacterial strains in soil, inclusion of bovine serum albumin (BSA) in the PCR reaction mix, and the use of excess enzyme. The AmpFISTR Blue PCR reaction mix includes BSA, and the use of 4.5 units of AmpliTaq Gold DNA polymerase is recommended.

4.1.5.8 Nonprobative Evidence

The Nonprobative Evidence guideline suggested by TWGDAM recommends the examination of nonprobative evidentiary samples. Six nonprobative cases were examined with the AmpFISTR Blue Kit. Following amplification, three cases (1–3) were analyzed using both the 377 DNA Sequencer and the 310 Genetic Analyzer, and the three other cases (4–6) were analyzed on only the 377 instrument.

Case 1 included two victim reference samples, two suspect reference samples, and two condom evidentiary specimens. Differential extractions were performed on swabbed extracts from each condom. The genotype obtained from the epithelial fraction of condom 1 matched the genotype of victim 1, and the genotype obtained from the epithelial cell fraction of condom 2 matched the genotype of victim 2 (refer to Fig. 7A). The sperm fraction from each condom showed a different male profile; one profile included a suspect while the other profile excluded both suspects.

Case 2 included a victim reference sample, a suspect reference sample (victim's father), a reference sample from the victim's boyfriend, and a pair of panties. A differential extraction had been performed on the sample from the panties. The genotype obtained from the epithelial fraction matched that of the victim reference sample (data not shown). There were also traces of the victim's type found in the sperm fraction. The major genotype found in the sperm fraction excluded the suspect and included the boyfriend.

Case 3 included a victim reference sample, a suspect reference sample, and both a sweater and sweatshirt belonging to the victim. Resulting profiles of the sweater and sweatshirt both matched the victim reference profile (data not shown). There were no unaccountable alleles and neither piece of evidence included the suspect.

Case 4 contained a victim reference sample, a suspect reference sample, and a vaginal swab from the victim. As shown in Fig. 7B, the genotype of the epithelial fraction matched that of the victim reference and also showed some minor component alleles that were consistent with the sperm fraction genotype. The genotype of the sperm fraction excluded the suspect, sharing only one allele in common at the D3S1358 locus.

Case 5 contained both a victim and a suspect reference sample and a vaginal swab from the victim. The genotype of the epithelial cell fraction matched that of the victim reference sample (data not shown). The sperm fraction genotype included the suspect.

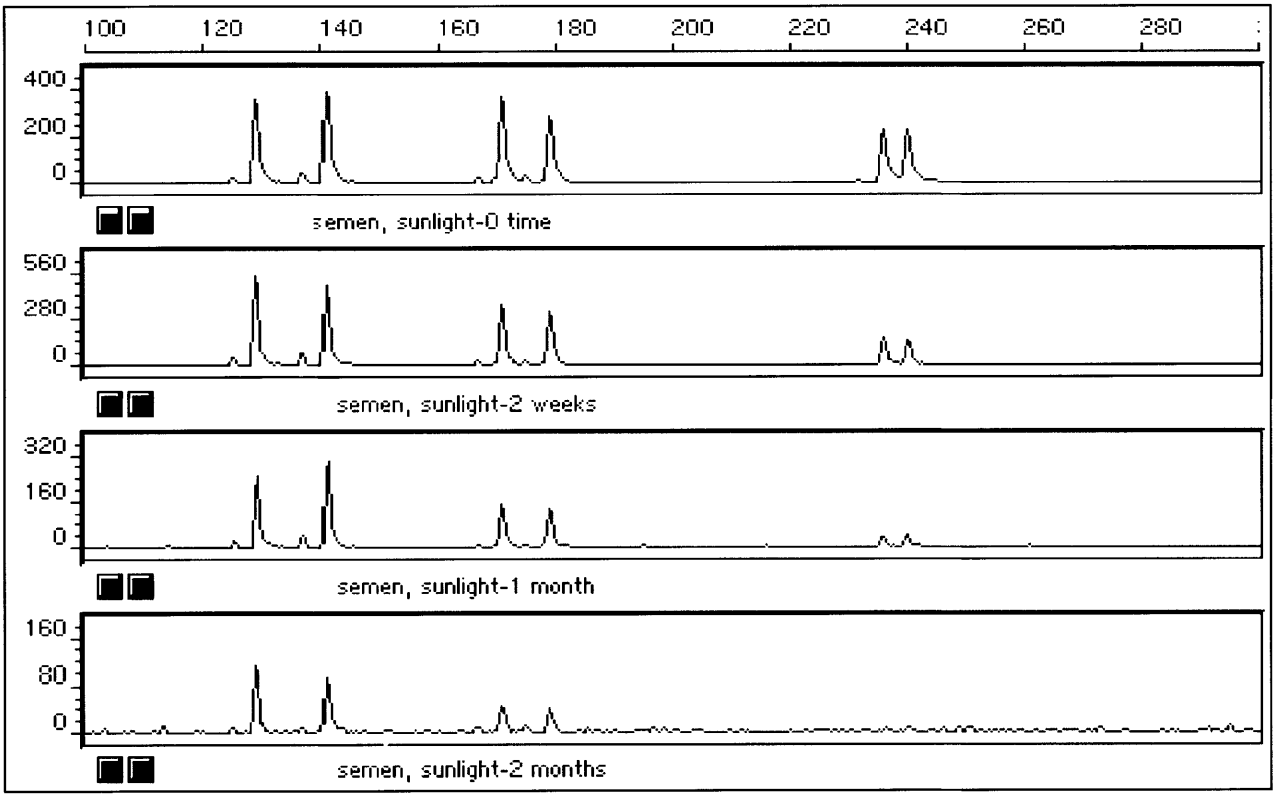

FIG. 5—*AmpFISTR Blue results of semen samples stored in sunlight for 0 time, 2 weeks, 1 month, and 2 months, run on the 377 DNA Sequencer.*

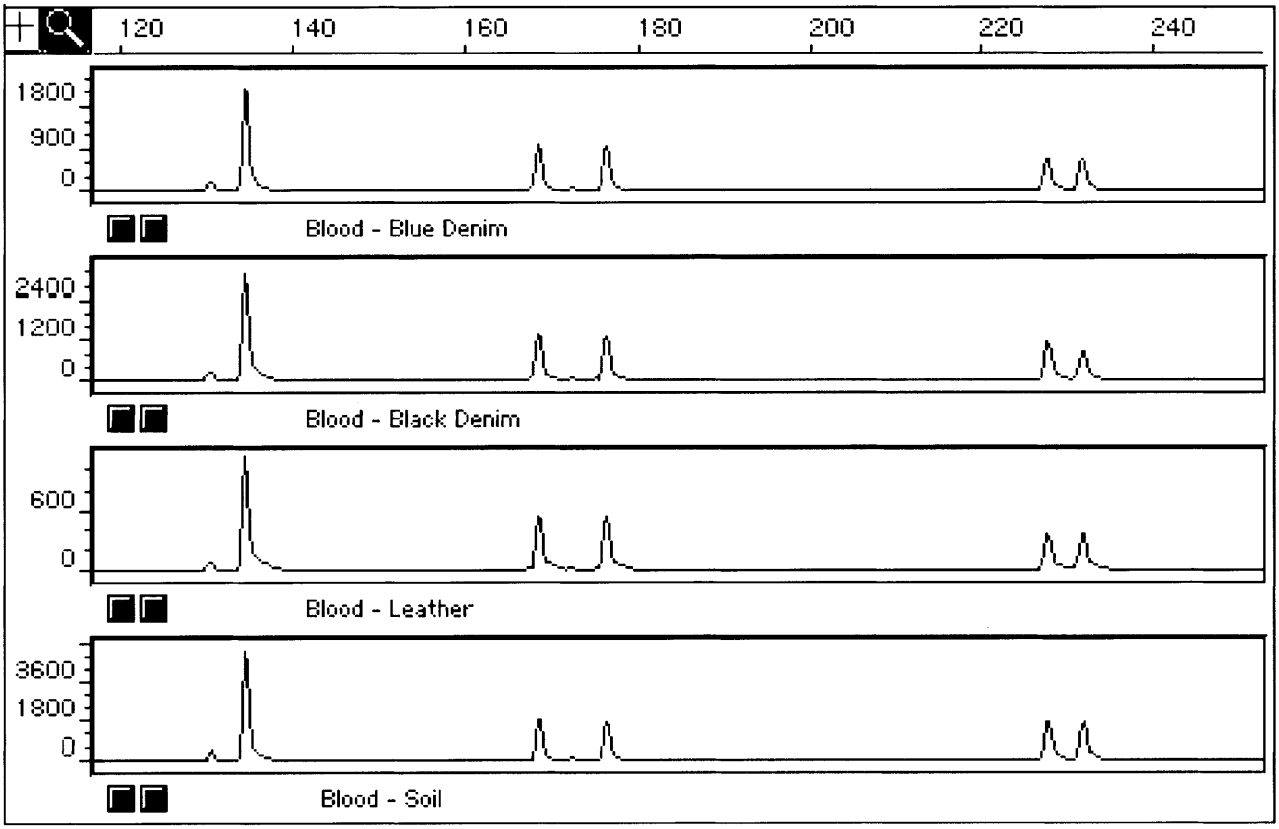

FIG. 6—*AmpFISTR Blue results of blood samples stored on or mixed with the following substrates: blue denim, black denim, leather, and soil. The amplified products were run on the 377 DNA Sequencer.*

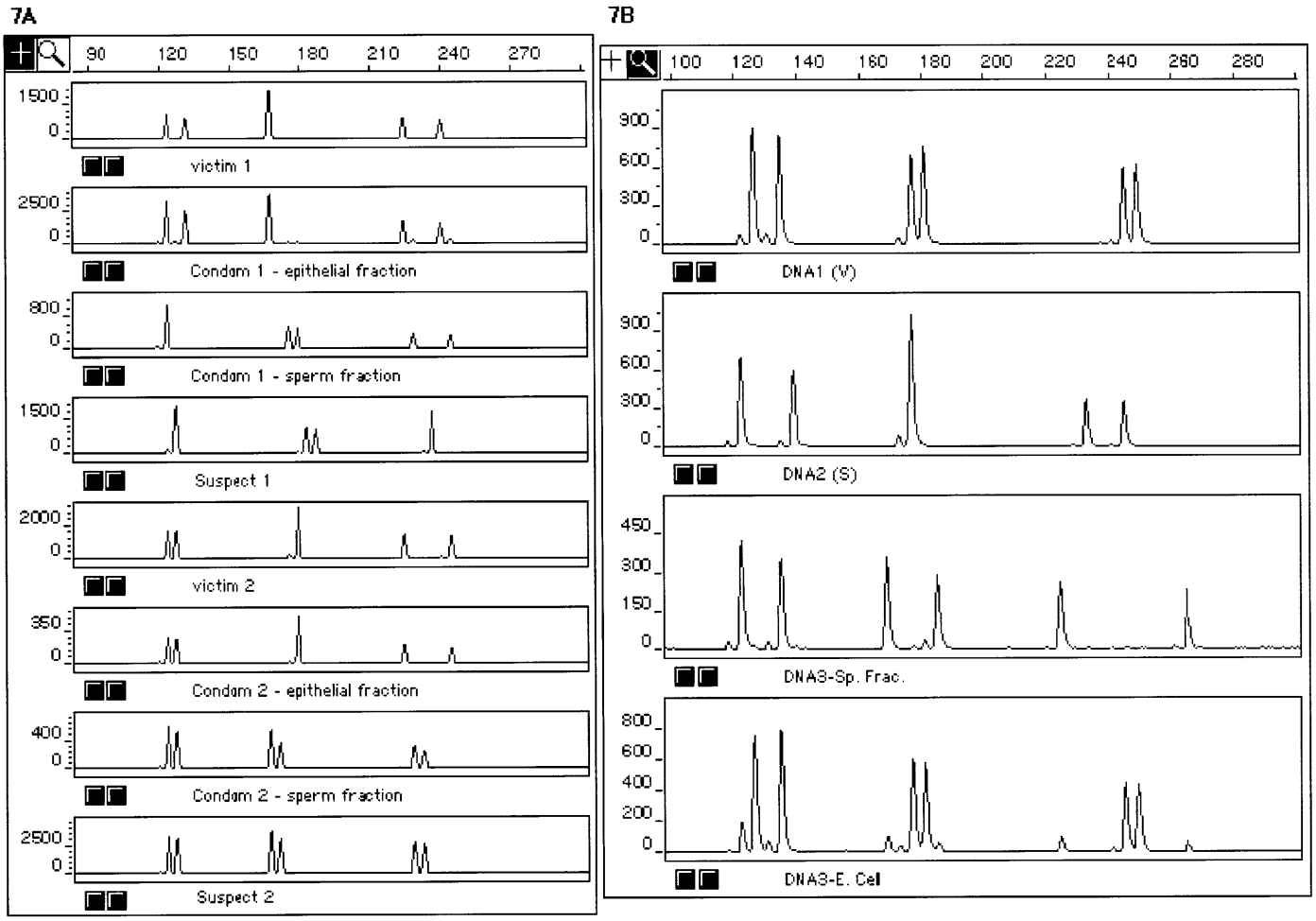

FIG. 7—*AmpFISTR Blue results of two nonprobative sexual assault cases. 7A shows case 1, run on the 310 Genetic Analyzer. Panels 1 (victim 1) and 5 (victim 2) are the results of the victim reference samples, and panels 4 (suspect 1) and 8 (suspect 2) are those of the suspects. The results of amplification of the sperm and epithelial fractions extracted from condom 1 are shown in panels 2 and 3. Similarly, panels 6 and 7 show that of condom 2. 7B shows case 4, which was run on the 377 DNA Sequencer. The first panel represents the victim reference sample, indicated as DNA1 (V). The second panel represents the suspect reference sample, indicated as DNA2 (S). The results of amplification of the sperm and epithelial fractions extracted from the vaginal swab are shown in panels 3 (DNA3-Sp. Frac.) and 4 (DNA3-E. Cell), respectively.*

Case 6 involved the attempted identification of the source of human remains using DNA extracted from a bone. The resulting AmpFISTR Blue genotype was compared to that of a putative mother, sister, and brother. The genotype from the bone contained one allele in common with the putative mother at each locus (see Fig. 8). Because there was no putative father reference sample available, the siblings were included in the attempted identification. The putative siblings both contained, in common with the bone, the alleles at vWA and FGA, which were distinct from those accounted for by their mother. These alleles of the putative siblings must be inherited by their biological father, who may then also be the biological father of the individual from whom the bone was recovered. The D3S1358 allele of the bone foreign to the putative mother was also foreign to both siblings; this is an obligate allele to the biological father of the unidentified individual. This result did not exclude this family as the biological family of the unidentified individual because the putative siblings contained the same allele not in common with their mother, indicating only one obligate allele to the biological father from these two siblings. Therefore, if the father of all three individuals is the same man, his genotype at D3S1358 would be heterozygous and contain both of the obligate alleles. Collectively, the results indicate an inclusion of the putative family as the biological family of the unidentified individual.

4.1.5.9 Nonhuman Studies

TWGDAM suggests examining DNA samples obtained from nonhuman sources to characterize the species specificity of a DNA assay that has been designed for human use. Due to the nature of forensic samples, it is very important to establish species specificity, particularly in regard to bacteria. One DNA sample from each of the following nonhuman organisms was examined: primates included gorilla, chimpanzee, and orangutan and nonprimates included bacteria (Salmonella, Citrobacter, and 2 separate sources of E. coli), yeast (S. cerevisiae), mouse, cat, dog, pig, chicken, and cow. Two and a half ng of DNA from the primates and 50 ng of DNA from the nonprimates were examined (yeast and bacterial copy number was equivalent to approximately 50 ng of human DNA).

The primate samples all amplified, producing fragments within the 100 to 300 bp region (data not shown). According to sequencing results and as might be expected from an evolutionary standpoint, the primate PCR products are a result of amplification of the same loci; an analysis of the sequence repeat motifs of these amplified

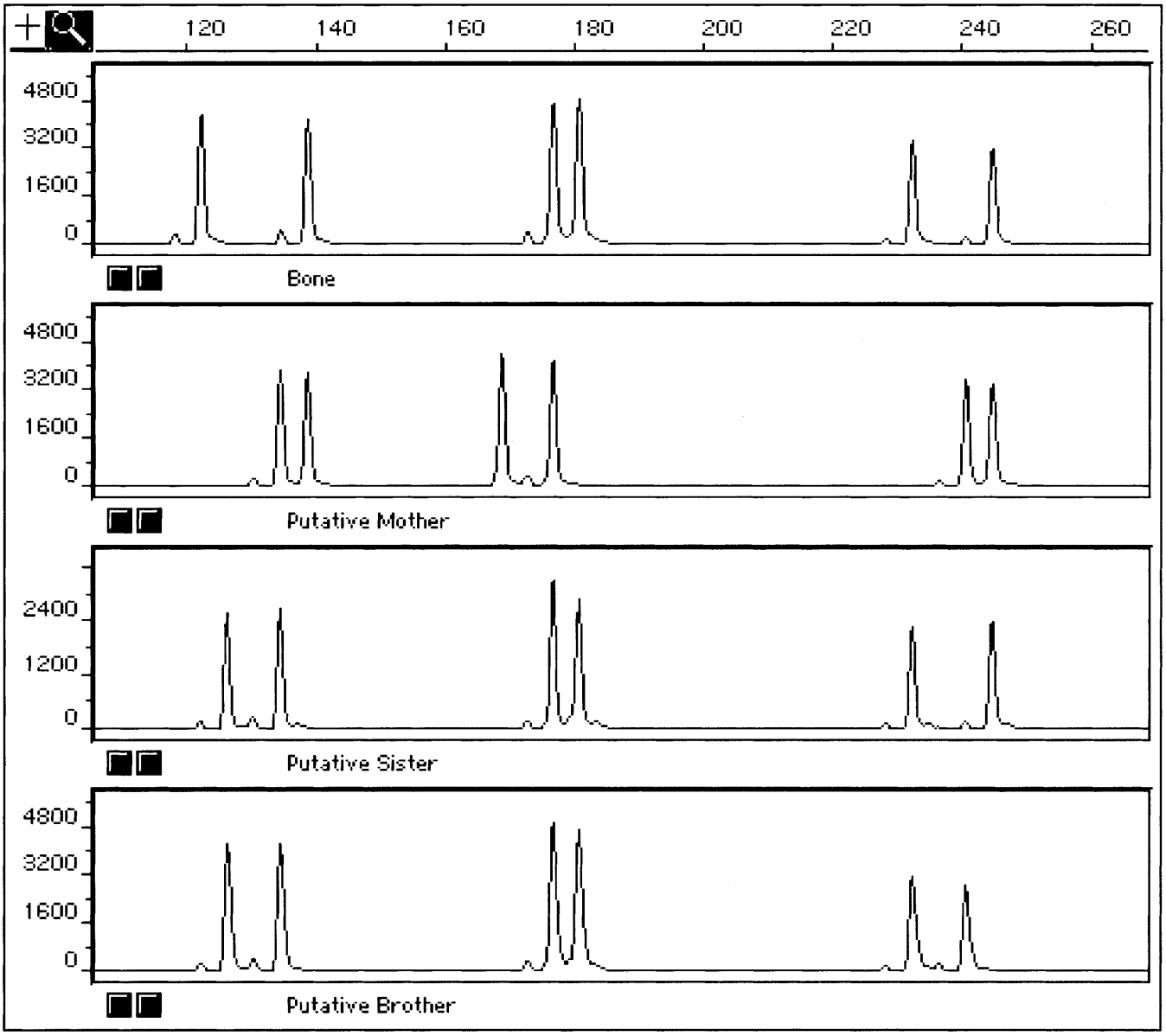

FIG. 8—*AmpFISTR Blue results of a nonprobative human remains identification case (case 6), run on the 377 DNA Sequencer. The results of amplification of DNA extracted from a bone of the remains are shown in panel 1 and those of the putative mother, sister, and brother reference samples are shown in panels 2, 3, and 4, respectively.*

fragments will be discussed in a future manuscript. An examination of multiple gorilla, chimpanzee, and orangutan samples would be interesting to determine if several alleles exist at each locus and how the alleles compare to human allele sizes on both the 377 DNA Sequencer and the 310 Genetic Analyzer.

Nonprimate DNA samples did not show any detectable PCR product. These results are particularly important in regard to E. coli, given the abundance of this organism not only in the human normal flora but also in the environment in general. It may be beneficial to examine specificity with a more thorough representation of bacterial and yeast species of the human normal flora.

4.1.5.10 Minimum Sample

When the total number of template copies added to a PCR is extremely low, the amplified alleles of a heterozygous individual may be unbalanced. This can be due to sampling of the DNA when added to the reaction or to a founder effect in the early cycles of the reaction; these are commonly referred to as PCR stochastic effects (44). Because such imbalance can affect the accuracy of a genotype result, the TWGDAM Minimum Sample guideline was suggested, which recommends establishing ''the quantity of DNA needed to obtain a reliable result.''

The minimum sample objective for the AmpFISTR Blue amplification protocol was that little or no signal be detected for DNA samples containing approximately 35 picograms (pg) or less, which is roughly equivalent to ten copies of DNA template, or five cells. To serve as an indicator of genomic DNA quantity, a peak height threshold below which results were interpreted with caution was additionally determined from the following experiments. Serial dilutions were performed using AmpFISTR Control DNA 9947A and another control DNA (sample B34, described under 4.4.1.5 Amplification), both of which were heterozygous at all three loci. Dilutions of amplified genomic DNA were: 1.0, 0.5, 0.25, 0.125,

0.063, 0.0313, 0.016, and 0.008 ng. Amplifications were performed on both the Thermal Cycler 480 and the GeneAmp PCR System 9600; PCR products were examined on both the 377 DNA Sequencer and the 310 Genetic Analyzer. The results of 0.25 to 1.0 ng were clearly typable with peak heights of approximately 150 RFU and greater (data not shown). At 0.125 ng and less, the peak heights in both samples were not significantly above the background ($<$ 150 RFU) or were undetectable. At 0.0313 ng specifically, peaks were extremely low or undetectable, and thus, DNA quantities as low as approximately 35 pg did not produce a typable result. Based on these results, we employed a peak height threshold of 150 RFU, below which peaks were interpreted with caution. Laboratories should determine a minimum peak height threshold for their instruments using high quality, single source genomic DNA samples which provides them with the desired sensitivity while not allowing for detection of low copy DNA. This is particularly important as the overall sensitivity of the assay may vary between laboratories.

4.2.1 Inheritance

In the development of a DNA assay, basic characteristics of the loci should be examined, including demonstration of the mode of inheritance by family studies, as suggested by TWGDAM. This is to insure independent assortment of alleles and, thus, to verify Mendelian inheritance.

The CEPH family DNA sets are ideal for studying inheritance patterns. These DNA sets represent 39 families of Utah Mormon, French Venezuelan, and Amish descent collected by the Centre d'Etude du Polymorphisme Humain (CEPH). The CEPH family DNA sets have been extensively studied throughout the world and are routinely used to characterize mode of inheritance of various DNA loci. Each family set contains three generations, generally including four grandparents, two parents, and several offspring.

The mode of inheritance of the AmpFISTR Blue loci was examined using 1 ng of DNA from the following four CEPH family sets: #884 (12 offspring), #1340 (6 offspring), #1341 (8 offspring), and #1345 (7 offspring). The genotype results confirmed independent assortment of AmpFISTR Blue alleles and, therefore, supported inheritance according to Mendelian rules (data not shown), as has been observed by other laboratories (28,30,31).

Additionally, the TWGDAM committee advises that the loci have a low frequency of mutation and recombination. A PCR product sizing assay can detect mutations and recombination events that affect the efficiency of amplification, which results in poor or lack of amplification of an allele, or the length of the PCR product. While one mutation has previously been observed at the vWA locus (45), no mutations or instances of recombination were observed at any of the AmpFISTR Blue loci in the 82 meiotic events tested in the four CEPH families.

4.4.1.3 Amplification—PCR Reaction Components and Thermocycling Parameters

The Amplification guideline 4.4.1.3 suggests the determination of the concentration of each critical PCR reaction component and the thermocycling parameters required to produce the necessary degree of specificity. In developing D3S1358, vWA, and FGA into a triplex PCR system, much effort was devoted to obtaining specific and sensitive results, reproducibly. This is crucial as the analyst should be able to reliably interpret, and therefore explain, every peak from a forensic sample result.

The PCR reaction components of the AmpFISTR Blue Kit have been optimized to give reliable performance by testing each over a wide range of concentrations. These components included Tris·HCl (pH 8.3), KCl, deoxynucleotide triphosphates, primers, AmpliTaq Gold DNA Polymerase, $MgCl_2$, BSA, and sodium azide. The optimal concentration for each component was established to be in the middle of a window that meets the reproducible performance requirements of specificity and sensitivity. Once the optimal concentration was determined for a single component, the others were tested sequentially until it was determined that each component was at the optimal concentration relative to the others in the reaction mix (data not shown). In achieving this level of reproducibility, the assay allows for minor measurement errors and variations in mixing in the amplification setup without resulting in a compromise to the efficiency and integrity of the reaction. The primers were additionally developed to produce balanced peak heights between the loci with noncompromised, single source DNA samples. Balance facilitates the interpretation of results from DNA samples that may be degraded, inhibited, and/or mixed with DNA from other donors, as exemplified in the data presented in this manuscript.

Similar to the PCR reaction mix components, the thermal cycling parameters are set in the middle of optimal ranges. A window of at least ± 2°C was tested around each setpoint. Specific PCR product with maximal sensitivity and balance between the loci was reproducibly achieved. Thermal cycler parameters were established for amplification of the AmpFISTR Blue Kit in the DNA Thermal Cycler 480 and GeneAmp PCR Systems 9600 and 2400. The ± 2°C window encompasses, and thus meets, the Perkin Elmer instrument specifications for each thermal cycler. Shown in Fig. 9 are representative results of amplification over a wide range of annealing temperatures on the GeneAmp PCR System 9600, including the ± 2°C window of 57°C and 61°C.

4.4.1.4 Amplification—Number of Cycles

The suggestion of the TWGDAM committee in Amplification guideline 4.4.1.4 is to examine the number of cycles necessary to produce a reliable genotype result. The cycle number affects reliability since it significantly affects the sensitivity of the assay and, therefore, the potential for PCR stochastic effects. AmpFISTR Blue reactions were amplified for 27, 28, 29, and 30 cycles on both the DNA Thermal Cycler 480 and GeneAmp PCR System 9600. Using 28 cycles, low quantities of DNA (less than 35 pg) produced extremely low peaks or were not detectable at all when the amplified products were examined on the 377 DNA Sequencer and 310 Genetic Analyzer (data not shown). Thus, 28 cycles were found to give optimal sensitivity and is the recommended cycle number for the AmpFISTR Blue Kit.

4.4.1.5 Amplification—Differential and Preferential Amplification

The Amplification guideline 4.4.1.5 recommends that the potential for differential (and preferential) amplification be assessed. Recall that differential amplification is defined in this manuscript as the difference in amplification of each locus within the AmpFISTR Blue system. Preferential amplification, on the other hand, is defined here as the difference in amplification efficiency of two alleles at a single locus. While differential amplification may simply lead to a loss of information when a locus becomes completely undetectable (as with degraded DNA), preferential amplification is a serious concern in that severe preferential amplification can lead to a mistyping. A mistyping can occur when one allele of a heterozygous type becomes undetectable and the genotype is interpreted as a homozygote. Typically observed peak height ratios in heterozygous samples were characterized not only to assist

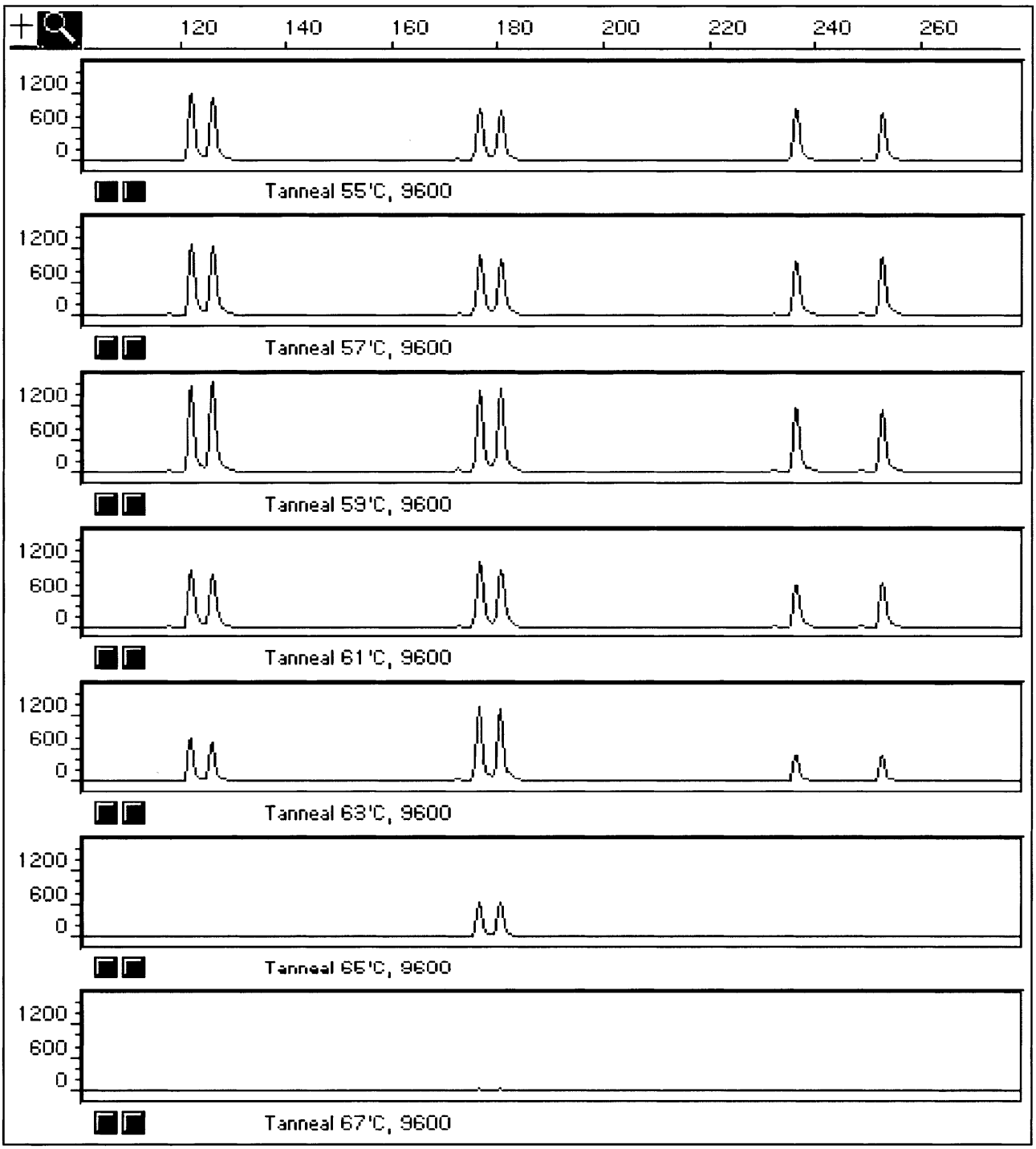

FIG. 9—*Effects of variation of annealing temperature on AmpFISTR Blue results from a GeneAmp PCR System 9600, run on the 377 DNA Sequencer. One and a half ng of a genomic DNA sample (from a Chelex extraction of a buccal cell swabbing) was amplified at the indicated annealing temperatures: 55°C, 57°C, 59°C, 61°C, 63°C, 65°C, and 67°C. The recommended annealing temperature is 59°C.*

in mixture recognition and interpretation but also to establish a quantitative measure for identifying occurrences of preferential amplification (<70% peak height ratio).

Preferential amplification of alleles in systems based on length polymorphisms is most likely to be observed when the alleles differ significantly in base pair size. Since the STR loci contained in the AmpFISTR Blue Kit have small size ranges, the potential for preferential amplification was expected to be low. However, because there are some rare alleles of the locus FGA which are longer in size than the typical size range, we obtained a sample (B34) that is heterozygous for FGA where the alleles are separated by 90 bp (240 and 330 bp). While such a genotype is very rare, this unique sample was ideally suited for inclusion in studies of preferential amplification.

There are several parameters which may affect amplification efficiency and, therefore, cause differential or preferential amplification with forensic samples. The following three conditions were examined: presence of inhibitors in a DNA sample, degraded DNA, and amplification denaturation and annealing temperatures. Sample B34 was included in the inhibition study.

To examine the effect of a PCR inhibitor on the potential for differential and preferential amplification, hematin was utilized. This is a heme derivative thought to be similar to that which is coextracted with DNA from blood-containing samples (46,47) and is a known inhibitor of PCR. One ng of the AmpFISTR Control DNA 9947A, sample B34, and a third genomic DNA sample, KG, was amplified in the presence of varying concentrations of hematin: 0, 4, 7, 10, 14, 17, 20, 24, 27, 30, and 34 μM. As the concentration of hematin was increased, the overall yield of products was reduced, and differential amplification was observed, as illustrated at 17 μM in panel 2 of Fig. 10. The order in which the AmpFISTR Blue loci became undetectable was different than that observed with degraded DNA; FGA became undetectable first, followed by D3S1358, then vWA. This may be due to slightly stronger primer binding at vWA, as this inhibition pattern is also observed at high annealing temperatures (as shown in Fig. 9).

These distinct patterns of differential amplification may be useful in determining whether lack of amplification or weak amplification is due to degradation or inhibition. Knowing which parameter is influencing the results may aid in determining how to proceed with the sample. Often, more signal may be obtained from degraded DNA samples if more DNA is added, whereas in the case of inhibition, the sample is often diluted to weaken the effect of the inhibitor, thus enhancing the efficiency of the amplification.

Preferential amplification of vWA and FGA alleles was observed only, yet reproducibly, with sample KG when amplified in the presence of the higher levels of hematin tested; panel 2 of Fig. 10 illustrates the degree of preferential amplification observed

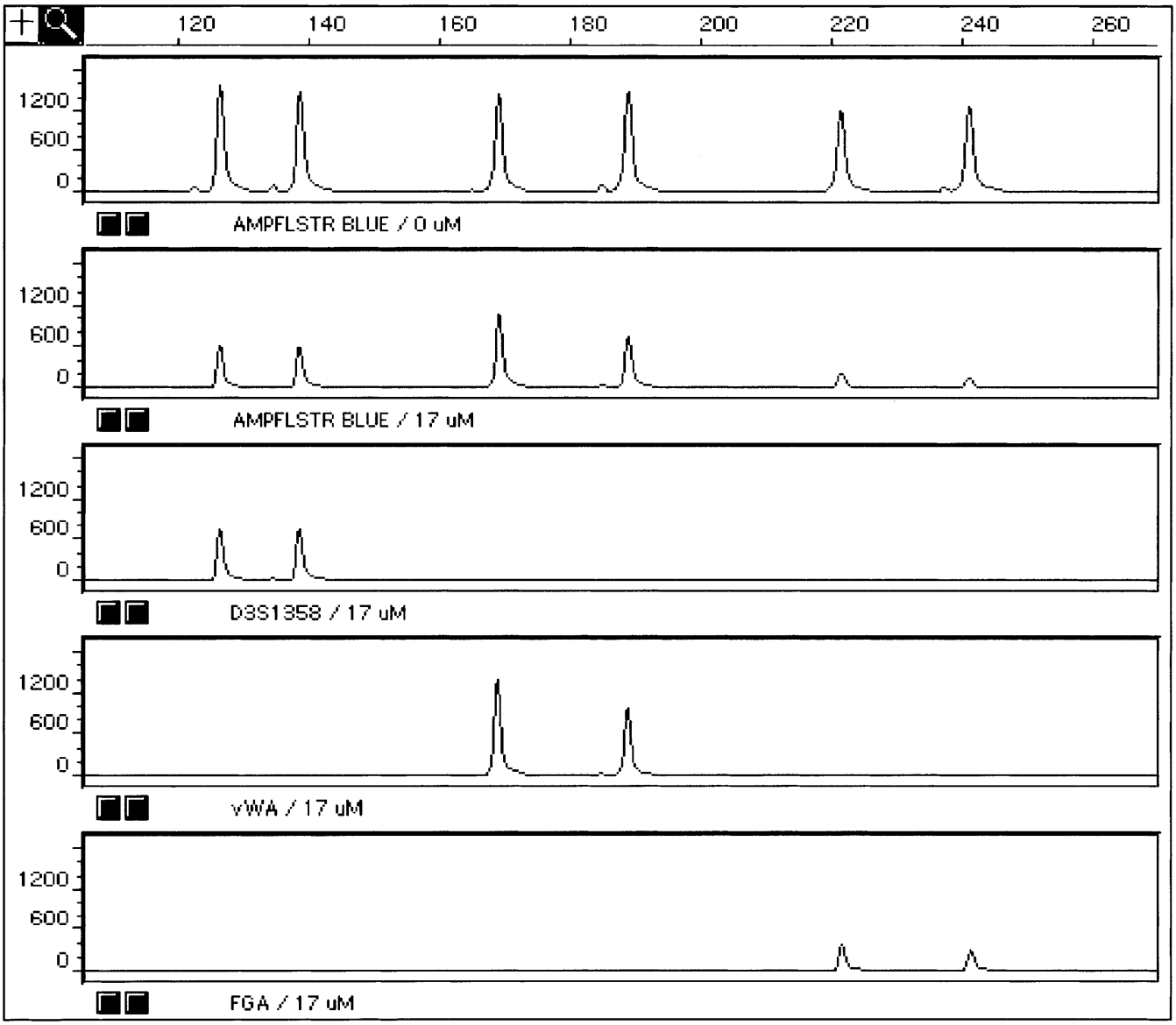

FIG. 10—*Single locus versus AmpFISTR Blue (triplex) amplifications in the presence of 17 μM hematin, run on the 377 DNA Sequencer. The first panel contains the AmpFISTR Blue results without hematin (0 μM). The second panel contains the AmpFISTR Blue results in the presence of 17 μM hematin, and panels 3 to 5 contain those of each locus separately, as indicated.*

at vWA when amplified with 17 μM hematin. This result is curious as the alleles at vWA and FGA are not widespread, unlike the FGA alleles in sample B34 which did not preferentially amplify.

Degradation was the second condition examined in testing for the occurrence of differential and preferential amplification. The differential amplification results are presented in the DNase I study described under guideline 4.1.5.6 Environmental Studies. Preferential amplification of the degraded DNA was not observed at any of the time points (see Fig. 3B for a representative example).

Thirdly, the effects of denaturation and annealing temperatures and hold times on the amplification of the AmpFISTR Blue loci were examined using 1 to 2 ng of the AmpFISTR Control DNA 9947A and other high quality genomic DNA samples. The denaturation temperatures tested were 92, 94, and 96°C. These were tested for both 30 s and 1 min hold times (data not shown). The annealing temperatures tested were 55 to 67°C, all for 1 min hold times. The majority of these were tested on each the DNA Thermal Cycler 480 and the GeneAmp PCR Systems 9600 and 2400. None of the tested annealing and denaturation temperatures induced differential amplification, except the following: at 63°C, the yields of both the D3S1358 and FGA alleles were reduced relative to those of vWA, and at 65°C, alleles of both D3S1358 and FGA were often not detectable (see Fig. 9 for GeneAmp PCR System 9600 results). Note that these temperatures are several degrees above the recommended annealing temperature (59°C). Preferential amplification was not observed in these experiments.

Based on the experiments performed to evaluate preferential amplification under stressing conditions, its occurrence was quite low. This is likely due to the relatively small size range of each locus. When preferential amplification was observed, there was never an instance in which the degree of preferential amplification affected the reliability of a resulting genotype.

Unlike preferential amplification, differential amplification was observed in several instances involving compromised samples or nonoptimal amplification conditions (significantly increased annealing temperature). Because the AmpFISTR Blue Kit has been developed to produce relatively equal peak heights between each of the loci, the recognition of differential amplification is relatively straight forward.

4.4.1.6 Amplification—Single Locus vs. Multilocus Amplification

The Amplification guideline 4.4.1.6 states that when ''more than one locus is amplified in one sample mixture, the effects of such amplification on each system (alleles) must be addressed and documented.'' The AmpFISTR Blue Kit is designed to amplify three STR loci in one reaction, and thus, single locus vs. triplex amplification studies were performed. DNA samples were amplified in three separate reactions each containing primers for only one AmpFISTR Blue locus and in a fourth reaction containing primers for all three AmpFISTR Blue loci (triplex). Samples tested included the following: 1 ng of AmpFISTR Control DNA 9947A, three samples to which varying levels of hematin were added (see Amplification Guideline 4.4.1.5), and the DNase I degraded sample described under guideline 4.1.5.6 Environmental Studies (all time points were tested). In all instances, the same genotype result was obtained whether the DNA samples were amplified for each locus separately or coamplified in the AmpFISTR Blue triplex reaction. Similarly, there was no significant difference in peak heights, even with respect to the extent of preferential and differential amplification observed, as exemplified in panels 2 to 5 of Fig. 10.

Thus, amplifications of the AmpFISTR Blue Kit (triplex) occurred with relatively equivalent efficiency as compared to those of each locus separately. As a result, single locus amplifications conferred no benefit over coamplifying the AmpFISTR Blue loci as a triplex. Conversely, a coamplification of the three loci will minimize the quantity of forensic sample consumed.

4.4.2.1 Amplification—Standards for Direct Characterization and 4.5.3 Internal Validation of Established Procedures

The TWGDAM 4.4.2.1 Amplification guideline recommends that appropriate standards for interpreting alleles be established for DNA assays which involve direct characterization of PCR product. In defining appropriate standards to use for interpreting AmpFISTR Blue alleles, a protocol for sizing and genotyping was considered. The main criterion was that the protocol provide accurate genotypes reproducibly as this is imperative for forensic application. This involved determining the sizing precision of the detection system, which is the suggestion of the 4.5.3 Internal Validation of Established Procedures guideline.

For obtaining base pair sizing results, the GeneScan-350 [ROX] Internal Lane Size Standard was evaluated. The GS-350 size standard contains 12 single-stranded fragments of 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340 and 350 bases and, therefore, is designed for sizing DNA fragments in the 35 to 350 bp range. The fragments are labeled with the dye ROX, which emits at a wavelength distinctly different from 5-FAM. This permits the standard to be used as an in-lane size standard, and thus, lane-to-lane and injection-to-injection variations are normalized. The AmpFISTR Blue Allelic Ladder (labeled with 5-FAM) was developed to provide an accurate means for conversion of base pair sizing results to genotypes. As with the amplified samples, the allelic ladder was run with the in-lane size standard, and genotyping was then accomplished by comparing sample allele sizes to the sizes obtained for the allelic ladder alleles. The allelic ladder contains the common alleles at the three loci: D3S1358 alleles 12–19, vWA alleles 11–21, and FGA alleles 18–30. The allele designations correspond to the number of four base pair repeat units present in each allele (48); the ladder includes a 2-bp variant allele at FGA that is designated as 26.2. The nomenclature has been confirmed by sequencing each allele in the allelic ladder (manuscript in preparation).

To examine precision in base pair sizing using the GS-350 in-lane size standard, within-gel and within-capillary precision studies were performed on the 377 DNA Sequencer and the 310 Genetic Analyzer. PCR product from sets of 31 population database samples along with three replicates of AmpFISTR Blue Allelic Ladder were run repeatedly. Standard deviations in sizing results were determined to be <0.15 bp for each allele. Hence, the GS-350 standard generated extremely precise sizing results (26,39).

A size window of three standard deviations was applied to the 0.15 bp S.D. to define a size range around each allele in the allelic ladder. In applying a window of ± 0.45 bp (3×0.15 bp), 99.7% of unknown alleles of the same size are statistically predicted to size within the window. In practice, any sample allele that sized within ± 0.5 bp of an allelic ladder allele was assigned the corresponding allele designation. Figure 11 illustrates the one base pair window, and precision, on the 377 DNA Sequencer.

Because the calculated sizes of the reference allelic ladder were determined from the same run as those of the samples to be genotyped, the one base pair window was a ''floating window.'' The floating window was necessary because sizes determined for

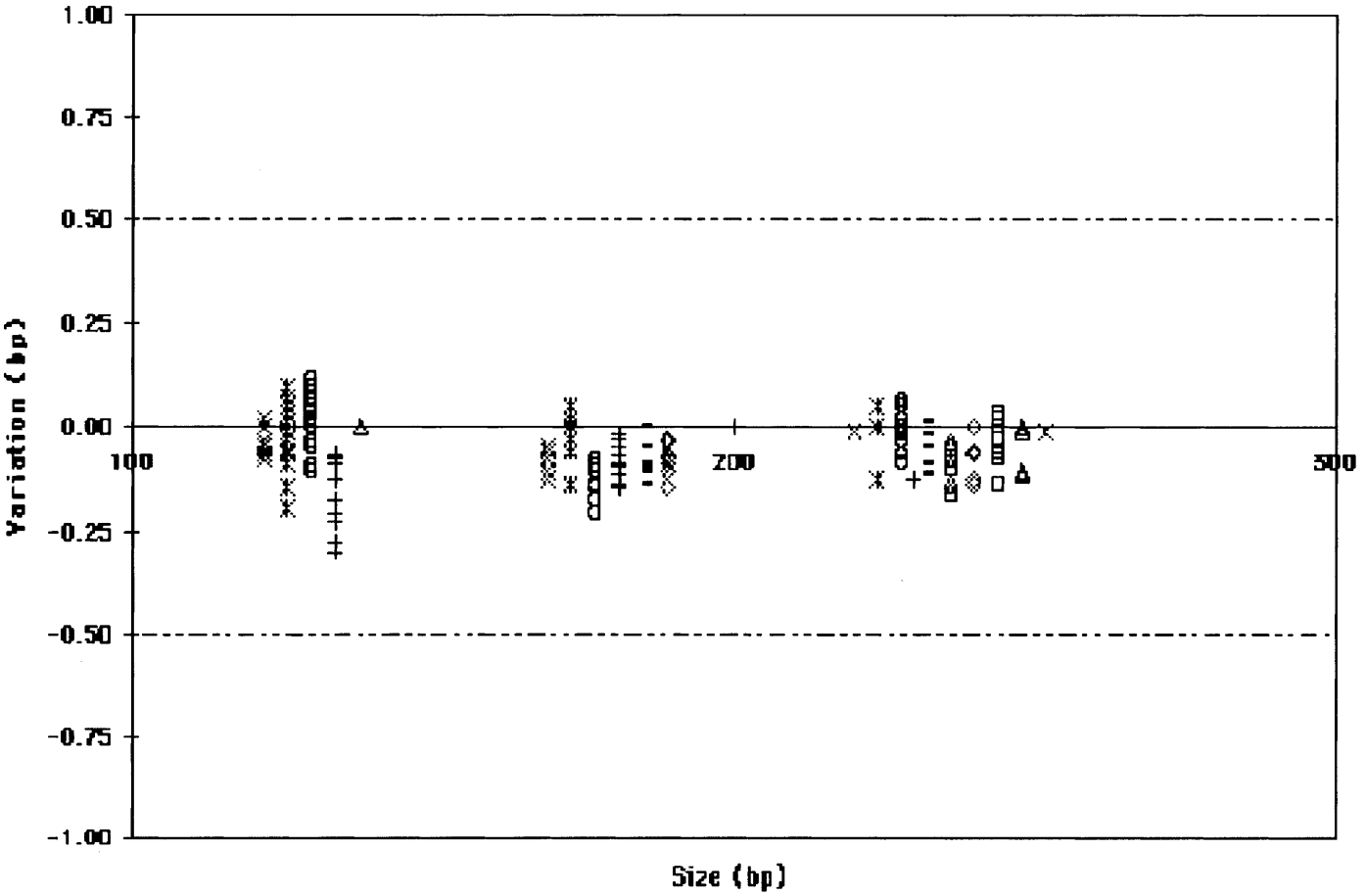

FIG. 11—*Precision results obtained from a single, representative run on the 377 DNA Sequencer. The X-axis represents the discrete sizes of allelic ladder alleles from one lane. Plotted on the Y-axis are the corresponding deviations in sizing obtained for 31 database samples and two other lanes of allelic ladder. The dashed lines represent a one base pair window around the reference allelic ladder (the X-axis).*

alleles compared between runs sometimes varied by $> \pm 0.5$ bp. By performing sizing comparisons ''with-in'' runs only, the ± 0.5 bp window was easily maintained. Furthermore, one base pair precision will routinely allow for the detection and accurate genotyping of potential rare variant alleles that differ in size from an allelic ladder allele by only a single base. If every potential allele (as defined by variation in length, not sequence) can be differentiated, the discrimination power of the system is maximized. For a more thorough explanation of sizing and genotyping, refer to Gill et al. (49) and Lazaruk et al. (26).

Conclusion

The AmpFISTR Blue PCR Amplification Kit is simple to use and highly discriminating. The results of the TWGDAM studies indicate that the assay is accurate and reliable for forensic applications. It is particularly important that the results were reproducibly specific and sensitive; spurious peaks and artifacts were not observed in any of the TWGDAM study results. In using the AmpFISTR Blue Kit with the 377 DNA Sequencer or the 310 Genetic Analyzer, very precise sizing and accurate genotyping can routinely be achieved. To further improve the level of discrimination, throughput, and sample conservation, the simultaneous 4-color detection capability of these instruments can be utilized. This makes possible the multiplexing of STR loci with overlapping size ranges, such as with the AmpFISTR Profiler™ and Profiler Plus™ PCR Amplification Kits, 10-locus coamplification kits which include the AmpFISTR Blue loci.

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DNA samples, including sample B34; and finally, Dan Carlson (PE Applied Biosystems) for figure preparation assistance.

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Additional information and reprint requests:
Jeanette M. Wallin
PE Applied Biosystems Divn
850 Lincoln Centre Dr.
Foster City, CA 94404