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

TWGDAM Validation of AmpFℓSTR™ PCR Amplification Kits for Forensic DNA Casework

REFERENCE: Holt CL, Buoncristiani M, Wallin JM, Nguyen T, Lazaruk KD, Walsh PS. TWGDAM validation of AmpFℓSTR™ PCR amplification kits for forensic DNA casework. J Forensic Sci 2002;47(1):66–96.

ABSTRACT: Laboratory procedures used in short tandem repeat (STR) analysis were subjected to various scenarios that assessed reliability and identified potential limitations. These validation studies were designed as recommended by the Technical Working Group on DNA Analysis Methods (TWGDAM) and the DNA Advisory Board (DAB) (17,18). Various DNA samples were amplified by the polymerase chain reaction (PCR) using AmpFℓSTR™ PCR Amplification Kits (i.e., AmpFℓSTR Green I, Profiler™, Profiler Plus™, and COfiler™ kits), detected with ABI Prism instrumentation, and analyzed using GeneScan and Genotyper software. Data acquired in these studies reinforced an existing body of knowledge and expertise regarding application and interpretation of STR typing in the forensic science community. Consistent STR genotypes were detected in various body tissues and fluids. Inter-laboratory comparisons produced concordant genotype results. Quantitative interpretational aids for DNA mixtures were characterized. Ability of the typing systems to type potentially compromised samples reliably was evaluated. Nonprobative case evidentiary DNA was successfully amplified, genotyped, and interpreted. Potential limitations or cautionary factors in the interpretation of minimal fluorescence intensity were demonstrated. Differential amplification between loci was observed when PCR was inhibited; preferential amplification typically was not. Single AmpFℓSTR locus amplification did not offer consistent benefit over AmpFℓSTR multiplexing, even in cases of DNA degradation or PCR inhibition. During rigorous evaluation, AmpFℓSTR PCR Amplification Kits reproducibly yielded sensitive and locus-specific results, as required in routine forensic analyses.

KEYWORDS: forensic science, DNA typing, AmpFℓSTR, STR, Amelogenin, TWGDAM validation, PCR

Successful application of comparative DNA genotyping in forensic laboratories has evolved from restriction fragment length polymorphism (RFLP) analysis to polymerase chain reaction (PCR)-based DNA typing (e.g., HLA DQA1 and D1S80 loci). Comparative forensic genotyping has again been thrust forward as known short tandem repeat (STR) markers and established laser-induced fluorescence technology have crossed from their widespread use in academic research, clinical applications, etc., and entered forensic science. As in routine genome mapping and linkage studies, multiplex amplification of several STR loci, in one tube, reduced manual processing time but also provided additional benefits to the forensic community, such as sample conservation and minimized contamination risk. Fluorescent STR typing procedures provided simple, rapid, and reliable processing of forensic samples. These PCR systems, whether custom made or supplied commercially, have been adopted into casework labs and DNA databank efforts around the world.

Various STR locus combinations provided the forensic community with a range of choices employing standardized and robust AmpFℓSTR™ procedures. Five STR multiplexes, AmpFℓSTR kits, were designed with one set of amplification and detection conditions through adaptation of locus-specific PCR primer sequences and empirical performance screening (Table 1, see Ref 16). Primer sets were developed that promote sensitive, balanced, and specific signal for 13 STR loci, and the gender related locus Amelogenin. These, when blended in different combinations, yielded the five multiplexes. Kit reagents included PCR reaction mix, AmpliTaq Gold™ DNA polymerase, PCR primer set, positive control DNA (cell line 9947A), allelic ladder, and mineral oil. Kit components and chemistry are identical between the kits, aside from the combination of loci amplified and contained in the allelic ladder.

Reliability of each STR typing kit for forensic applications was systematically evaluated in studies suggested by the Technical Working Group on DNA Analysis Methods (TWGDAM) committee (17) and by the DNA Advisory Board (DAB) (18). While STRs represent a genetic typing milestone in forensic settings, interpretational boundaries and limitations exist. Delineated through validation, knowledge of these has contributed to sound interpretation skills applied by forensic analysts. Validation of AmpFℓSTR multiplex performance, represented by the AmpFℓSTR Blue kit (D3S1358, vWA, and FGA), was published previously (19). Subsequently, AmpFℓSTR PCR primer combinations were expanded. For example, the three AmpFℓSTR Green I STR primer sets were substituted for the three AmpFℓSTR Blue STR primer sets, loci were added to the AmpFℓSTR Blue multiplex to create the AmpFℓSTR Profiler™ and Profiler Plus™ kits, and loci were added to the AmpFℓSTR Green I multiplex to create the AmpFℓSTR COfiler kit (Table 1). TWGDAM Guideline 4.1.3 states that, when new STR loci are added to a validated STR procedure, appropriate studies of limited scope (e.g., population studies, human DNA control value determination) must be performed. AmpFℓSTR COfiler kit validation complied with guideline 4.1.3; this validation standard was exceeded in evaluation of the AmpFℓSTR Green I, Profiler, and Profiler Plus multiplexes, as each of these were subjected to the full set of TWGDAM validation studies. Results of all experiments described herein, conducted on each kit, were either identical or so similar that they had no impact on genotype scoring or on drawing meaningful conclusions. Experimental results serve to reinforce and extend the current knowledge base re-

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

Received 10 July 2000; and in revised form 25 Jan. and 7 May 2001; accepted 7 May 2001.

TABLE 1—*AmpFℓSTR PCR amplification kits.*

Locus (reference)	AmpFℓSTR Blue	AmpFℓSTR Green I	AmpFℓSTR Profiler™	AmpFℓSTR Profiler Plus™	AmpFℓSTR COfiler™
D3S1358 (1)	X		X	X	X
vWA (2)	X		X	X	
FGA (3)	X		X	X	
TH01 (4,5)		X	X		X
TPOX (6)		X	X		X
CSF1PO (7)		X	X		X
D5S818 (8)			X	X	
D13S317 (8)			X	X	
D7S820 (9,10)			X	X	X
D8S1179 (11)				X	
D21S11 (12)				X	
D18S51 (11)				X	
D16S539 (13)					X
AMEL (14,15)		X	X	X	X

garding appropriate application and interpretation of fluorescent STR typing in a caseworking environment.

Methods

Sample Preparation and DNA Extraction

Body fluid stains for Standard Specimens (TWGDAM 4.1.5.4), Reproducibility (TWGDAM 4.1.5.4), Mixed Specimens (body fluids, TWGDAM 4.1.5.5), Environmental (sunlight and temperature, TWGDAM 4.1.5.6), Matrix Studies (TWGDAM 4.1.5.7), and Nonprobative Evidence studies (TWGDAM 4.1.5.8) were prepared on cheesecloth and extracted by criminalists in the DNA Unit of the Santa Clara County District Attorney's Crime Laboratory (San Jose, CA) and/or at the California Department of Justice DNA Laboratory (Berkeley, CA). A bone (femur) sample was prepared as a powder with a hacksaw. Samples were stored at −15 to 25°C for up to four years prior to PCR. All samples were extracted using an organic phenol/chloroform protocol (20) (mixed stains containing semen by differential lysis), with the exception of the following Chelex® resin (21) extractions: six blood and saliva samples (Standard Specimens), four hairs (Standard Specimens), five liquid and dried blood samples (Reproducibility), one donor in purified DNA mixture study, sexual assault victim and suspect reference samples, one donor in hematin study (19), and four donors in denaturing temperature and annealing temperature studies. Single source samples used for peak height ratio and stutter percentage calculations were from random samples collected for population databases and have been reported previously (22). Partially degraded DNA sample series were prepared using 0.005 units/μL Deoxyribonuclease I (DNAse I; Gibco BRL, Gaithesburg, MD) as described previously (19). DNA extracts were quantified using the QuantiBlot® Human DNA Quantitation Kit (Perkin Elmer, Norwalk, CT) (23).

AmpFℓSTR PCR Amplification Kit Reactions

Using AmpFℓSTR Green I, Profiler, Profiler Plus, or COfiler PCR Amplification Kit reagents and suggested protocols, a target DNA amount of 1.0 to 2.5 ng was amplified in 50 μL reaction volumes, unless otherwise noted. For DNA extracts treated with DNAse I, 4 ng template was amplified from the treated samples and 1.5 ng from the time zero control. Samples were amplified in either GeneAmp® Thin-Walled Reaction Tubes in the DNA Thermal Cycler 480 (Perkin Elmer, Norwalk, CT) or MicroAmp® Reaction Tubes with Caps in the GeneAmp PCR Systems 9600 or 2400 (Perkin Elmer). Amplification parameters were those recommended in each AmpFℓSTR User's Manual or Bulletin (24–27) and are the same for each thermal cycler model: initial denaturation (95°C, 11 min), followed by cycles of denaturation (94°C, 1 min), annealing (59°C, 1 min), and extension (72°C, 1 min). A final extension was performed at 60°C for 30 min (AmpFℓSTR Green I) or for 45 min (AmpFℓSTR Profiler, Profiler Plus, and COfiler). All three thermal cycler models were used in TWGDAM sections 4.4.1.4 (Cycle Number) and 4.4.1.9 (Nonhuman Studies).

Sample Electrophoresis and Data Analysis

AmpFℓSTR products were detected with ABI Prism™ 377 or 377XL DNA Sequencers and/or ABI Prism 310 Genetic Analyzers, using procedures and sample volumes as recommended in each AmpFℓSTR User's Manual or Bulletin (24–27), unless otherwise noted. Briefly, for ABI 377 runs, amplified PCR product and GeneScan™-350 or -500 [ROX] Internal Lane Size Standard (PE Applied Biosystems, Foster City, CA) were added to a mixture of dextran blue dye and deionized formamide (deionized as in Ref 26). Denatured samples were loaded on 5% Long Ranger (Pharmacia Biotech Inc., Piscataway, NJ) gels (36-cm well-to-read plates) and run for 2.25 h at 3000 V in 1X TBE running buffer. Data were collected using ABI PRISM 377 Collection software with GeneScan run module "GS Run 36F-2400" (and with "XL Scan" for 50-lane gels). For the ABI PRISM 310 Genetic Analyzer, amplified product and GeneScan-350 or -500 [ROX] Internal Lane Size Standard were added to deionized formamide and denatured. Samples were injected (5 s) and electrophoresed at 15 kV in Performance Optimized Polymer 4 (POP4™, PE Applied Biosystems) in 1X Genetic Analyzer Buffer with EDTA (PE Applied Biosystems). Data were collected using the ABI PRISM 310 Collection software with GeneScan run module "GS STR POP4 (1 mL) F". Electrophoretic results were analyzed using GeneScan Analysis Software and Genotyper® Software, as appropriate. Peak height ratios and stutter calculations were as in Ref 19, except that off-scale data were flagged automatically by GS 3.x software versions.

Results

The following studies of AmpFℓSTR PCR Amplification Kits (Table 1) are presented in the format of the TWGDAM guidelines.

Unless otherwise stated, allele peaks were interpreted when greater than or equal to 100 relative fluorescence units (RFU).

Standard Specimens 4.1.5.1 and Reproducibility 4.1.5.4

> "The typing procedure should have been evaluated using fresh body tissues, and fluids obtained and stored in a controlled manner. DNA isolated from different tissues from the same individual should yield the same type."
>
> "Prepare dried stains using body fluids from donors of known types and analyze to ensure that the stain specimens exhibit accurate, interpretable and reproducible DNA types or profiles that match those obtained on liquid specimens."

Table 2 summarizes the DNA samples used to evaluate genetic typing of standard specimens (fresh body tissues and cadaver samples) as well as reproducibility between liquid and dried blood samples. Genotypes were generated at the 13 AmpFℓSTR loci combinations in the AmpFℓSTR Green I (30 samples), Profiler (33 samples), and Profiler Plus (55 samples) kits. These were identical for a given individual, regardless of tissue/fluid type, liquid/dried state of the sample, or PCR primer combination (kit) used (data not shown).

Consistency 4.1.5.2

> "Using specimens obtained from donors of known type, evaluate the reproducibility of the technique both within the laboratory and among different laboratories."

For each of the AmpFℓSTR kits, two independent laboratories exchanged ten genomic DNA samples. Concordant results were reported for all 20 genotypes (data not shown). Reproducibility at PE Biosystems and at the California Department of Justice DNA Laboratory has been tested with several samples, hundreds of times, using each AmpFℓSTR kit.

Mixed Specimen Studies 4.1.5.5

> "Investigate the ability of the system to detect the components of mixed specimens and define the limitations of the system."

The following studies simulated forensic casework scenarios and assisted in understanding the identification and interpretation of DNA mixtures using the AmpFℓSTR kits (also see Ref 28).

Limit of Detection of the Minor Component

Purified DNA Samples Mixed in Defined Ratios—Mixtures of purified DNA, from two donors (1 ng total template DNA), at ratios from 1:20 to 20:1 were examined for AmpFℓSTR Profiler Plus (Fig. 1, JOE-labeled loci), Profiler, and Green I kits. The limit of detection of the minor component (≥100 RFU) was approximately 1:10, as reported previously (19). This limit is somewhat influenced by the specific combination of genotypes in each mixture.

Various Body Fluids Mixed in Defined Ratios—Various body fluids (blood:blood, saliva:blood, semen:blood, and semen:saliva), from two donors, were combined in defined ratios, by volume, from 50:1–1:50, extracted (differential extraction for stains containing semen), and genotyped using the AmpFℓSTR Profiler and Profiler Plus multiplexes. Typical results are shown in Figs. 2 to 5. As in mixtures of purified, quantitated DNA, the limit of detection occurred when the minor body fluid volume was present at approximately one-tenth that of the major genotype in blood:blood or saliva:blood mixtures, and in epithelial cell fractions of stains containing blood or saliva mixed with semen. In contrast to mixtures of purified, quantitated DNA samples, the concentration of donor DNA samples extracted from actual liquid body fluid mixtures is dependent on the density of nucleated cellular material present in each body fluid sample. Thus, DNA mixtures can be detected from significantly reduced volumes of body fluid(s) from some individuals. For example, blood genotype could be detected at some loci even at the saliva:blood ratio of 50:1, depending on the DNA concentration of the specific fluid samples collected (Fig. 3).

Sperm DNA carryover into the epithelial cell fraction became apparent when semen was mixed at a 1:1 ratio with either blood or saliva (Fig. 4*A* and 5*A*). In sperm fractions from these differential extractions, the male genotype was detectable from every semen:blood or semen:saliva mixture ratio, with no trace of female DNA (Figs. 4*B* and 5*B*). Of course, it is expected that STR typing from differential extractions will sometimes exhibit carryover of DNA between the two fractions, in either direction.

Sexual Assault Evidence—See Section 4.1.5.8, "Nonprobative Evidence," for a discussion of results.

Interpretation of Mixed DNA Samples Using Quantitative Data

Tools for Resolving Contributor Genotypes in Mixtures—Expected values for two quantitative variables (i.e., peak height ratio and polymerase stutter percent) that enhance ability to resolve contributors in a DNA mixture were calculated for AmpFℓSTR loci in single source samples (DNA samples described previously in Ref

TABLE 2—*Sample types analyzed in standard specimen and reproducibility studies.*

Sample State	Body Tissue	AmpFℓSTR Green I Kit	AmpFℓSTR Profiler Kit	AmpFℓSTR Profiler Plust Kit
Fresh Tissues	Blood, saliva, hair; semen or vaginal fluid	7 donors (21 samples)	6 donors (24 samples)	8 donors (30 samples)
Cadaver	Brain, kidney, liver, muscle	√	√	√
Cadaver	Bone	√	√	…
Cadaver	Skin	…	…	√
Liquid	Blood	2 donors	2 donors	5 donors (organic and Chelex)
Dried	Blood	2 donors	2 donors	5 donors (organic and Chelex)

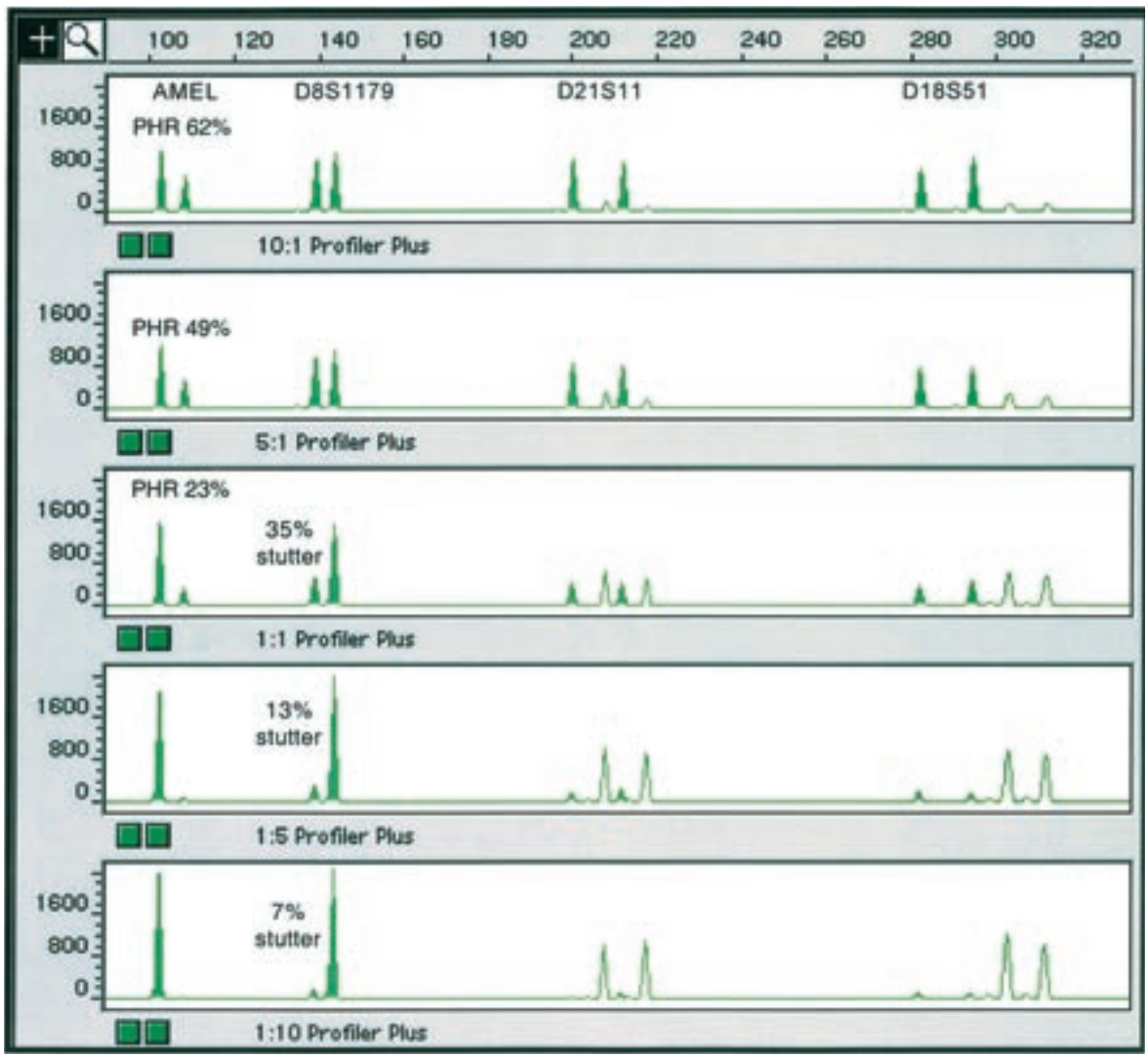

FIG. 1—*Detecting STR mixtures. Electropherograms of AmpFℓSTR Profiler Plus PCR products (JOE-labeled loci) detected on the ABI Prism 310 Genetic Analyzer and analyzed with GeneScan software. Template DNA from two donors, mixed in defined ratios (10:1, 5:1, 1:1, 1:5, 1:10), provide instances where allele number, peak height ratio (PHR), and stutter percent aided in mixture detection and allele assignment. For example, in the third panel (1:1 ratio), there are four alleles at both the D21S11 and D18S51 loci, PHR at Amelogenin (AMEL) is 23% and stutter measured at D8S1179 is 35%. Expected PHR in most unmixed samples of similar peak height is >70%; stutter percentage at D8S1179 is typically less than 12%. To assist viewing, allele peaks attributable to the major genotype shown in the first panel are shaded in subsequent panels. The X- and Y-axes indicate fragment size (bases) and signal intensity (RFU), respectively.*

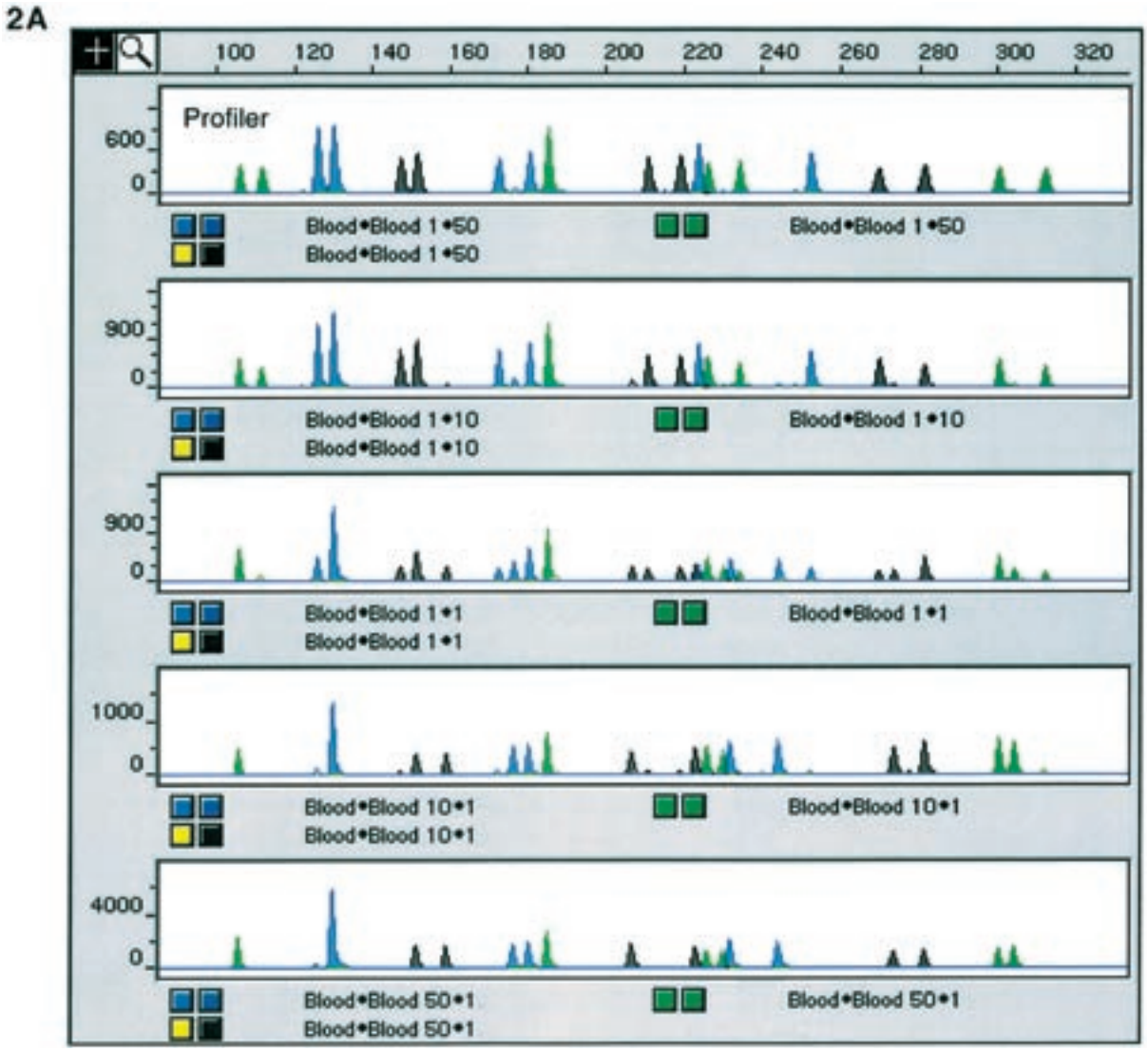

FIG. 2—*Body fluid mixtures: blood from two individuals. Electropherograms (GeneScan software) of PCR products from blood samples mixed in defined ratios (1:50, 1:10, 1:1, 10:1, 50:1) and amplified using the AmpF ℓSTR Profiler and Profiler Plus PCR Amplification Kits are shown in* 2A *and* 2B, *respectively. Figure* 2C *shows the individual donor profiles at the AmpF ℓSTR Profiler Plus loci; a closer view of Profiler Plus results from a one to one mixture of blood (by volume) from these donors is shown in* 2D. *Results in* 2A *were detected on the ABI Prism 377 DNA Sequencer while ABI Prism 310 Genetic Analyzer data are shown in* 2B, 2C, *and* 2D. *The X- and Y-axes indicate fragment size (bases) and signal intensity (RFU), respectively.*

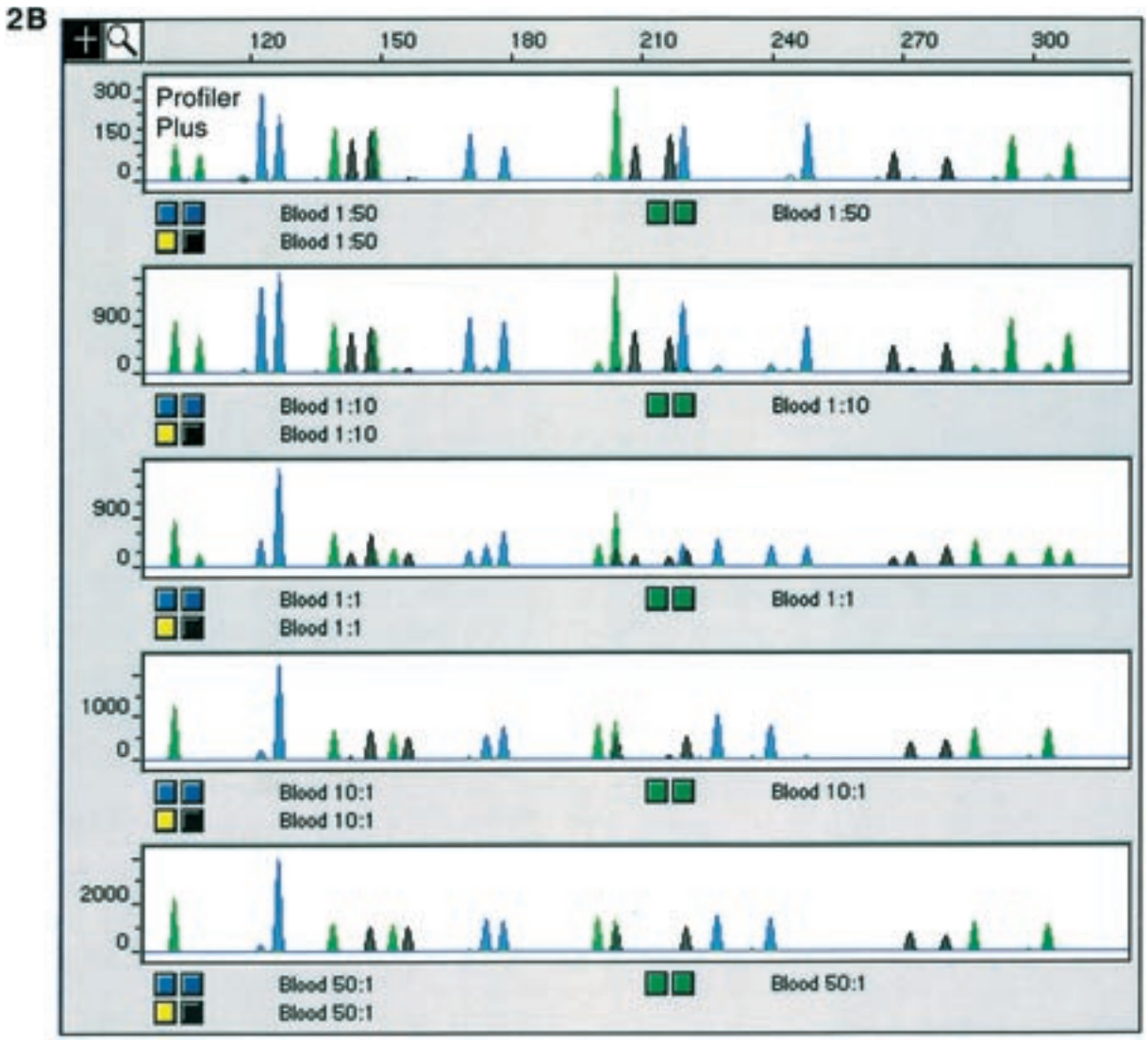

FIG. 2—*(continued)*

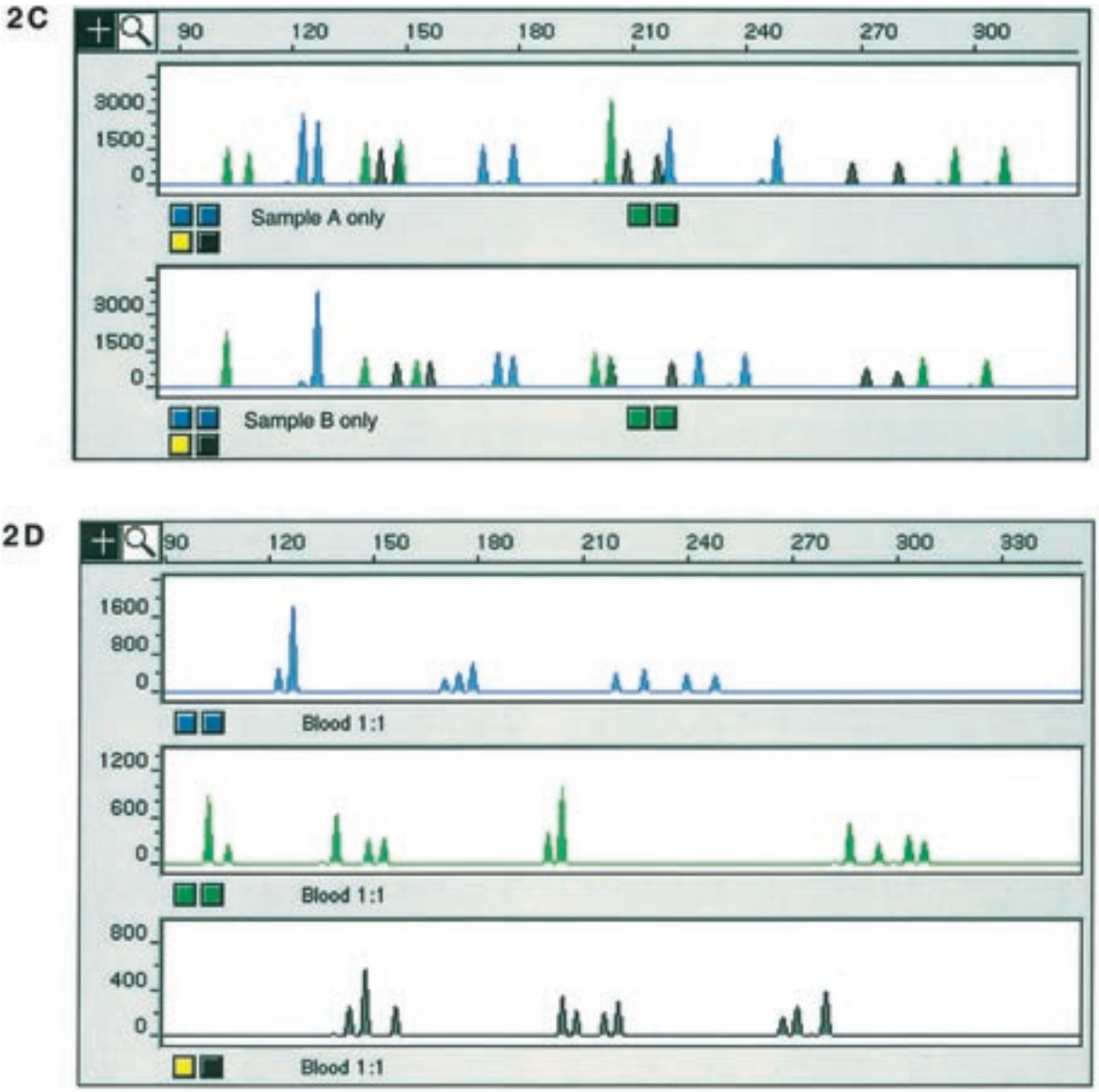

FIG. 2—*(continued)*

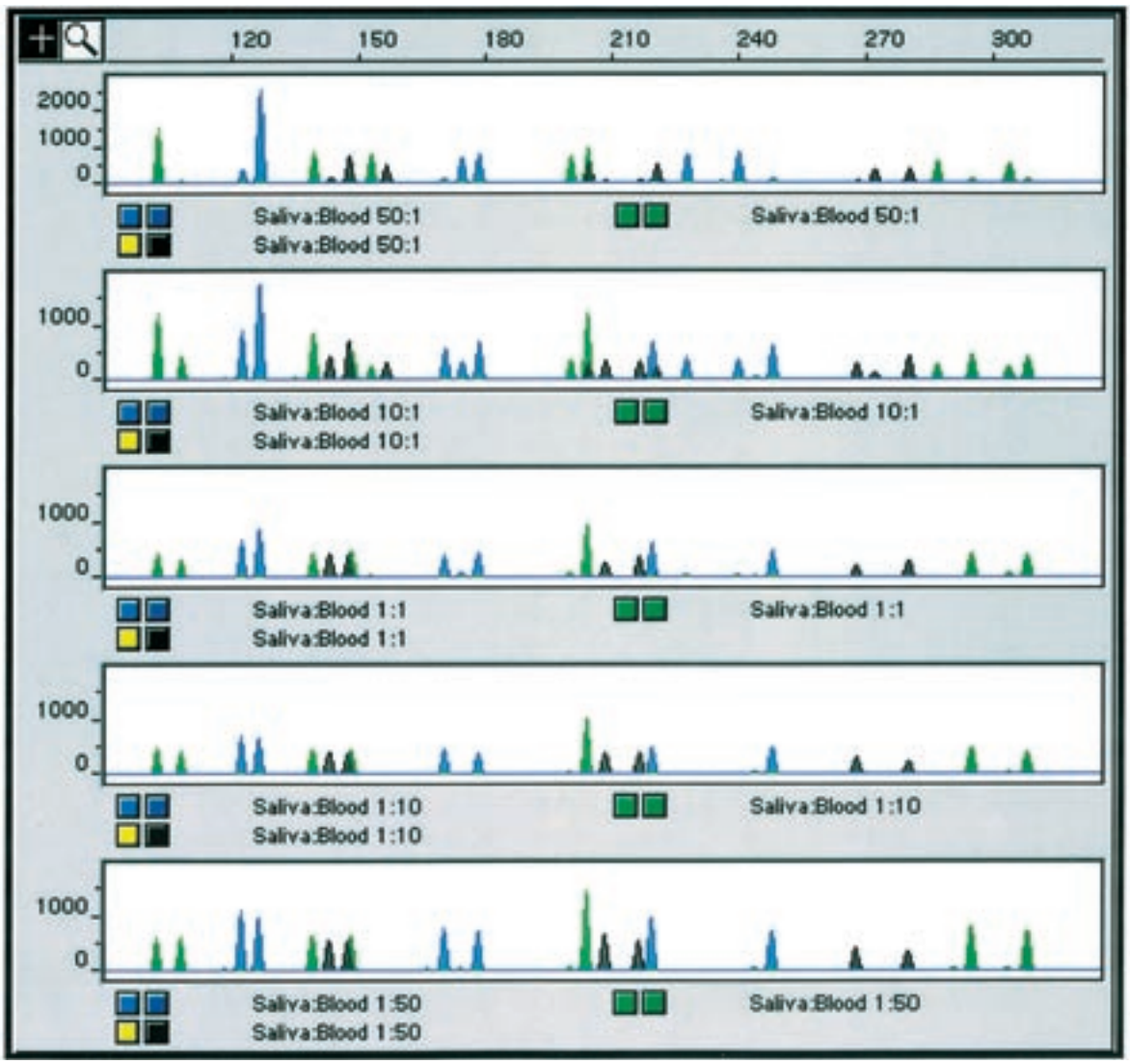

FIG. 3—*Body fluid mixtures: saliva and blood from two individuals. Electropherograms of PCR products from a saliva and a blood sample mixed in defined ratios (50:1, 10:1, 1:1, 1:10, 1:50) and amplified using the AmpFℓSTR Profiler Plus PCR Amplification Kit are shown, as detected on the ABI Prism 310 Genetic Analyzer and analyzed with GeneScan software. The X- and Y-axes indicate fragment size (bases) and signal intensity (RFU), respectively.*

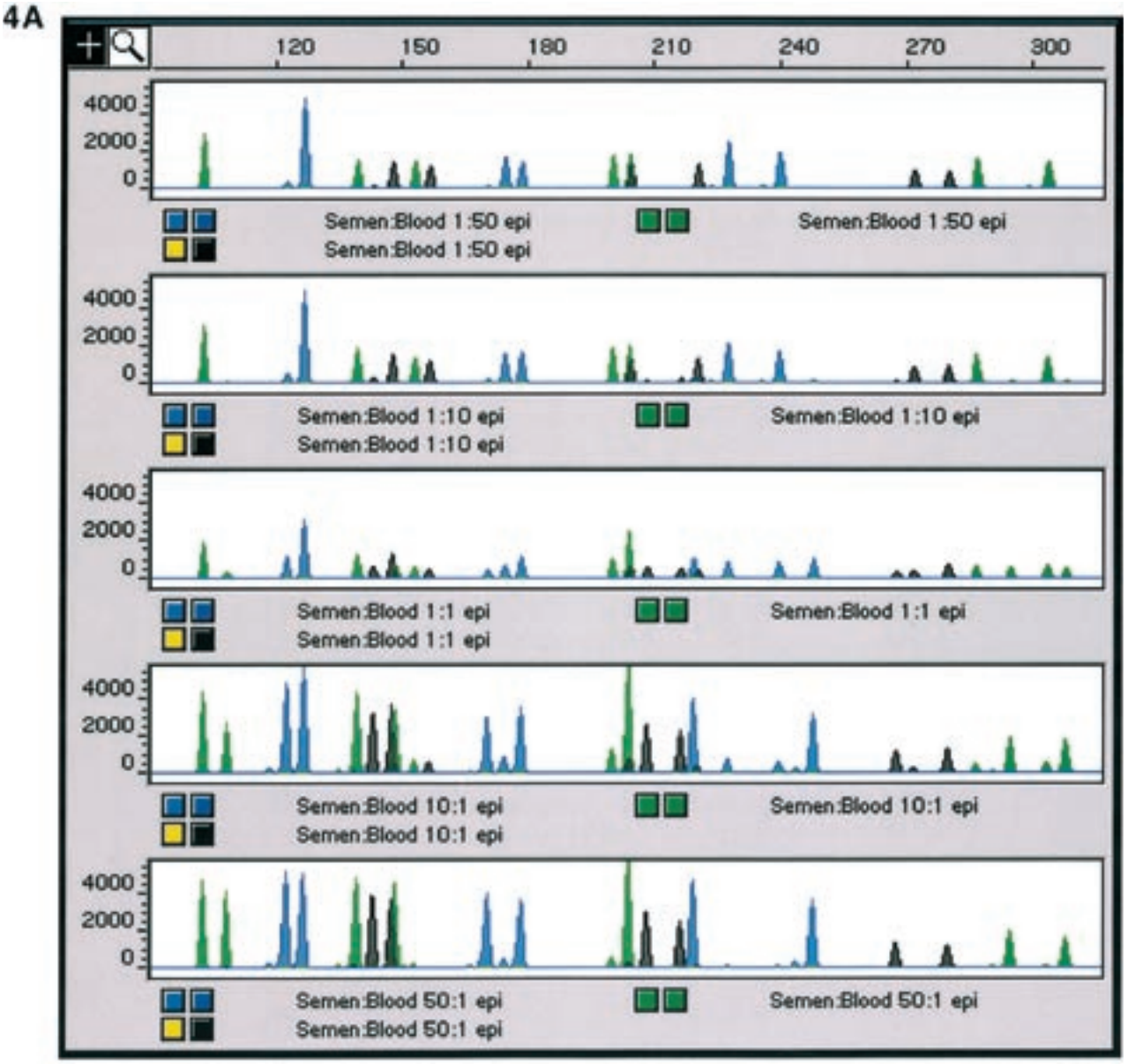

FIG. 4—*Body fluid mixtures: semen and blood from two individuals. Samples of semen and blood were mixed in defined ratios, by volume, (50:1, 10:1, 1:1, 1:10, 1:50) and differentially extracted to produce a nonsperm cell (epithelial cell,* 4A*) and a sperm cell DNA fraction (*4B*). Electropherograms of PCR products generated with the AmpFℓSTR Profiler Plus PCR Amplification Kit are shown, as detected on the ABI Prism 310 Genetic Analyzer and analyzed with GeneScan software. The X- and Y-axes indicate fragment size (bases) and signal intensity (RFU), respectively.*

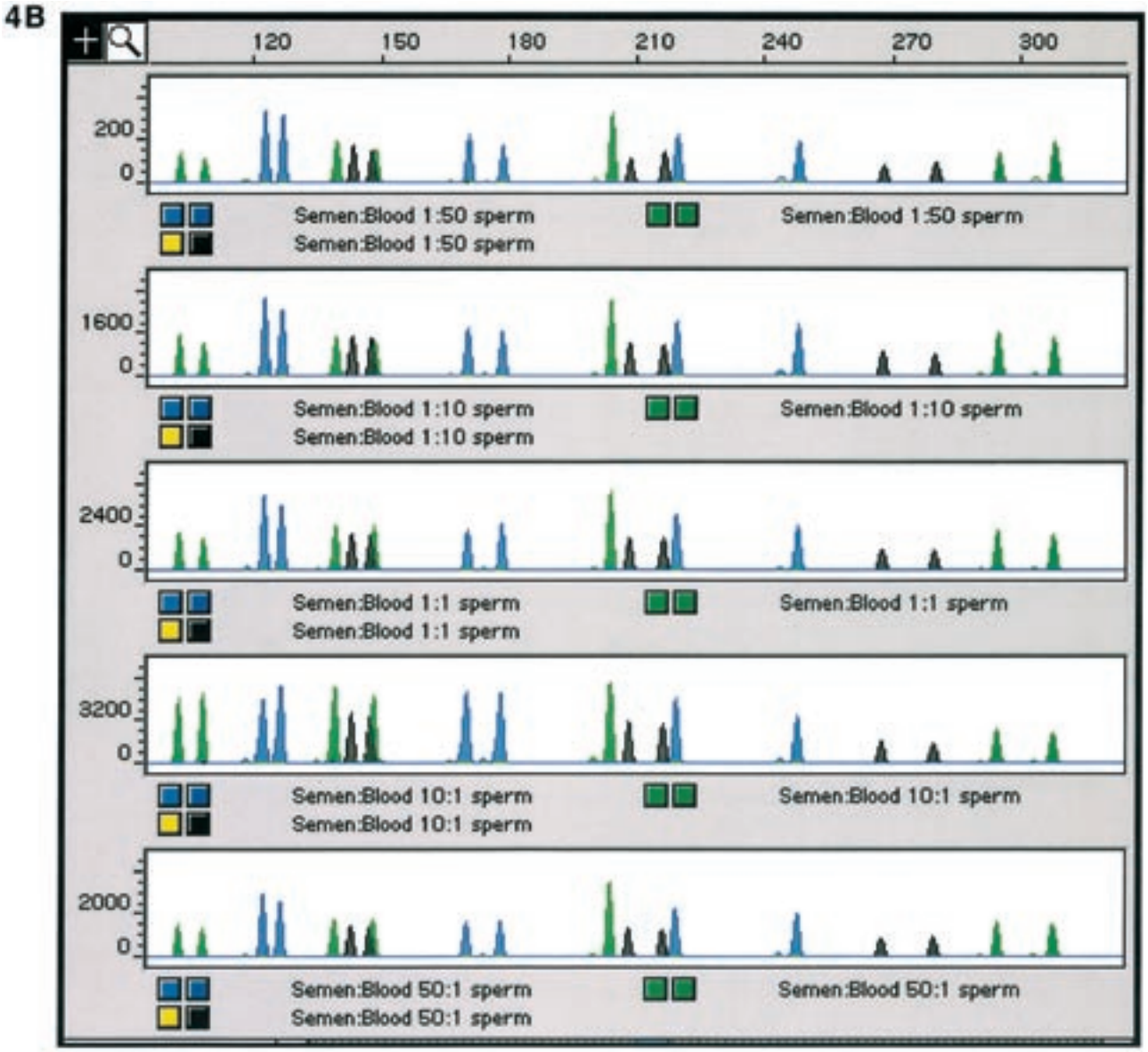

FIG. 4—*(continued)*

22). Such data are invaluable tools in DNA mixture detection and the subsequent resolution of genotype between individuals. Heterozygote peak height ratio (PHR) is defined as the height (RFU) of the lower peak divided by the height of the higher peak, expressed as a percentage. The proportion of DNA polymerase stutter relative to the main allele (stutter percent) was measured by dividing the height of the stutter peak by the height of the main allele peak (19). Because of sample throughput, a majority of these quantitative data were collected on the ABI Prism 377 DNA Sequencer. Smaller scale studies were run on the ABI Prism 310 Genetic Analyzer (>150 samples) and verified the ranges of PHR and stutter percent values as measured on the 377; choice of instrument platform does not pose an important factor in quantitation of these relative peak heights within a DNA sample (data not shown).

Balanced signal between alleles of a heterozygous pair were seen as PHRs averaged between 91% and 93% (Table 3) for all 13 STR loci and amelogenin. Thus, the average difference in peak heights was between 7 and 9%. The most extreme PHR, in 99.7% of all single source sample measurements, is expected to fall within three standard deviations of the mean. The lowest observed mean PHR of 91% (D21S11 and D18S51) and the highest standard deviation of 7% (D21S11), indicate that PHRs less than 70% are rare in normal, unmixed DNA samples. In this manuscript, balanced signal refers to heterozygous PHRs greater than 70%. Factors that can result in imbalanced signal at a locus include very low amounts of template DNA (see section 4.1.5.10), degraded DNA (see section 4.1.5.6), PCR inhibition (see sections 4.1.5.7 and 4.4.1.5), or a primer binding site mutation (16,29). When interpreting DNA mixtures, it is important to analyze and consider all loci, as a PHR <70% may be seen occasionally in a single source sample due to chance alone (see section 4.1.5.10 and Ref 28).

Stutter percentages were reproducible for each allele within an STR locus (Figs. 6 to 9; see Ref 19 for AmpFℓSTR Blue (D3S1358, vWA, FGA) stutter data and plots). Values for this expected PCR artifact were similar when measured in triplex reac-

tions (AmpFℓSTR Blue, Green I, or the three NED-labeled loci) as compared to AmpFℓSTR Profiler multiplex reactions (9 STR loci), from a subset of DNA samples (Table 4 and additional data not shown). Peak height ratio and stutter data were directly transposable to the DNA mixtures, body fluid mixtures, and adjudicated case studies presented in this manuscript.

Environmental Studies 4.1.5.6

> "Evaluate the method using known or previously characterized samples exposed to a variety of environmental conditions. The samples should be selected to represent the types of specimens to be routinely analyzed by the method. They should resemble actual evidence materials as closely as possible so that the effects of factors such as matrix, age and degradative environment (temperature, humidity, UV) of a sample are considered."

Table 5 summarizes the DNA samples and treatments used to evaluate genetic typing of specimens exposed to potentially degradative environments (i.e., enzymatic digestion, sunlight, temperature). As expected and reported previously for PCR-based forensic typing methods, as the average genomic DNA length approached the size of the PCR target sequence, intact template became scarce and thus reduced the yield of PCR product (signal intensity). Of course, then, as the extent of DNA degradation increased, loci failed to amplify in order of decreasing size. For instance, the first loci to show reduced yield were the longest, D18S51 and CSF1PO, followed by D7S820, D16S539, FGA, and so forth in AmpFℓSTR Profiler Plus amplifications. In no instance was a change in genotype observed. Addition-

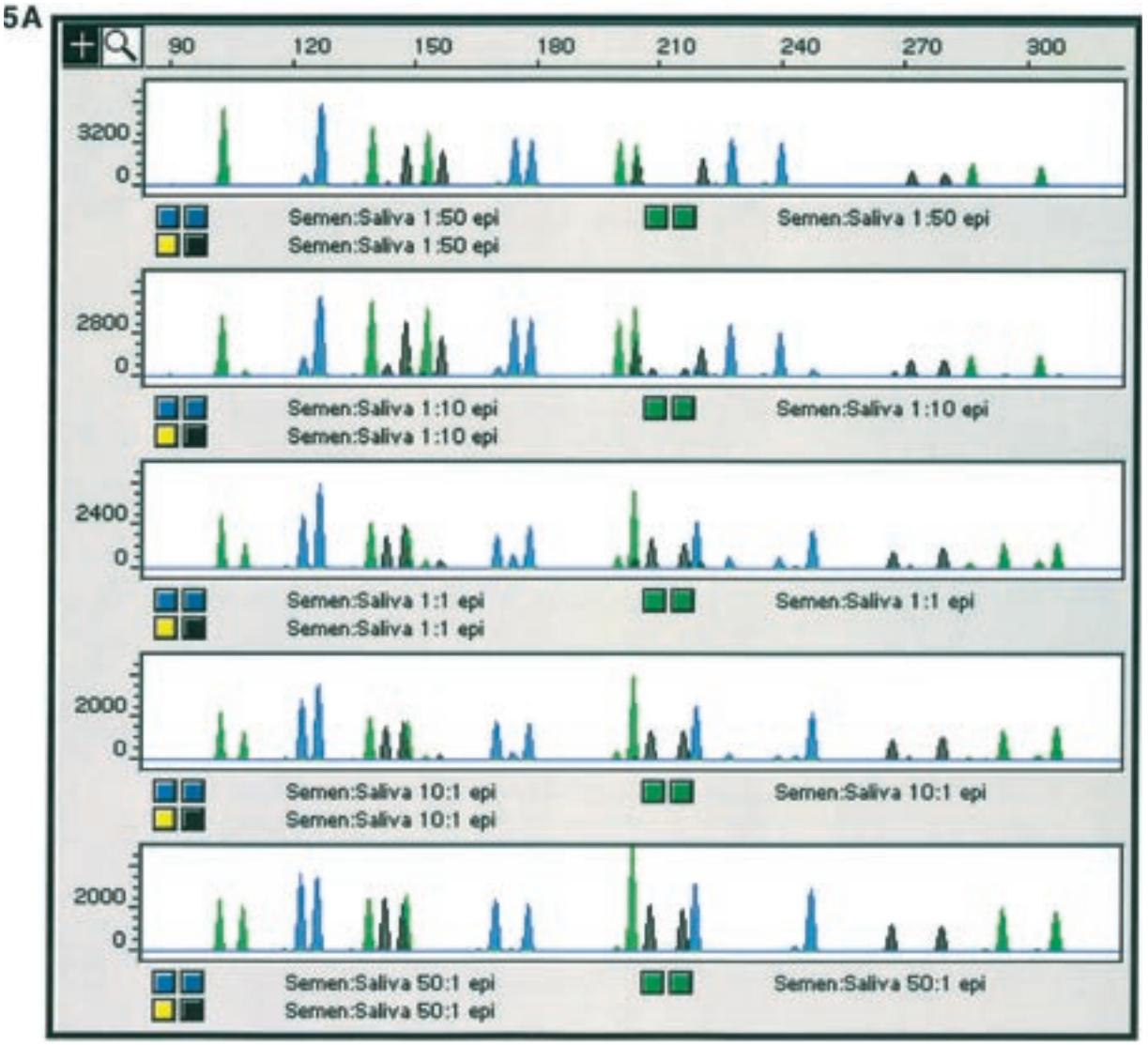

FIG. 5—*Body fluid mixtures: semen and saliva from two individuals. Samples of semen and saliva were mixed in defined ratios, by volume, (1:50, 1:10, 1:1, 10:1, 50:1) and differentially extracted to produce a nonsperm cell (epithelial cell,* 5A*) and a sperm cell DNA fraction (*5B*). Electropherograms of PCR products generated with the AmpFℓSTR Profiler Plus PCR Amplification Kit are shown, as detected on the ABI Prism 310 Genetic Analyzer and analyzed with GeneScan software. The X- and Y-axes indicate fragment size (bases) and signal intensity (RFU), respectively.*

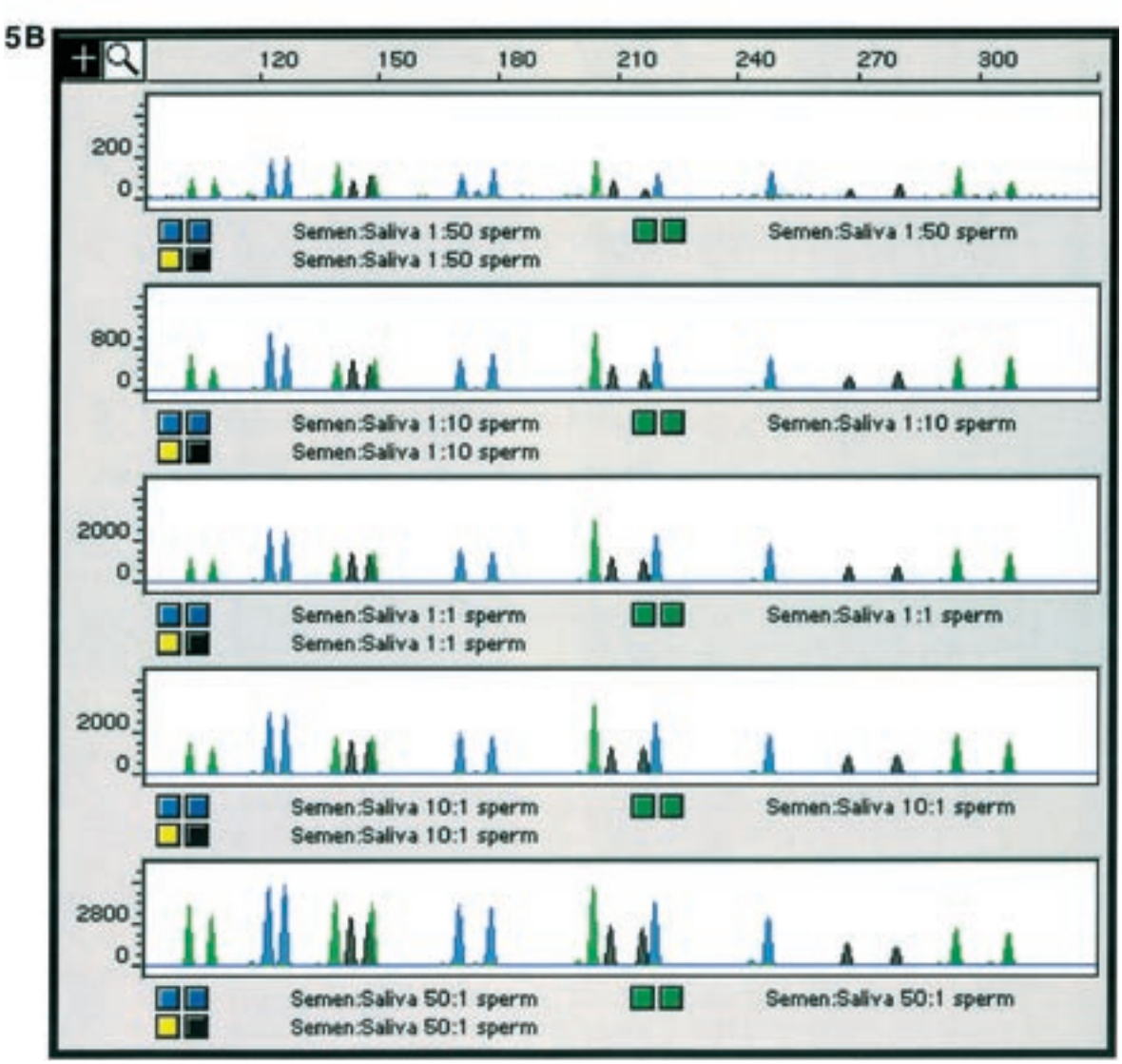

FIG. 5—*(continued)*

TABLE 3—*Summary of observed peak height ratios (PHR) at AmpFℓSTR loci.*

Locus	Peak Height Ratio	
	Mean ± SD*	N
D3S1358	93 ± 4%	68
vWA	93 ± 5%	74
D16S539	92 ± 6%	70
FGA	93 ± 5%	80
Amelogenin	92 ± 6%	78
TH01	92 ± 6%	70
TPOX	92 ± 6%	78
CSF1PO	92 ± 6%	84
D8S1179	92 ± 6%	93
D21S11	91 ± 7%	95
D18S51	91 ± 6%	100
D5S818	92 ± 5%	65
D13S317	93 ± 5%	73
D7S820	93 ± 6%	79

* SD = Standard deviation.

ally, while exhibiting the peak height pattern typical of partially degraded DNA, amplification of DNA in a semen stain stored at room temperature for more than three years yielded results at all ten AmpFℓSTR Profiler loci and all ten AmpFℓSTR Profiler Plus loci (Fig. 10). Furthermore, results reflected those obtained previously with the AmpliType® DQA1 PCR Amplification Kit (e.g., FGA and HLA DQA1 (242bp), of similar base pair size, failed to amplify at the same time point) (data not shown).

Matrix Studies 4.1.5.7

> "Examine prepared body fluids mixed with a variety of commonly encountered substances (e.g., dyes, soil) and deposited on commonly encountered substrates (e.g., leather, denim)."

Table 6 summarizes the DNA samples and matrices used to evaluate genetic typing of specimens exposed to various substrate ma-

terials; 16, 38, and 36 stains were analyzed with the AmpFℓSTR Green I, Profiler, and Profiler Plus kits, respectively. Whether stored for one week (Green I, Fig. 11*A*; Profiler and Profiler Plus, Fig. 11*B* and *C*), or for one year (Profiler Plus, Fig. 11*C*), complete genotypes were obtained for all 126 reactions. Nonspecific artifacts were not detected; samples all yielded genotypes identical to untreated reference samples. Of the AmpFℓSTR Profiler samples extracted after one year on the various substrates, a majority (12 of 14 bloodstains and 22 of 24 semen stains) yielded results for all ten AmpFℓSTR Profiler loci. Information was not generated at the CSF1PO and D7S820 STR loci from one blood sample deposited on leather for one year (Fig. 11*B,* second panel) and CSF1PO signal <100 RFU was detected from one blood sample deposited on blue denim (data not shown). Likewise, CSF1PO, or both CSF1PO and D7S820 failed to amplify, respectively, from one semen sample on dyed cloth upholstery and on nylon for one year (data not shown). It is not surprising that CSF1PO and D7S820 signal strength, two of the longest (bp) AmpFℓSTR loci, would be affected before other loci in the presence of either DNA degradation or PCR inhibition. For each of the four samples where eight or nine AmpFℓSTR Profiler loci amplified, a complete 10-locus genotype was obtained from the other donor on the same matrix (Fig. 11*B,* bottom panel).

Nonprobative Evidence 4.1.5.8

"Examine DNA profiles in nonprobative evidentiary stain materials. Compare the DNA profiles obtained for the known liquid blood versus questioned blood deposited on typical crime scene evidence."

Analysis of nonprobative evidence specimens may be considered the most practical of the validation sample sets as they represent the ultimate application of the testing procedure. DNA extracts from ten adjudicated cases (one unidentified remains, two homicides, and seven sexual assaults) were analyzed using one or more of the AmpFℓSTR kits, as shown in Table 7. When a case was tested using multiple kits (Table 7), genotypes from AmpFℓSTR loci that overlap between kits were identical.

STR typing results from both evidentiary samples in nonprobative Case 1 included the suspect and excluded the victim as a possible source of the bloodstains. Conversely, in Case 2, the evidentiary bloodstain STR profiles were the same as that of the victim; the suspect was excluded as a possible contributor.

Samples containing semen were subjected to differential extraction (nonsperm and sperm cell fractions) in Cases 3 to 9. Complete partitioning between cell fractions was accomplished in sexual assault Cases 4, 6, and 7 (Fig. 12, FAM-labeled loci). In Case 3, victim nonsperm DNA was carried over into the sperm cell fraction from the victim's panties. The sperm cell STR profile (major genotype) excluded the suspect and was the same as the STR profile of a known boyfriend. The suspect in Case 4 was excluded as a possible source of sperm; suspect had no alleles in common with those of the sperm fraction. For Case 5, the DNA profile of the sperm on a vaginal swab and the DNA profile of the nonsperm fraction minor component on a penile swab (incomplete partitioning) were

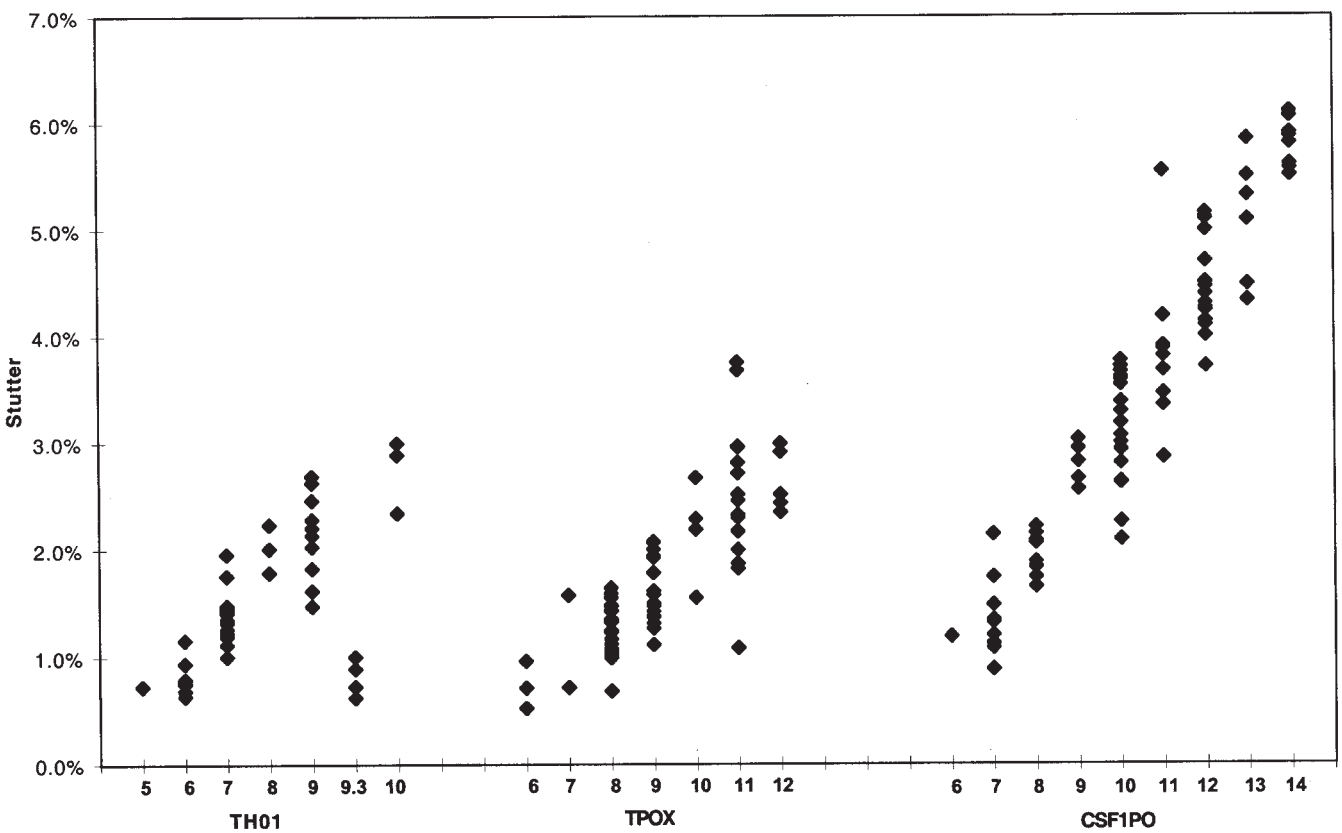

FIG. 6—*Stutter percentages for the AmpFℓSTR Green I STR loci, TH01 (min 0.6%, max 2.9%, SD 0.4%,* n *47), TPOX (min 0.5%, max 3.8%, SD 0.4%,* n *70), and CSF1PO (min 0.9%, max 6.1%, SD 0.4%,* n *83) measured in single source DNA samples. Default stutter filters in AmpFℓSTR Genotyper templates are 7% for TH01 and TPOX and 9.1% for CSF1PO (32). The X- and Y-axes indicate allele number and stutter percent, respectively. Min = minimum measured value, max = maximum measured value, SD = average standard deviation across all alleles measured,* n *= number of observations.*

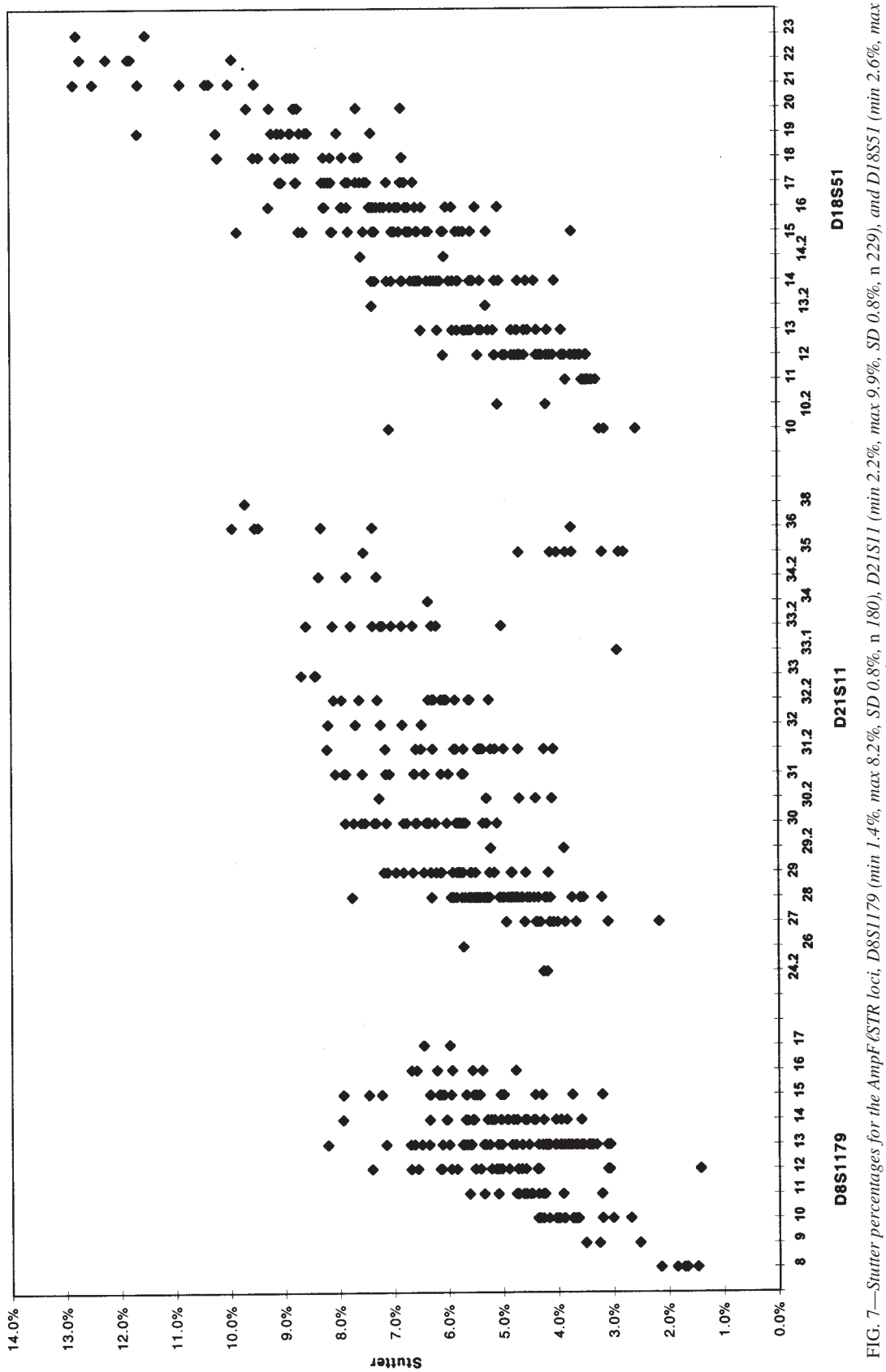

FIG. 7—*Stutter percentages for the AmpFℓSTR loci, D8S1179 (min 1.4%, max 8.2%, SD 0.8%,* n *180), D21S11 (min 2.2%, max 9.9%, SD 0.8%,* n *229), and D18S51 (min 2.6%, max 12.8%, SD 0.8%,* n *235) measured in single source DNA samples. Default stutter filters in AmpFℓSTR Genotype templates are 10.7% for D8S1179, 13% for D21S11, and 15.3% for D18S51 (32). The X- and Y-axes indicate allele number and stutter percent, respectively. Min = minimum measured value, max = maximum measured value, SD = average standard deviation across all alleles measured,* n *= number of observations.*

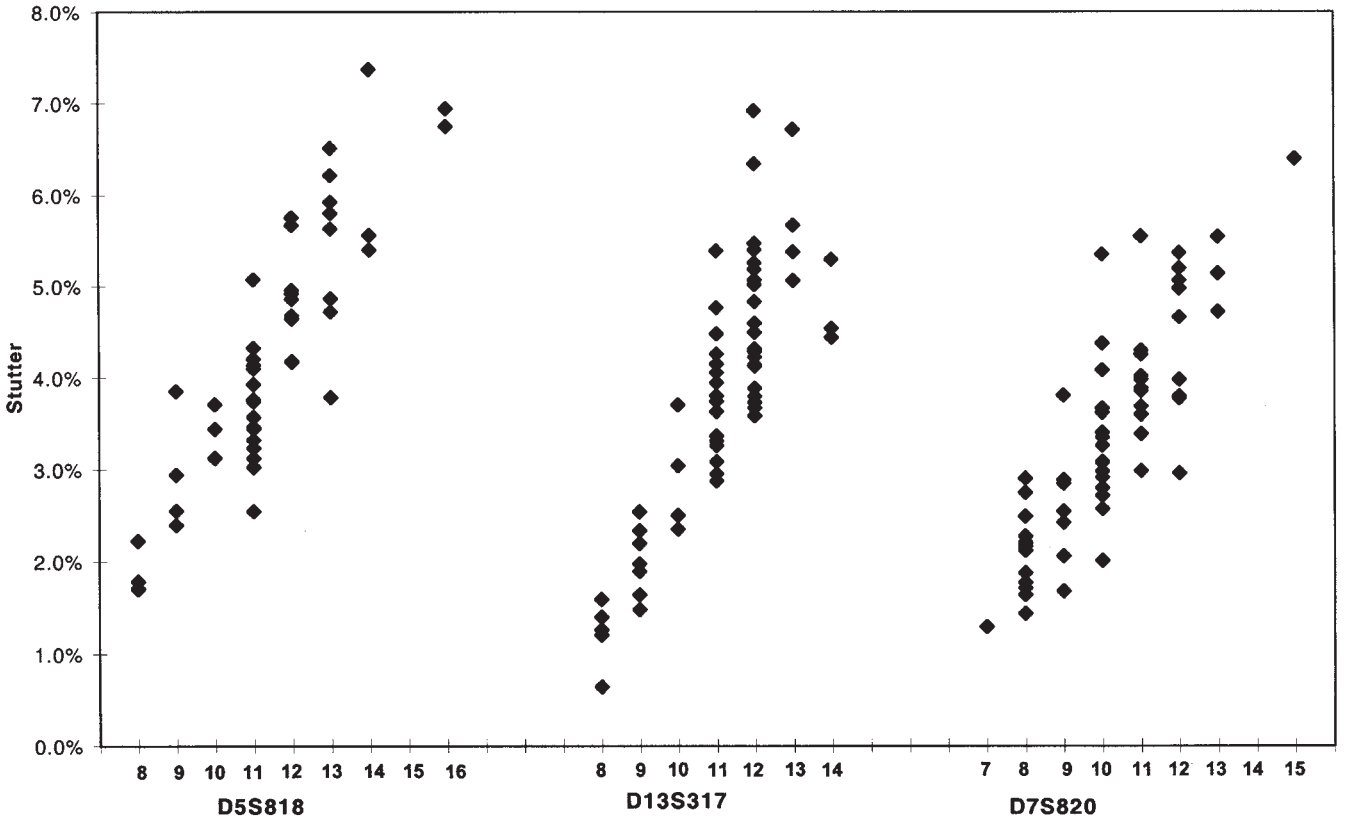

FIG. 8—*Stutter percentages for the AmpFℓSTR loci, D5S818 (min 1.7%, max 7.4%, SD 0.6%,* n *51), D13S317 (min 0.6%, max 6.9%, SD 0.6%,* n *60), and D7S820 (min 1.3%, max 6.4%, SD 0.6%,* n *95), measured in single source DNA samples. Default stutter filters in AmpFℓSTR Genotyper templates are 10.7% for these three loci (32). The X- and Y-axes indicate allele number and stutter percent, respectively. Min = minimum measured value, max = maximum measured value, SD = average standard deviation across all alleles measured,* n *= number of observations.*

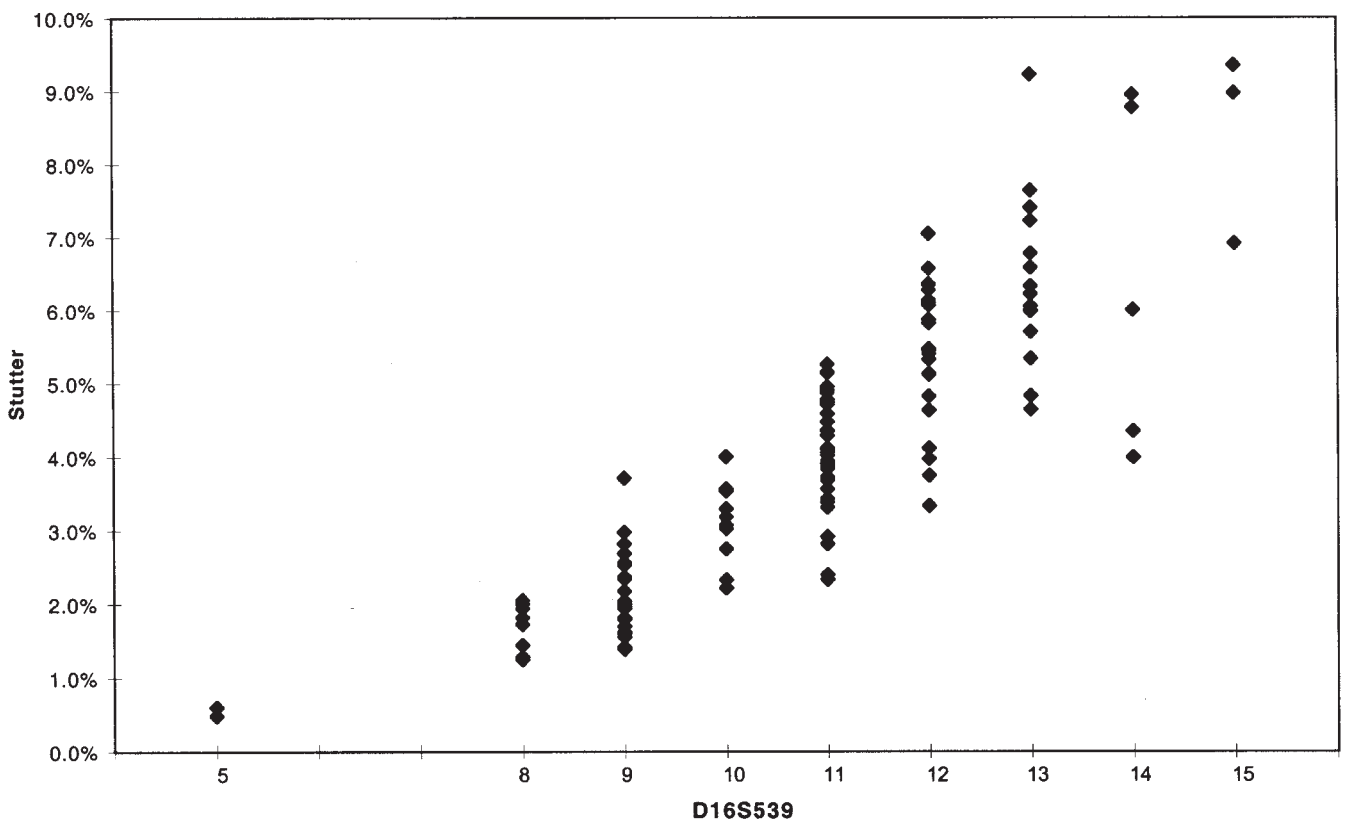

FIG. 9—*Stutter percentages for the AmpFℓSTR locus D16S539 (min 0.5%, max 9.4%, SD 0.9%,* n *130) measured in single source DNA samples. The default stutter filter in AmpFℓSTR Genotyper templates is 13% for D16S539 (32). The X- and Y-axes indicate allele number and stutter percent, respectively. Min = minimum measured value, max = maximum measured value, SD = average standard deviation across all alleles measured,* n *= number of observations.*

TABLE 4—*Stutter percentages at AmpFℓSTR loci in triplex and multiplex PCR reactions.*

			AmpFℓSTR Kit		
			Blue (triplex)	Green (triplex)	Profiler (multiplex)
Locus	Alleles	N	Max Stutter ± SD		
D3S1358	15, 18	27	6.7 ± 0.4	…	7.3 ± 0.4
vWA	14, 16, 17, 19	37	8.8 ± 0.3	…	8 ± 0.3
FGA	19, 21, 24	33	6.9 ± 0.3	…	7.4 ± 0.4
TH01	7, 9.3	35	…	2.3 ± 0.3	2.7 ± 0.3
TPOX	8, 11	19	…	3.3 ± 0.4	2.7 ± 0.4
CSF1PO	9, 10, 11, 12	39	…	4.7 ± 0.4	5.2 ± 0.4

N = total number of observations.

TABLE 5—*Sample types analyzed in environmental studies.*

Sample Type	Treatment	Timepoints*	AmpFℓSTR Green I	AmpFℓSTR Profiler	AmpFℓSTR Profiler Plus
DNA	DNAse I	0, 0.5, 1, 4, 8 min	1 donor	1 donor	1 donor
Bloodstains	Sunlight, shade 4°C, RT, 37°C, 65°C	0, 1w, 1, 2, 6, 12 mo	1 donor	…	…
Bloodstains	Sunglight, shade RT, 37°C	0, 1, 4 mo, 1y	…	2 donors	2 donors
Semen Stains	Sunlight, shade 4°C, RT, 37°C, 65°C	0, 1w, 1, 2, 6, 12 mo	1 donor	…	…
Semen Stains	Sunlight, shade RT, 37°C	0, 1, 4 mo, 1y	…	2 donors	2 donors

* Room temperature (RT), minutes (min), week (w), month (mo), year (y).

identical to those of the suspect and victim, respectively. The suspects were included as possible semen donors in Cases 6 and 7; no suspect was developed in Case 8. The sperm fraction from Case 8 revealed a mixture of semen donor DNAs, in possible accordance with the victim's account of multiple assailants (Fig. 13, FAM-labeled loci). Alleles foreign to the victim in Case 9 were not detected in the nonsperm cell fraction; sperm fraction major genotype (incomplete partitioning) included the suspect (Fig. 14, FAM-labeled loci).

Case 10 involved unidentified human remains recovered after two months outdoors. Because the father of the missing person in question was unavailable, samples were collected from the putative mother and three putative siblings (two brothers and one sister). Each sample was typed with the AmpFℓSTR Green I and Profiler Plus kits; typing results from a questioned bone were compared with the putative family members (Fig. 15, NED-labeled loci). At every Green I and Profiler Plus locus, one allele was shared between the bone and the putative mother. At least one sibling possessed the nonmaternal allele at each locus (see Fig. 15, additional data not shown). Results coincided with initial typing with the AmpFℓSTR Blue kit (19). Additionally, amelogenin typing of the questioned bone indicated a female individual, the same sex as the putative victim. AmpFℓSTR typing results thus included a daughter of the mother and father of the tested siblings as a possible donor of the questioned bone.

Minimum Sample 4.1.5.10

"Where appropriate, establish quantity of DNA necessary to obtain a reliable typing result."

When template DNA copy number is low, stochastic PCR effects (i.e., chance allele sampling) may result in a substantial imbalance in fluorescent signal between the PCR products (two fluorescent allele peaks) of a heterozygote. Sensitivity studies were conducted for each AmpFℓSTR kit to understand the response to low template copy number at AmpFℓSTR loci (data not shown, see Ref 16). Additional studies using the AmpFℓSTR Profiler Plus kit were conducted as well. Human DNA templates consisted of three known proficiency test samples (CTS-98-3 #1, #2, and #3), 9947A (AmpFℓSTR kit control), and heterozygote standard D14749 (Serological Research Institute, Hayward CA). Serial dilutions of templates (2, 1, 0.5, 0.25, and 0.125 ng) were amplified and detected using the ABI Prism 310 Genetic Analyzer. 9947A and D14749 amplifications were prepared in triplicate and each was injected three times.

Resulting fluorescent peaks were categorized with respect to peak height. Peak heights ratios (PHR, see section 4.1.5.5) were measured from heterozygous loci and plotted (Fig. 16). As peak height decreased, a concomitant reduction in the minimum and mean fluorescent signal intensity balance between alleles (heterozygotes), measured by peak height ratio (PHR), occurred (Fig. 16 and Table 8). While more likely to occur at lower signal intensities, imbalanced heterozygote peak heights (<70% PHR), were not seen in every amplification that yielded weak signal. Between 150 and 300 RFU, a peak height ratio of <60% was measured twice in 140 analyzed allele pairs (234, 410 RFU) and (214, 368 RFU). In the fluorescent signal range of 300 to 1000 RFU, a peak height ratio of <70% was measured in 3 of 233 analyzed allele pairs, as follows: (316, 547 RFU), (420, 668 RFU), and (436, 663 RFU).

Analysis of empirical data revealed that a single allele peak of <100 RFU *could* have a sister allele <50 RFU (for the following discussion signal less than 50 RFU is considered at or near background noise). In every instance where the peak height of one allele of a heterozygote fell below 50 RFU, it was still visible in the electropherogram. *Visual detection* of only one allele peak at a truly heterozygous locus was not seen until fluorescent signal of both alleles was less than 50 RFU. While these data describe a conservative aspect of the 100 RFU "cautionary zone," close scrutiny of a single allele peak less than 100 RFU, for the potential of a sister allele of very low signal, may be in order. Individual laboratories may find it useful to determine the lower limit of their detection system independently.

Amplification—Number of Cycles 4.4.1.4

> "The number(s) of cycles necessary to produce reliable results must be determined."

AmpFℓSTR reactions (1.5 ng DNA template) were amplified for 27, 28, 29, and 30 cycles (AmpFℓSTR Green I and Profiler, DNA Thermal Cycler 480 and GeneAmp PCR System 9600) or

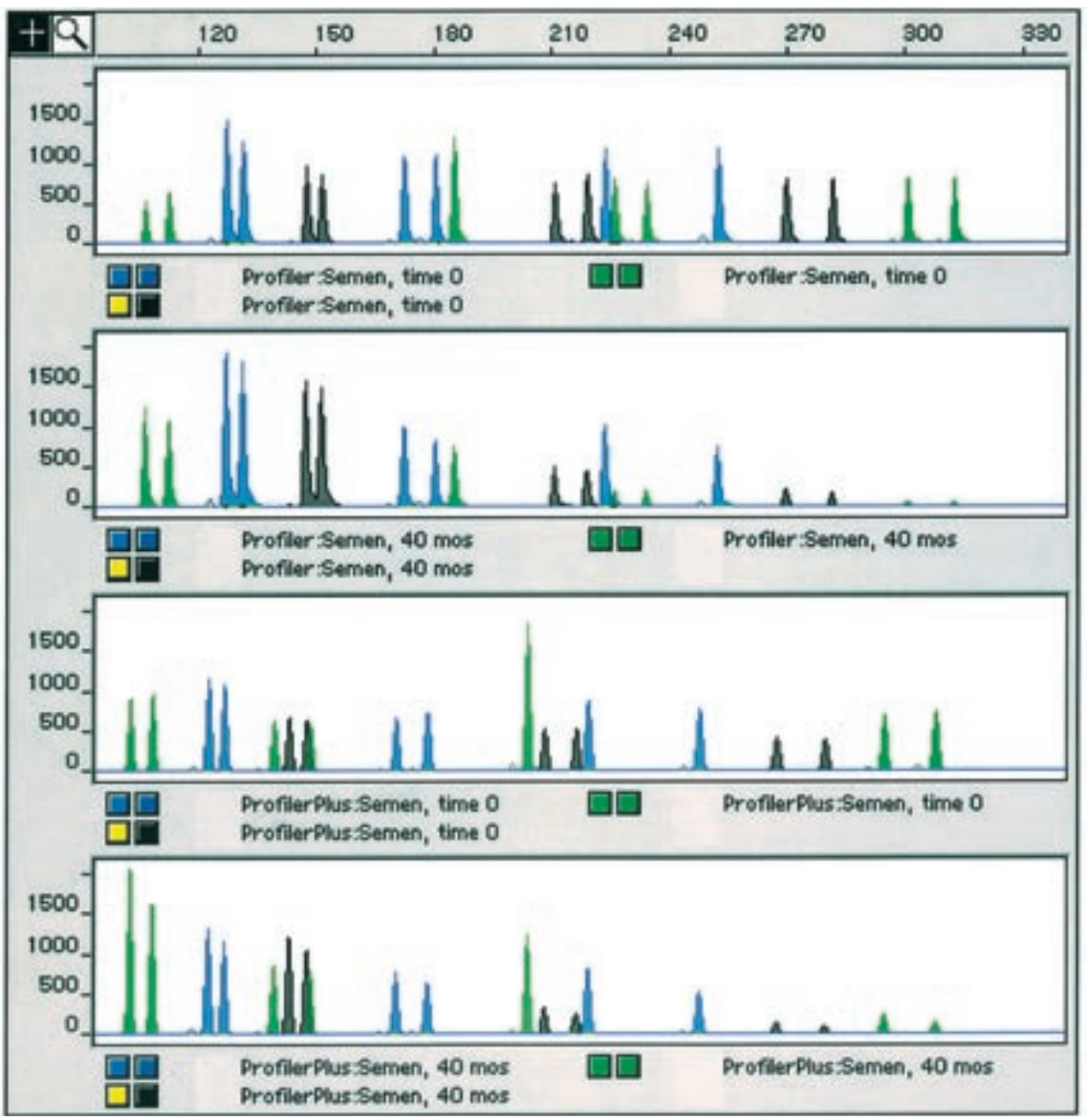

FIG. 10—*Environmental study: Age. Electropherograms (GeneScan software) of PCR products from untreated DNA (panels one and three) and DNA from a semen stain stored at room temperature for more than 40 months (panels two and four) are shown. The top two panels show results from AmpFℓSTR Profiler PCR Amplification Kit amplifications (ABI Prism 377 DNA Sequencer); Profiler Plus kit results detected on the ABI Prism 310 Genetic Analyzer are shown in the bottom two panels. The X- and Y-axes indicate fragment size (bases) and signal intensity (RFU), respectively.*

TABLE 6—*Sample types analyzed in matrix (substrate) studies.*

Sample (Timepoint(s))*	Matrix†	AmpFℓSTR Green I	AmpFℓSTR Profiler	AmpFℓSTR Profiler Plus
Blood (1w)	Bk & b denim, carpet, cotton panty, swab, leather, leaves, newspaper, wool	1 donor	…	…
Blood (1w, 1y)	Wool, cotton, nylon, leather, b denim, glass, metal	…	2 donors	2 donors
Semen (O/N)	Bk & b denim, carpet, cotton panty, newspaper, nylon, soil	1 donor	…	…
Semen (1w, 1y)	Wool, cotton, nylon, leather, b denim, acetate, vinyl, upholstery, facial tissue, condoms‡, latex glove	…	2 donors	2 donors
Semen (1w, 1y)	Dyed cloth upholstery (red)	…	2 donors	…

* All at room temperature (RT) either overnight (O/N), or for 1 week (w) and 1 year (y).
† Black (bk), blue (b).
‡ One with a spermicide (5% nonoxynol-9) and one with a water soluble lubricant.

for 27, 28, 29, 30, and 31 cycles (AmpFℓSTR Profiler Plus, DNA Thermal Cycler 480 and GeneAmp PCR Systems 9600 and 9700). Peak heights greater than 100 RFU (see 4.1.5.10 Minimum Sample) were obtained for all cycle numbers tested (data not shown). After 31 cycles, signal at some loci exceeded the dynamic range of the ABI Prism 310 Genetic Analyzer and 377 DNA Sequencer fluorescent detection system (8191 RFU, raw data) (data not shown). No tested cycle number produced nonspecific fluorescent peaks. The manufacturer's recommended cycle number gave optimal sensitivity on ABI Prism instruments (see Ref 16 for additional information regarding dynamic range and sensitivity levels).

Amplification—Differential and Preferential Amplification 4.4.1.5

> "Potential for differential amplification must be assessed and addressed."

Differential amplification is here defined as increased amplification of a locus, compared with the other loci labeled with the same dye color, in a coamplified system. This difference may cause information loss if a primer set that is susceptible to weak signal is included in a multiplex. Preferential amplification is used here to describe a difference in the amplification efficiency of two alleles at a single locus, present in quantities sufficient for amplification (i.e., this is not stochastic sampling; see section 4.1.5.10), that produces less than 70% PHR in a single source sample (see section 4.1.5.5 Mixed Specimen Studies). We investigated the potential for preferential amplification (~1–2 ng template DNA) by measuring PHRs in a cross section of multilocus genotypes (Table 4). In no case was preferential amplification within a locus observed. Effects of a range of thermal cycling denaturation and annealing temperature on differential and preferential amplification are presented in Ref 16. Effects of three additional variables are presented below.

Widespread Allele Sizes

Observation of preferential PCR amplification of alleles, in systems that are scored based on length polymorphism, may be more likely between alleles that differ significantly in base pair size. Since many STR loci have small size ranges (AmpFℓSTR average of ~40 bp), the potential for preferential amplification is relatively low. We identified two DNA samples that contained unusually "widespread" alleles and evaluated their amplification in AmpFℓSTR Profiler and Profiler Plus reactions. FGA alleles in one sample were separated by 90 bp and D18S51 alleles in a different sample were separated by 44 bp. In one atypical amplification out of several replicates, a PHR of ~52% was seen between widespread FGA alleles (~500RFU for the 240 bp allele, ~260 RFU for the 330 bp allele) from 1 ng template DNA. Balanced peak heights were seen in the same experiment with 0.5 ng or 2.5 ng template and in additional 1 ng amplifications (i.e., imbalance was not reproducible; see section 4.1.5.5), suggesting that the peak height imbalance may not be due to preferential amplification, but to chance. Balanced signal was detected between the "widespread" D18S51 alleles.

Challenging PCR by Inhibition

Duplicate AmpFℓSTR Green I, Profiler, Profiler Plus, and COfiler amplifications of two DNA samples and of AmpFℓSTR Control DNA 9947A were performed in the presence of varying concentrations of hematin (0 to 24 μM), as described previously (19). Differential amplification was observed, by definition, as PCR was inhibited and longer loci failed to amplify at the higher hematin levels. Moreover, as the concentration of hematin was increased, the overall yield of PCR products was reduced (Fig. 17). Preferential amplification was observed once in the presence of 24 μM hematin, at the vWA locus (i.e., peak height ratio ~50%), in an AmpFℓSTR Profiler reaction; both alleles were >100 RFU and easily visible (data not shown here, see Figure in Ref 25). Amplification of all alleles in this sample with sizes greater than the vWA alleles (TH01, D13S317, FGA, TPOX, D7S820, and CSF1PO) was inhibited.

Degraded DNA

Differential amplification results are presented in "Environmental Studies" section. Preferential amplification of alleles within an STR locus was not observed at any timepoint following enzymatic treatment with DNAse I.

Amplification—Single Locus Versus Multilocus Amplification 4.4.1.6

> "Where more than one locus is amplified in one sample mixture, the effects of such amplification on each system (alleles) must be addressed and documented."

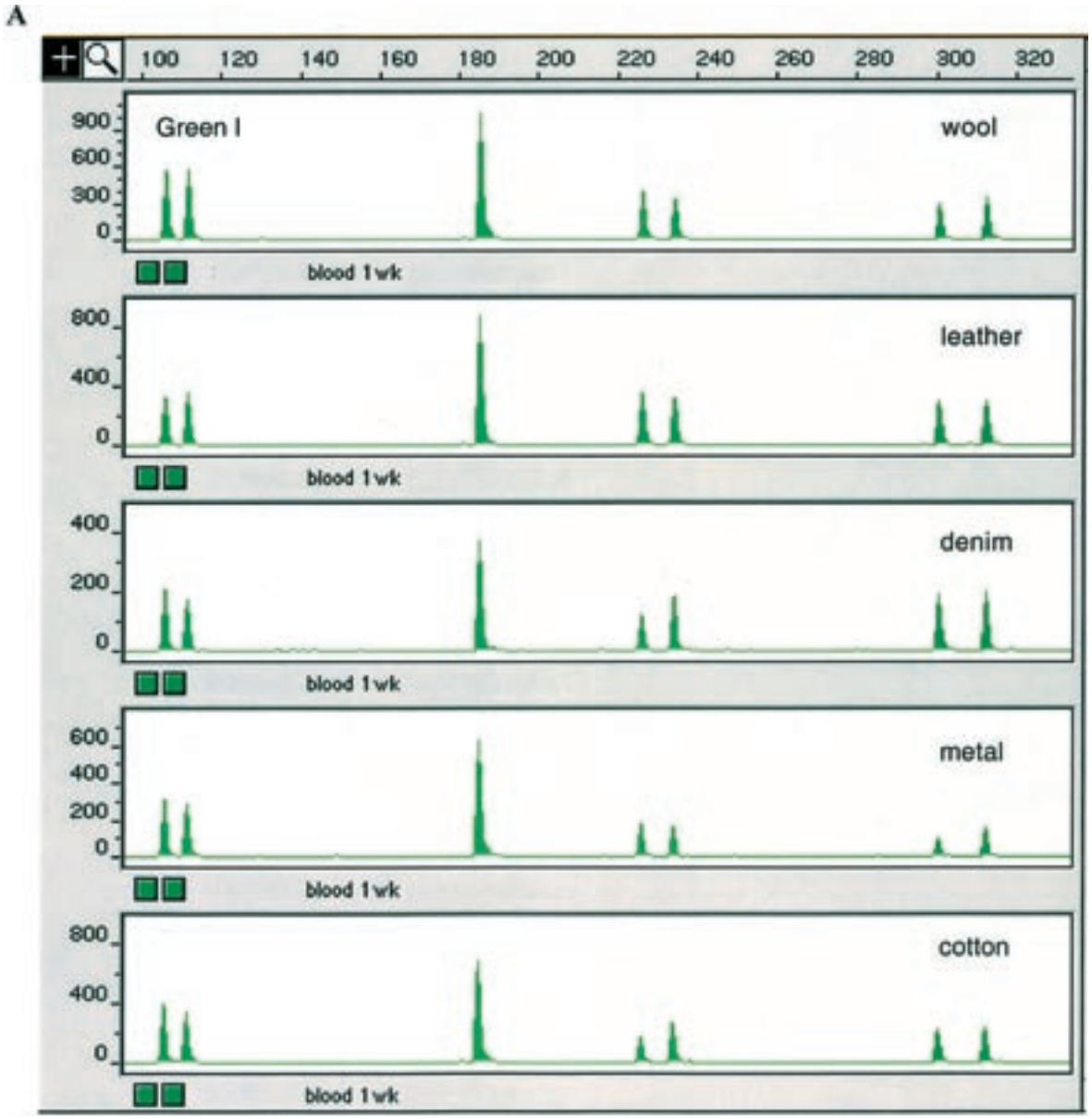

FIG. 11—*Matrix and age study. Panel* (A) *shows electropherograms (GeneScan software) of PCR products (AmpFℓSTR Green I kit) from bloodstains (one donor) deposited on a variety of substrate materials overnight and detected on the ABI Prism 377 DNA Sequencer. Results from bloodstains from two donors are shown in* (B) *and* (C) *for the AmpFℓSTR Profiler and Profiler Plus kits, respectively. Blood was deposited on leather for one week (first and third panels) and for one year (second and fourth panels). ABI Prism 377 DNA Sequencer data are shown in* (B)*; results in* (C) *were detected on the ABI Prism 310 Genetic Analyzer. The X- and Y-axes indicate fragment size (bases) and signal intensity (RFU), respectively.*

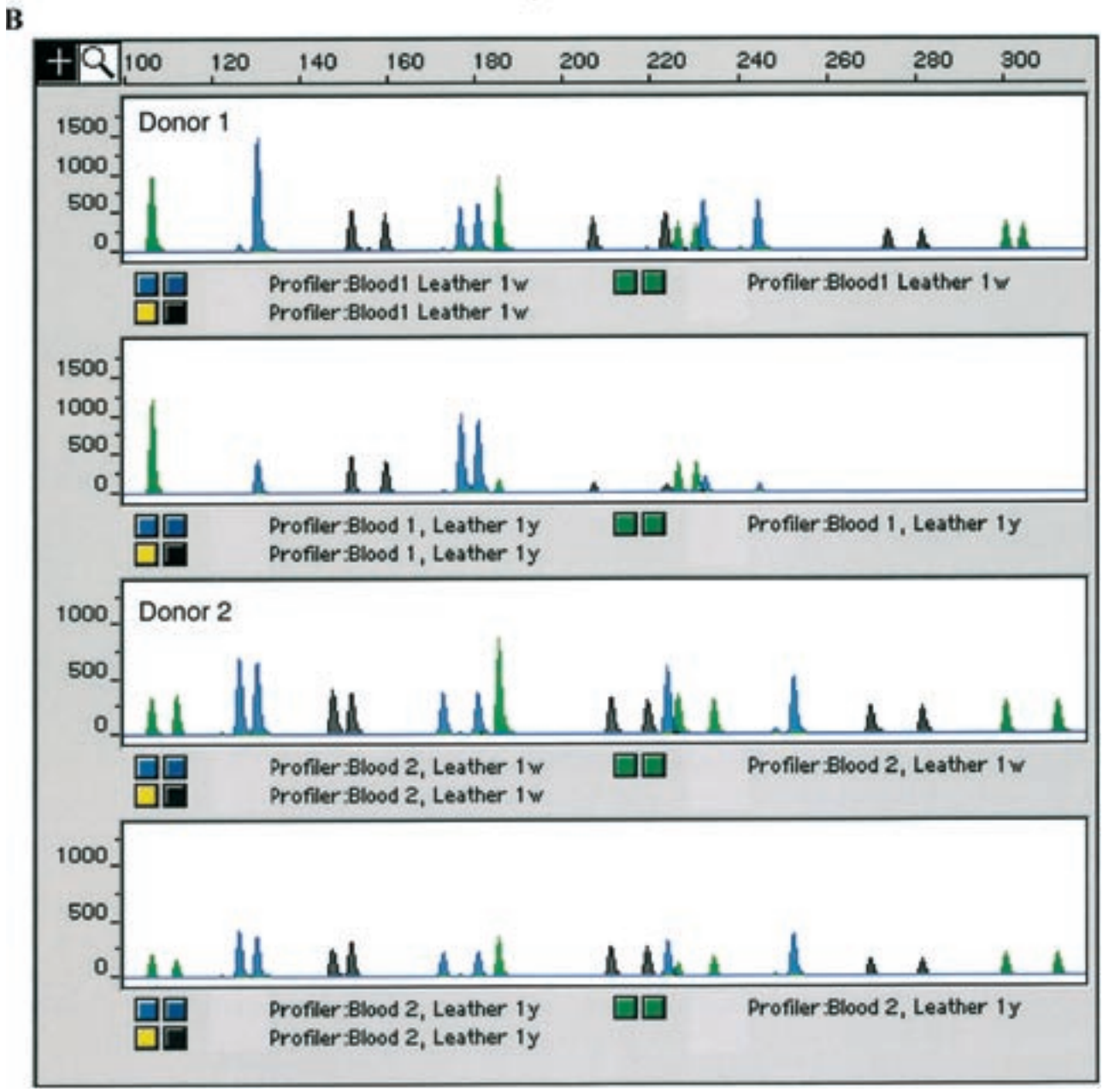

FIG. 11—*(continued)*

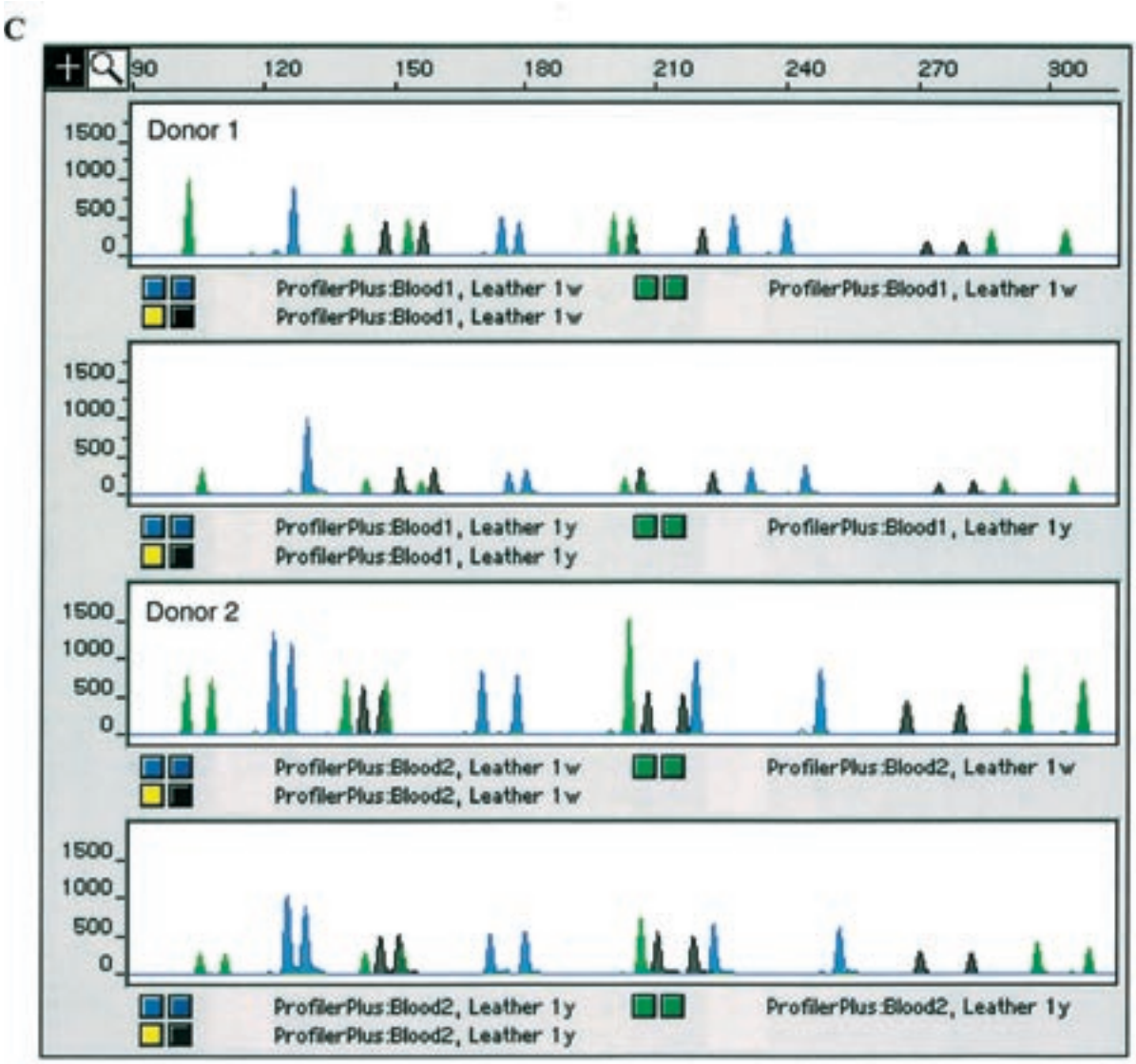

FIG. 11—*(continued)*

TABLE 7—*Adjudicated case samples in nonprobative evidence studies.*

Case	Evidence Items	Reference Samples*	AmpFℓSTR Green I	AmpFℓSTR Profiler	AmpFℓSTR Profiler Plus	AmpFℓSTR COfiler
1	Blood on jeans pocket Substrate control	(S), (V)	√	…	…	…
2	Sweater, sweatshirt	(S), (V)	√	…	…	…
3	(V) panties	(S), (V), (C)	√	…	…	…
4	Vaginal swab	(S), (V)	√	…	…	…
5	Vaginal swab (S) penile swab	(S), (V)	√	…	…	…
6	Vaginal swab	(S), (V)	…	√	√	√
7	Vaginal swab	(S), (V)	…	√	√	√
8	Vaginal swab	(V)	…	√	√	√
9	Vaginal swab	(S), (V)	…	√	√	…
10	Questioned bone	Putative mother, three siblings	√	…	√	…

* Suspect (S), Victim (V), Consensual sexual partner (C).

DNA samples were amplified in separate reactions containing primers for only one AmpFℓSTR locus as well as in reactions containing primers for each locus combination represented by the AmpFℓSTR Green I, Profiler, Profiler Plus, and COfiler kits (Table 1). Samples included control DNA 9947A with and without PCR inhibitor (see section 4.4.1.5) and DNA from a single timepoint of DNAse I degradation (see section 4.1.5.6). Even when a locus was amplified in the presence of hematin, either alone or coamplified, significant differences were not seen (genotype or approximate peak height) (Fig. 18). Benefit in performing single locus amplifications was not demonstrated for any combination of kit primers.

Discussion

As reported previously of the AmpFℓSTR Blue PCR Amplification Kit (19), TWGDAM/DAB validation studies have addressed issues and concerns associated with forensic STR analysis. AmpFℓSTR validation studies were brought full-circle through practical application of STR typing of nonprobative evidence samples. Additionally, characterization of AmpFℓSTR Green I, Profiler, Profiler Plus, and COfiler PCR Amplification Kit performance and descriptions of potential limitations were provided. Standard specimens, consistency, and reproducibility experiments demonstrated reliable and reproducible data regardless of tissue type, state, or specific analyst. Environmental abuse and matrix studies showed that chemical/environmental insults (i.e., exposure to substances commonly encountered in a forensic context), if having any effect at all, do not alter genotype but rather may result in overall loss of signal. Reliability of typing DNA derived from similar samples has been demonstrated in many previous studies and was overviewed recently (28).

A forensic sample containing DNA from two sources is comprised (at a single locus) of the following possible genotype combinations: (1) heterozygote + heterozygote, no overlapping alleles (four peaks), (2) heterozygote + heterozygote, one overlapping allele (three peaks), (3) heterozygote + heterozygote, two overlapping alleles (two peaks), (4) heterozygote + homozygote, no overlapping alleles (three peaks), (5) heterozygote + homozygote, overlapping allele (two peaks), (6) homozygote + homozygote, no overlapping alleles (two peaks), (7) homozygote + homozygote, overlapping allele (one peak). Thus mixed genetic information at a locus may be indicated by an apparent increase in ploidy (i.e., more than two alleles), as well as by an imbalanced peak height ratio (PHR) and/or significantly increased stutter peak (19,30), if a weak minor component allele is 4 bp less than a major component allele (Fig. 1). Specific genotype combinations and input DNA ratios contained in a mixture determined to what degree contributing genotypes of the major and minor component(s) are resolvable at each locus. The ability to obtain and compare quantitative values for allele peak heights provided data that was invaluable to resolution of mixed genotypes (Fig. 1). Such objective information may be contrasted with classical band intensity comparisons on stained gels (e.g., RFLP, D1S80). Ultimately, the analyst in the context of each particular case must determine the likelihood that any sample is a mixture, as well as donor(s) genotype assignments.

Though more rare in results greater than 1000 RFU, a peak height ratio (PHR) less than 70% is possible in a single source sample. Because decreased peak height ratio can sometimes aid in resolving alleles between individuals in a DNA mixture, it is important to understand the difference between a shared allele and an imbalance caused by another factor(s) (see section 4.1.5.5). Imbalanced peak height(s) should be evaluated in the context of all available information regarding the forensic sample. Of course, imbalanced amplification caused by stochastic allele sampling in PCR, DNA degradation, PCR inhibition, and/or a mutation under a primer, is not an indicator of a DNA mixture.

Genotyper software genotypes alleles automatically, relative to AmpFℓSTR allelic ladders (22,31). To facilitate differentiation of peaks that are purely stutter from those that additionally contain a minor allele in a mixture, a quantitative stutter threshold can be applied when genotyping data. The "Kazam" macro, contained within AmpFℓSTR Genotyper templates, may be used to capture and filter allele labels from stutter peaks near the percentage range expected for single source samples. AmpFℓSTR stutter filter thresholds built around the maximum value observed in validation studies ± three standard deviations (SD) (see legends to Figs. 6 to 9) are conservative, as 99.7% of all stutter percentages are predicted to fall within three SDs of the mean value (32). It is important to remember that the software filter is a tool for DNA analysts; final interpretation is based on all available information, including professional judgement and experience. Within each AmpFℓSTR locus, a trend was seen in that percent stutter generally increased with allele length, attributable to increase in repeat unit number. Individual laboratories may wish to develop more rigorous quantita-

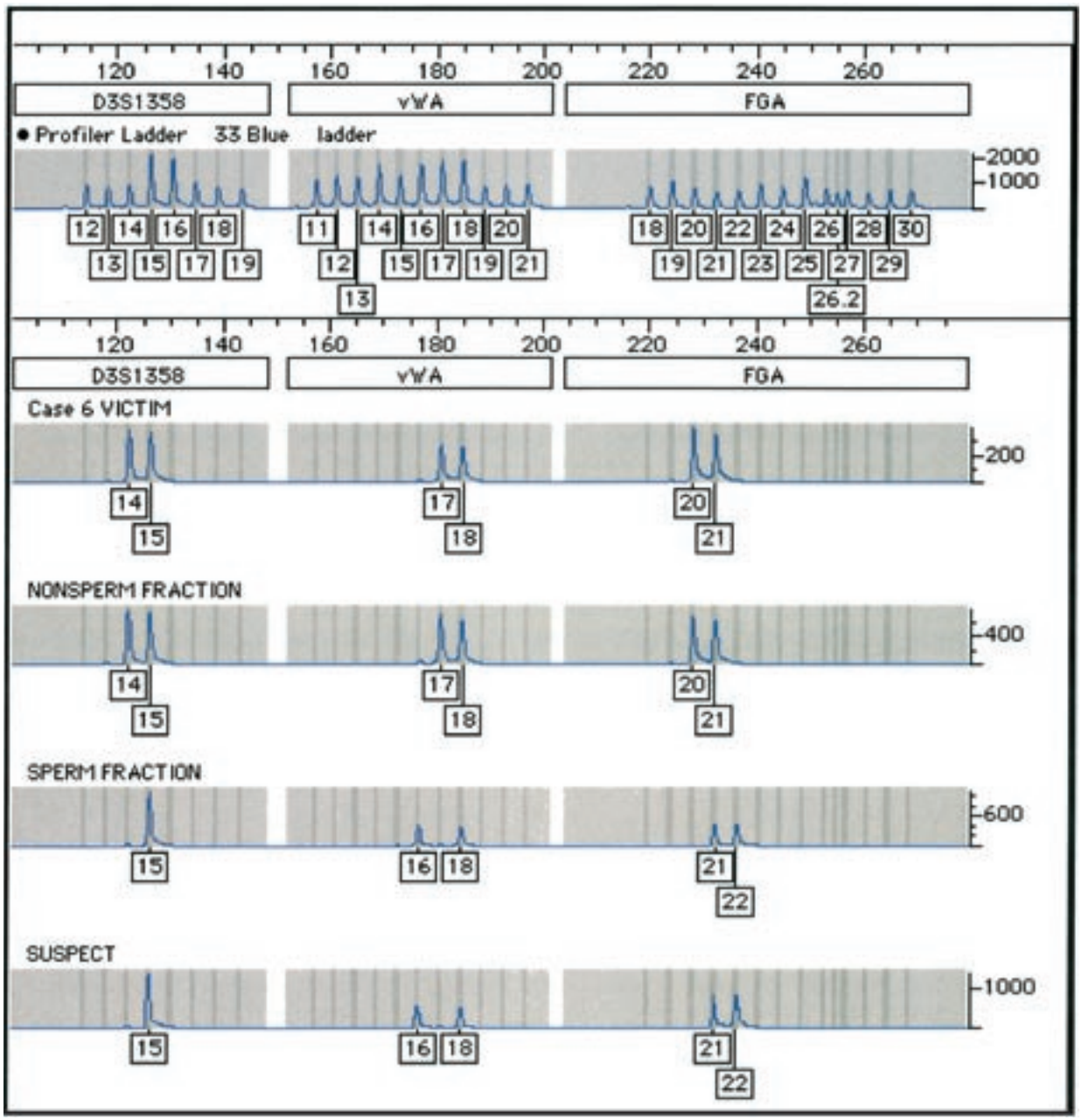

FIG. 12—*Nonprobative sexual assault evidence: Case #6. Genotyper software plots of AmpF ℓSTR Profiler PCR Amplification Kit results (ABI Prism 377 DNA Sequencer data). Panels (from top to bottom) show the FAM-labeled loci in the AmpF ℓSTR Profiler Allelic Ladder, victim reference sample, non-sperm cell and sperm cell fractions of a differential DNA extraction from a vaginal swab, and suspect reference sample. Genotyper software automatically labeled AmpF ℓSTR alleles within ± 0.5 bp bins, relative to the allelic ladder. The X- and Y-axes indicate fragment size (bases) and signal intensity (RFU), respectively.*

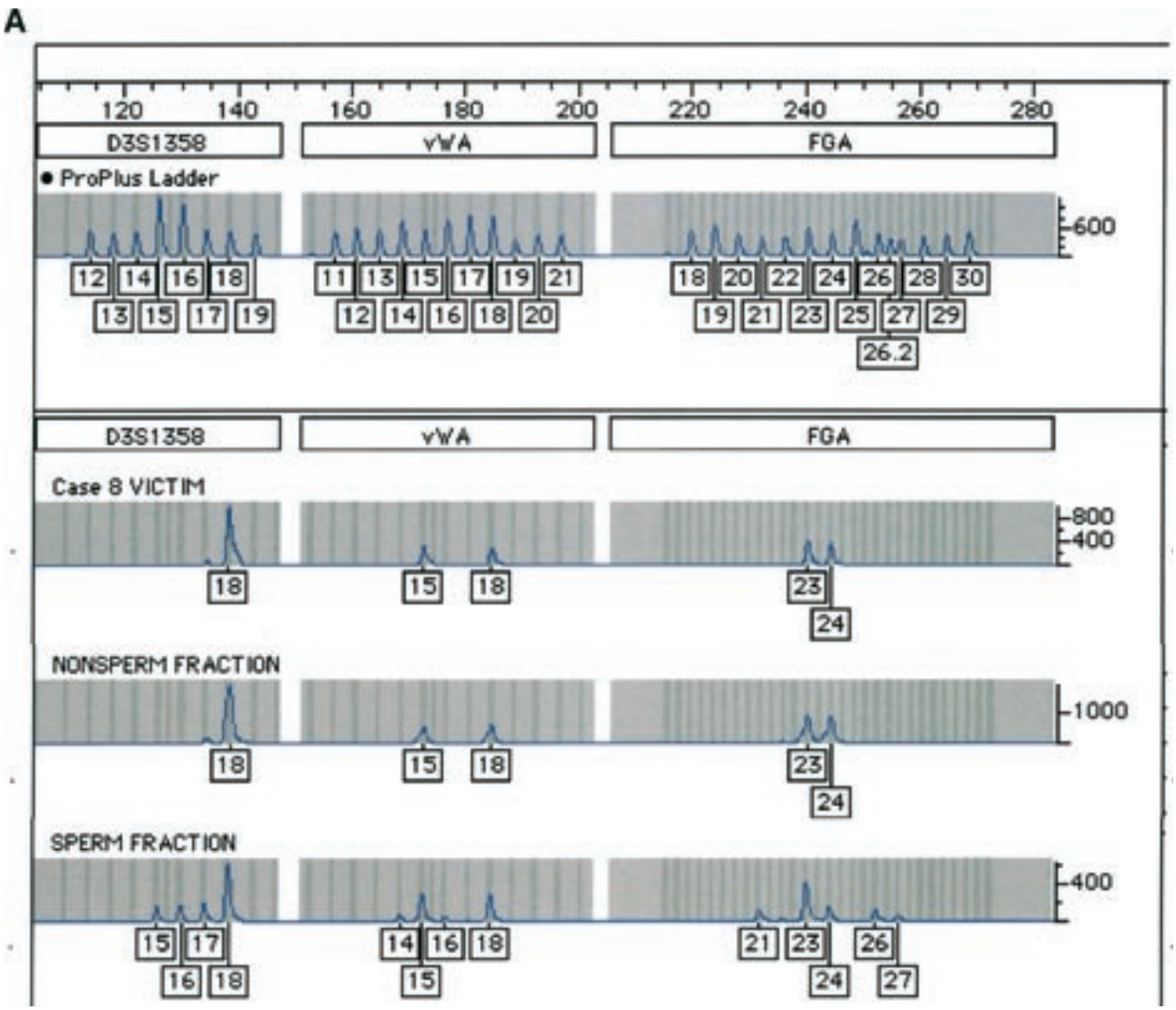

FIG. 13—*Nonprobative sexual assault evidence: Case #8. Genotyper software plots of AmpFℓSTR Profiler Plus* (A) *and COfiler* (B) *PCR Amplification Kit results. Panels (from top to bottom) show the FAM-labeled loci in AmpFℓSTR Allelic Ladders, victim reference sample, and the nonsperm cell and sperm cell fractions of a differential DNA extraction from a vaginal swab. Genotyper software automatically labeled AmpFℓSTR alleles within ± 0.5 bp bins, relative to the allelic ladder. ABI Prism 377 DNA Sequencer data are shown in* (A)*; results in* (B) *were detected on the ABI Prism 310 Genetic Analyzer. The X- and Y-axes indicate fragment size (bases) and signal intensity (RFU), respectively.*

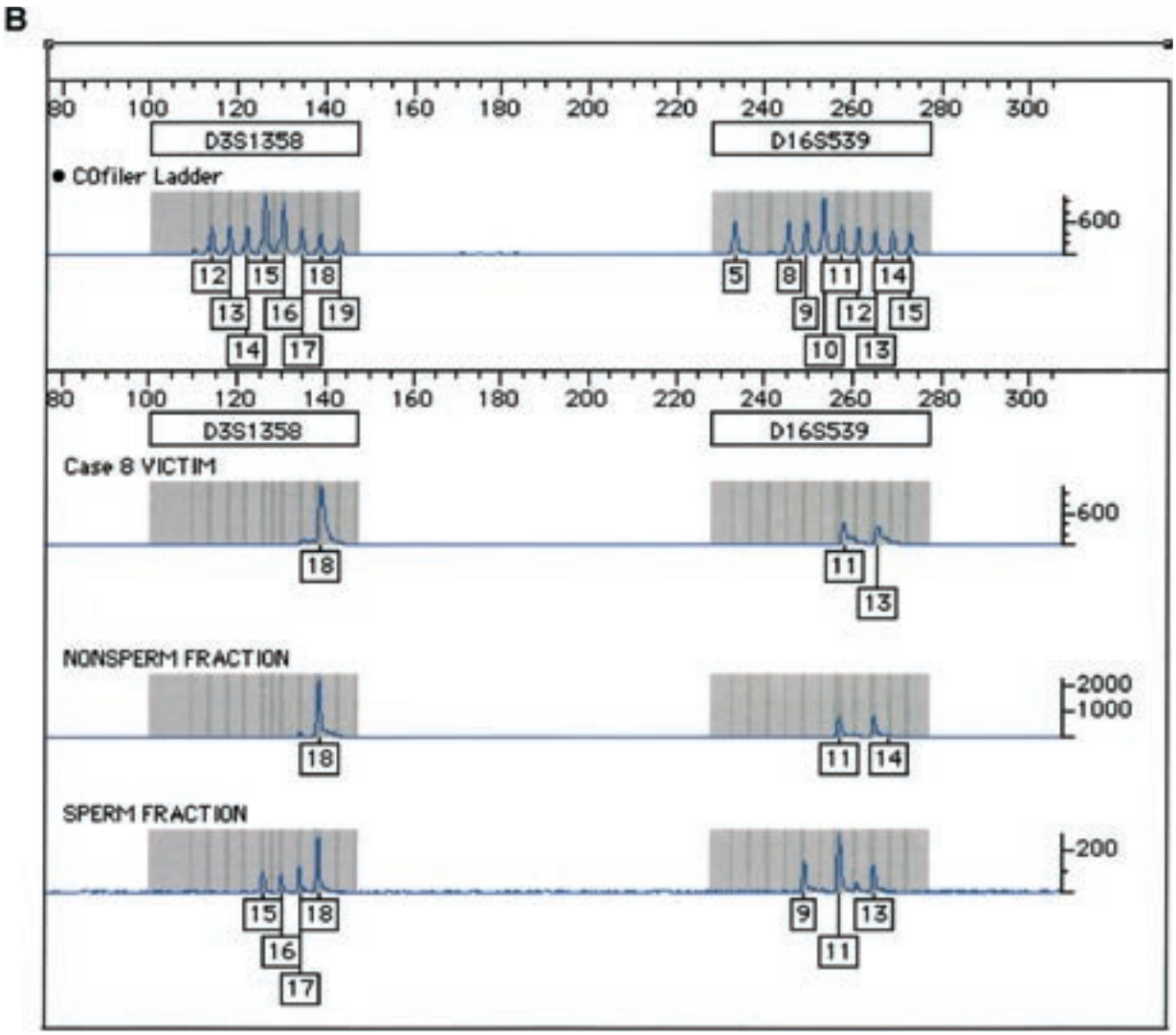

FIG. 13—*(continued)*

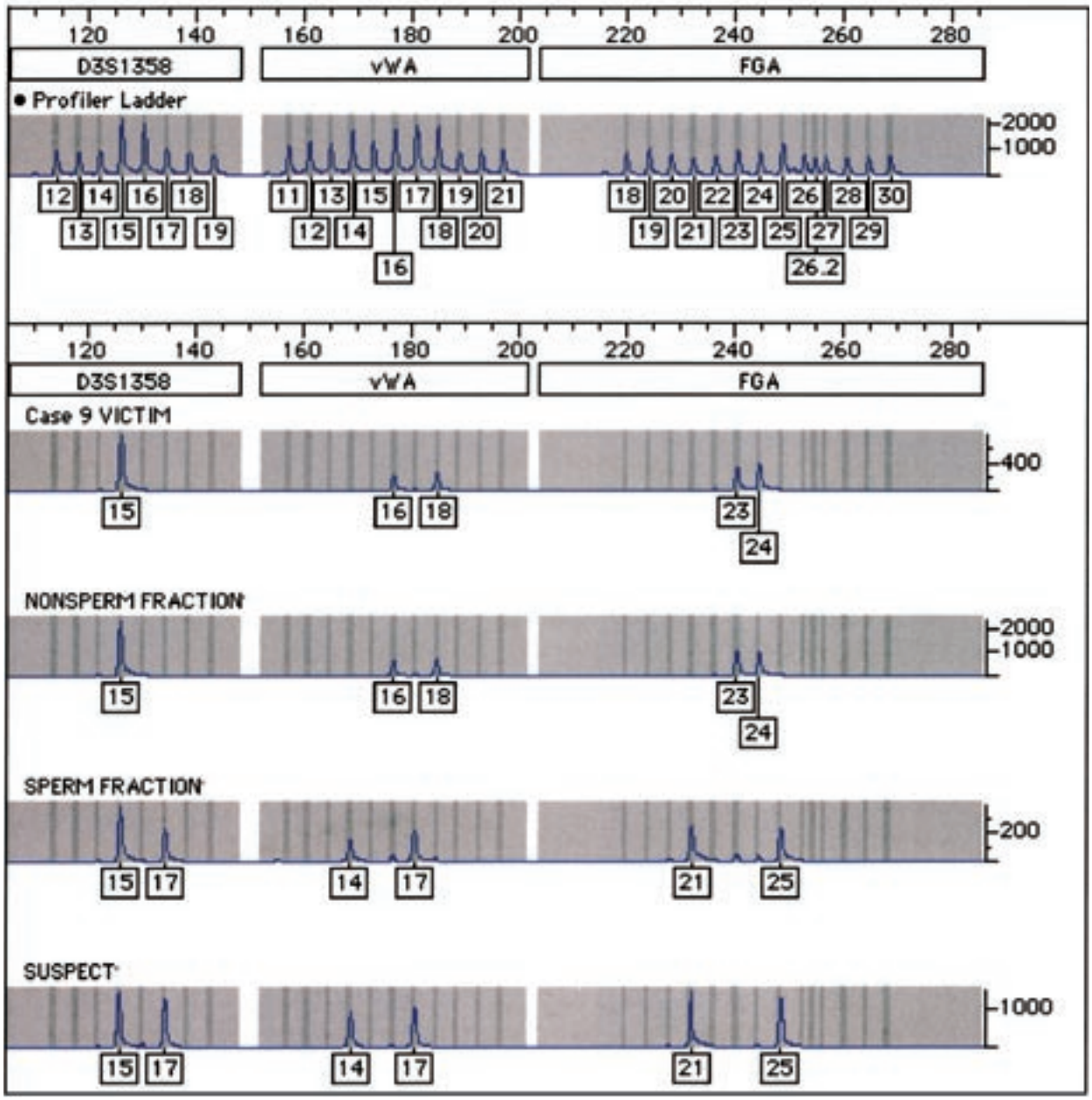

FIG. 14—*Nonprobative sexual assault evidence: Case #9. Genotyper software plots of AmpFℓSTR Profiler PCR Amplification Kit results (ABI Prism 377 DNA Sequencer data). Panels (from top to bottom) show the FAM-labeled loci in AmpFℓSTR Allelic Ladders, victim reference sample, nonsperm cell and sperm cell fractions of a differential DNA extraction from a vaginal swab, and suspect reference sample. Genotyper software automatically labeled AmpFℓSTR alleles within ± 0.5 bp bins, relative to the allelic ladder. The X- and Y-axes indicate fragment size (bases) and signal intensity (RFU), respectively.*

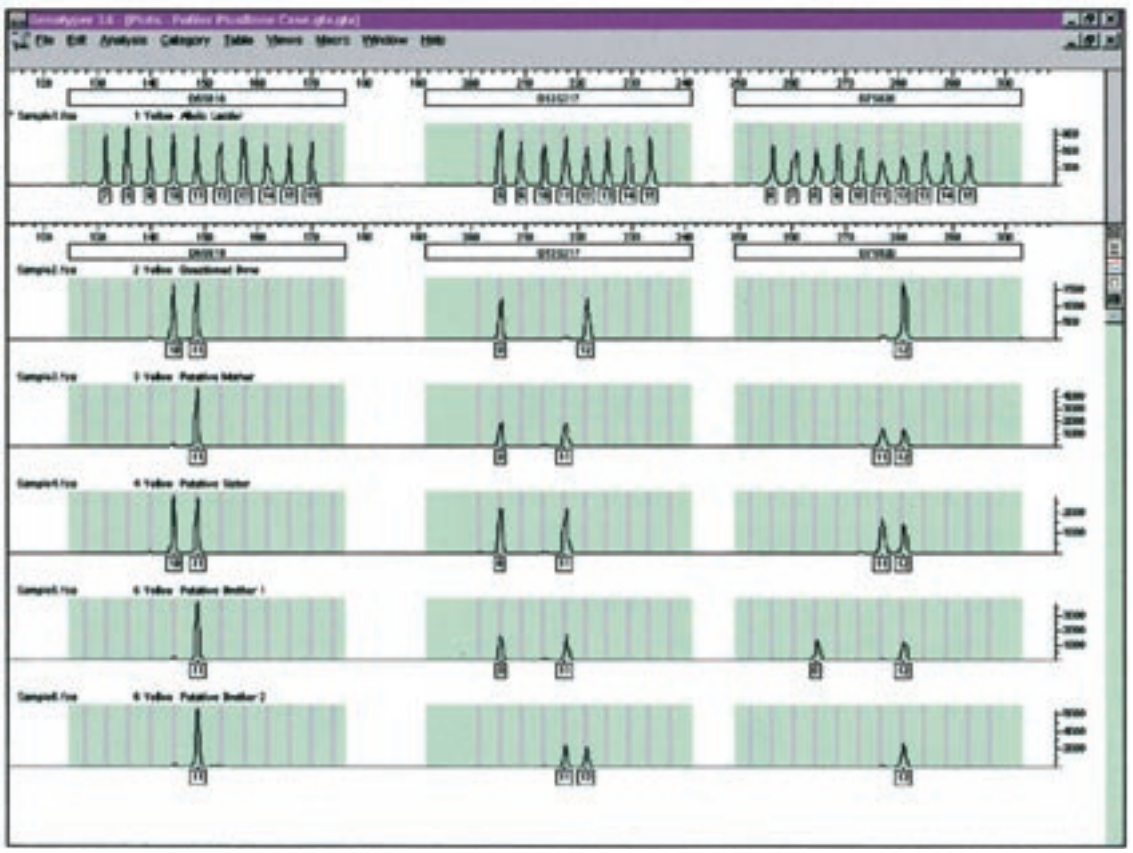

FIG. 15—*Nonprobative unidentified human remains evidence (Case 10). Genotyper software plots of AmpFℓSTR Profiler Plus PCR Amplification results (ABI Prism 310 Genetic Analyzer data). Panels (from top to bottom) show the NED-labeled loci in the AmpFℓSTR Profiler Plus Allelic Ladder, questioned bone (femur), putative mother, putative sister, and two putative brothers. Genotyper software automatically labeled AmpFℓSTR alleles within ± 0.5 bp bins, relative to the allelic ladder. The X- and Y-axes indicate fragment size (bases) and signal intensity (RFU), respectively.*

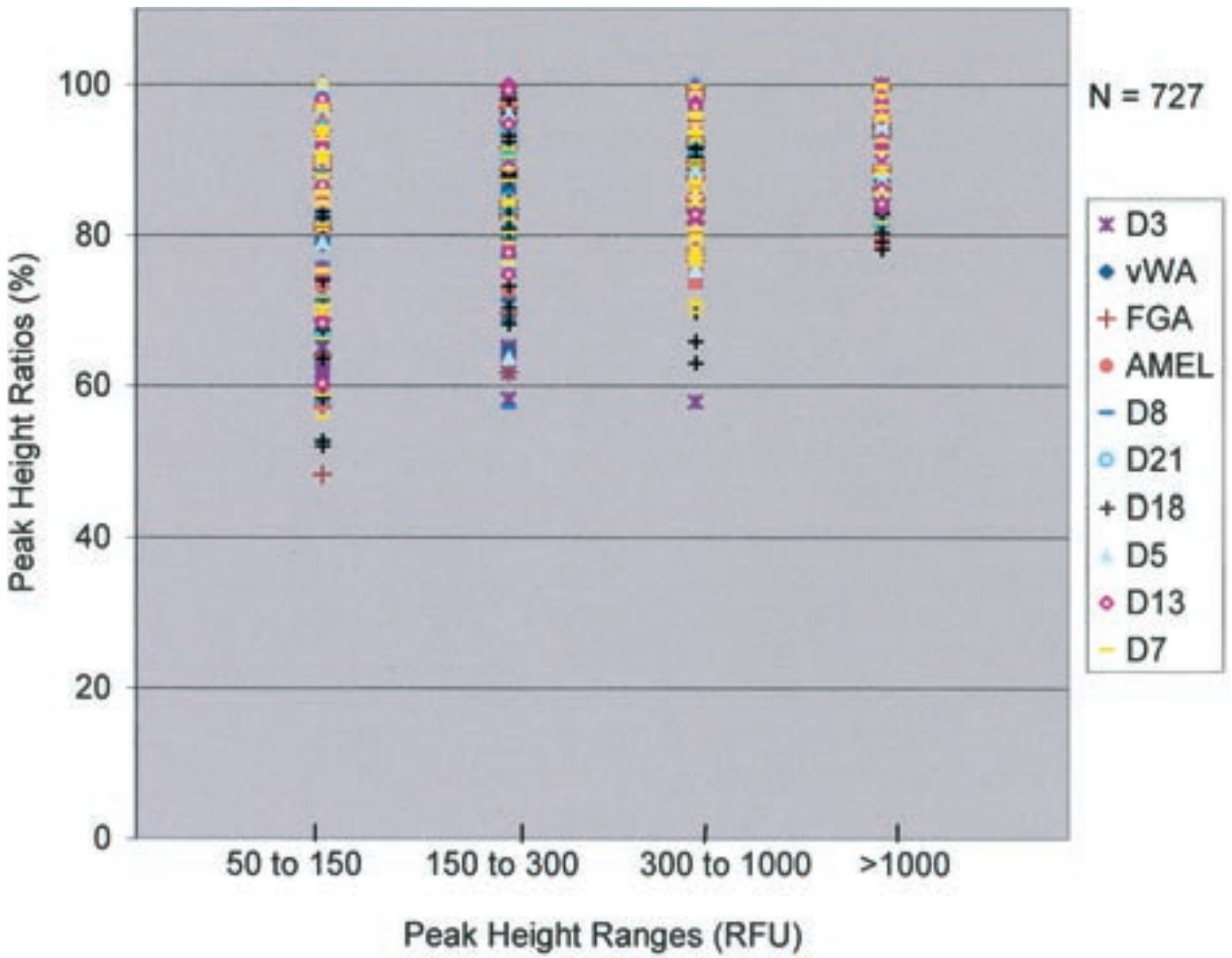

FIG. 16—*Peak height ratios (percentages, Y-axis) from 727 heterozygous allele pairs. Data from AmpFℓSTR Profiler Plus reactions categorized into four ranges of fluorescent signal intensity (relative fluorescence units (RFU), X-axis).*

TABLE 8—*Peak height ratio measurements at different fluorescent intensity ranges.*

	Relative Fluorescence Units (RFU)			
PHR	50–150	150–300	300–1000	>1000
Observed Minimum	48%	57%	58%	78%
Theoretical Minimum (Mean—3 StDev)	40%	54%	63%	75%
Mean	80%	85%	88%	92%
N	145	140	233	209

tive stutter interpretation by considering stutter values of each allele at a locus.

The AmpFℓSTR kits performed reliably and reproducibly within a range of template DNA concentrations. Results reinforced previous reports that fluorescent signal intensity, not strictly input DNA concentration, is important in evaluation of data quality. For example, in forensic DNA casework, a reaction with 1 to 2 ng of input DNA may produce peaks with weak signal (e.g., due to PCR inhibition or DNA degradation) while a reaction with less DNA (e.g., <1 ng template) may amplify well and produce interpretable signal. Data showed that valuable genotype information can be extracted from evidence DNA samples, available in only limited quantity and/or producing low fluorescence signal. Practical relevance of stochastic effects in PCR, lies in an extreme, yet potential, instance where only one allele peak at a heterozygous locus is visible. Therefore, importance of interpreting single peaks of low fluorescence signal with caution, and on a case-by-case basis, is recognized. Despite the potential for stochastic sampling effects, genotype may sometimes be discerned. Depending on factors such as overall data quality, nature of the sample, extent of the forensic question posed, etc., a conclusion may be drawn that the genotype is homozygous, or the possibility of a second allele may be accounted for through appropriate statistical estimation. In some situations analysts may find it suitable to designate a weak single peak as an allele but not to assign genotype, or to report that locus as inconclusive. Of course, if DNA template remains, re-amplification is sometimes beneficial. Regardless of the troubleshooting strategy employed or the extent of the interpretation, STR allele assignments and profile designation in forensic casework are considered in the context of all available data regarding the sample and/or profile, with sensitivity studies in mind, using practical scientific knowledge.

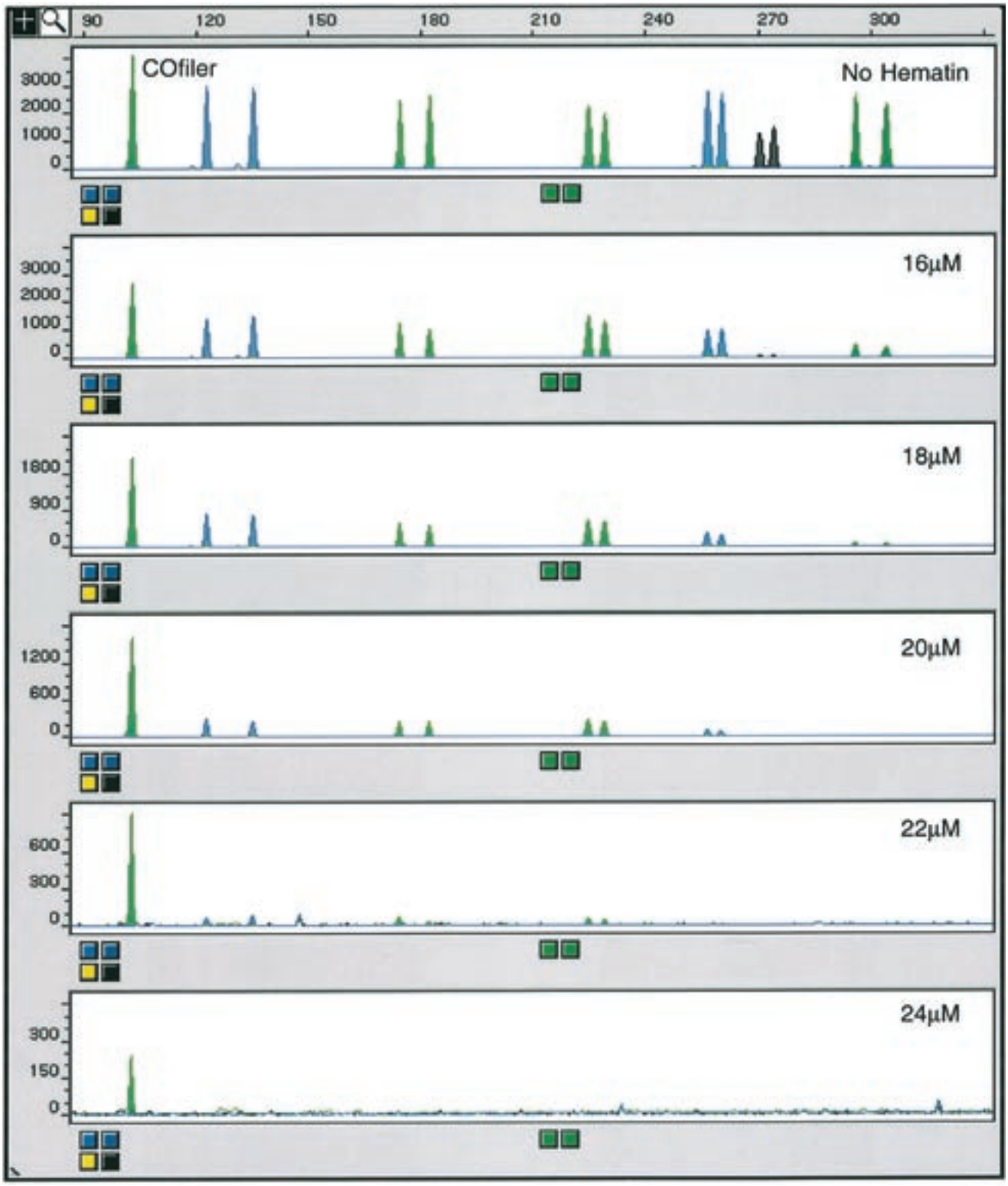

FIG. 17—*Challenging PCR by inhibition: Hematin. Electropherograms (GeneScan software, ABI Prism 310 Genetic Analyzer) of AmpF ℓSTR COfiler PCR products from untreated DNA (top panel) and from amplifications performed in the presence of various concentrations of the heme-like PCR inhibitor hematin (16, 18, 20, 22, and 24 µM, respectively) are shown. The X- and Y-axes indicate fragment size (bases) and signal intensity (RFU), respectively.*

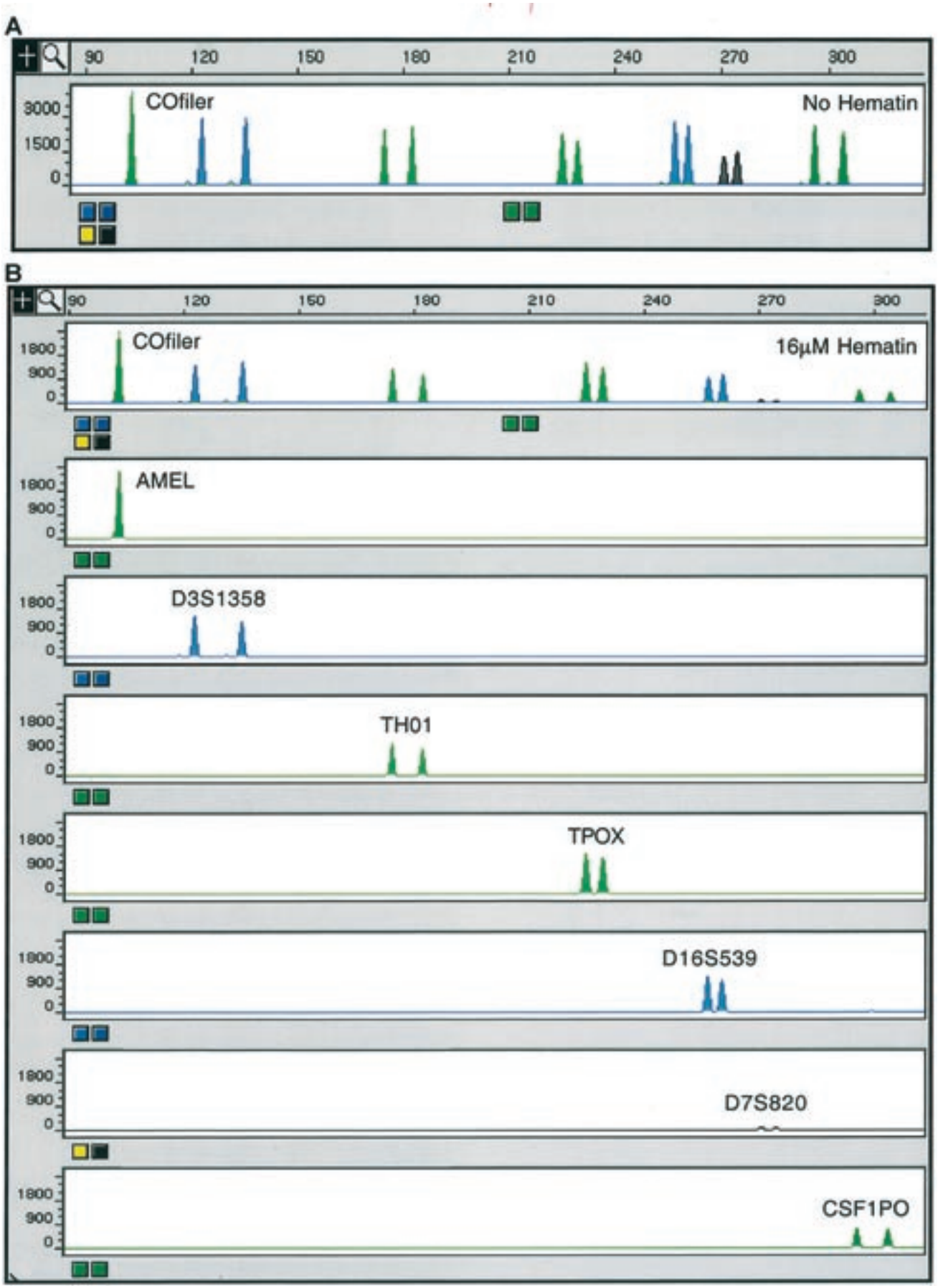

FIG. 18—*Challenging PCR by inhibition: Single and multilocus AmpFℓSTR amplifications.* (A) *shows electropherograms (GeneScan software, ABI Prism 310 Genetic Analyzer) of DNA amplified in the AmpFℓSTR COfiler multiplex without hematin. Results in* (B) *were generated in the presence of 16 µM hematin. Top panel in* (B) *shows an AmpFℓSTR COfiler reaction; single locus amplifications of the seven COfiler loci, in the presence of 16 µM hematin, are shown in the lower panels. The X- and Y-axes indicate fragment size (bases) and signal intensity (RFU), respectively.*

A complete picture of validation, according to TWGDAM/DAB, is seen when data in this manuscript are combined with additional publications as follows: 4.1.5.3 Population Studies (22), 4.1.5.9 Nonhuman Studies (16), 4.2.1 Inheritance, 4.2.2 Gene Mapping, 4.2.4 Polymorphism (22), 4.2.3 Detection (31), 4.4.1.1 primer sequences (16), 4.4.1.2 "Laboratory Conditions" (24–27), 4.4.1.3 "Reaction Conditions" (16), 4.4.2.1 Amplification—Standards for Direct Characterization (GeneScan-350 or -500 [ROX] Internal Lane Size Standards) (34) and (AmpFℓSTR Allelic Ladders) (22), 4.5.3 Internal Validation of Established Procedures (sizing precision, ±0.5-bp floating bin approach), (31,33). AmpFℓSTR PCR Amplification Kits clearly withstood rigorous validation challenges and met or exceeded all criteria necessary for use in forensic casework analysis.

Acknowledgments

The authors wish to acknowledge DNA Unit analysts at the Santa Clara County District Attorney Crime Laboratory (San Jose, CA), especially Lisa Brewer and Cynthia Hall, and at the California Department of Justice DNA Laboratory (Berkeley, CA) for preparing many of the validation stains/DNA samples that allowed successful and thorough evaluation of the AmpFℓSTR multiplexes. We also recognize analysts at the San Francisco Police Department Criminalistics Laboratory, especially Bonnie Cheng and Pamela Hofsass, for generating data for the sensitivity study.

References

1. Li H, Schmidt L, Wei MH, Hustad T, Lerman MI, Zbar B, et al. Three tetranucleotide polymorphisms for loci: D3S1352; D3S1358; D3S1359. Hum Mol Genet 1993;2:1327.
2. Mancuso DJ, Tuley EA, Westfield LA, Worrall NK, Shelton-Inloes BB, Sorace JM, et al. Structure of the gene for human von Willebrand factor. J Biol Chem 1989;264(33):19514–27.
3. Mills KA, Even D, Murray JC. Tetranucleotide repeat polymorphism at the human alpha fibrinogen locus (FGA). Hum Mol Genet 1992;1:779.
4. Polymeropoulos MH, Xiao H, Rath DS, Merril CR. Tetranucleotide repeat polymorphism at the human tyrosine hydroxylase gene (TH). Nucleic Acids Res 1991;19:3753.
5. Edwards A, Hammond H, Jin L, Caskey CT, Chakraborty R. Genetic variation at five trimeric and tetrameric repeat loci in four human population groups, Genomics 1992;12:241–53.
6. Anker R, Steinbrueck T, Donis-Keller H. Tetranucleotide repeat polymorphism at the human thyroid peroxidase (hTPO) locus. Hum Mol Genet 1992;1:137.
7. Hammond HA, Jin L, Zhong Y, Caskey CT, Chakraborty R. Evaluation of 13 short tandem repeat loci for use in personal identification applications. Am J Hum Genet 1994;55:175–89.
8. Hudson TJ, Stein LD, Gerety SS, Ma J, Castle AB, Silva J, et al. An STS-based map of the human genome. Science 1995;270:1945–54.
9. CHLC (Cooperative Human Linkage Center), accession number 511, http://www.chlc.org, The University of Iowa, #140 EMRB, Iowa City, IA, 52242; GenBank accession number G08616.
10. Green ED, Mohr RM, Idol JR, Jones M, Buckingham JM, Deaven LL, et al. Systematic generation of sequence-tagged sites for physical mapping of human chromosomes: application to the mapping of human chromosome 7 using yeast artificial chromosomes. Genomics 1991;11:548–64.
11. Barber MD, Parkin BH. Sequence analysis and allelic designation of the two short tandem repeat loci D18S51 and D8S1179. Int J Legal Med 1996;109:62–5.
12. Sharma V, Litt M. Tetranucleotide repeat polymorphism at the D21S11 locus. Hum Mol Genet 1992;1:67.
13. CHLC (Cooperative Human Linkage Center), accession number 715, http://www.chlc.org, The University of Iowa, #140 EMRB, Iowa City, IA, 52242; GenBank accession number G07925.
14. Nakahori Y, Takenaka O, Nakagome. A human X-Y homologous region encodes amelogenin, Genomics 1991;9:264–9.
15. Sullivan KM, Mannucci A, Kimpton CP, Gill P. A rapid and quantitative sex test: Fluorescence-based PCR analysis of X-Y homologous gene amelogenin. BioTechniques 1993;15:636–41.
16. Wallin JM, Holt CL, Lazaruk KD, Nguyen TH, Walsh PS. Constructing universal multiplex PCR systems for comparative genotyping. J Forensic Sci 2002;47(1).
17. Technical Working Group on DNA Analysis Methods. Guidelines for a quality assurance program for DNA analysis. Crime Lab Digest 1995; 22(2):21–43.
18. US Department of Justice, Federal Bureau of Investigation Washington, D.C. Quality assurance standards for forensic DNA testing laboratories. 1998.
19. Wallin JM, Buoncristiani MR, Lazaruk KD, Fildes N, Holt CL, Walsh PS. TWGDAM validation of the AmpFℓSTR Blue PCR Amplification kit for forensic casework analysis. J Forensic Sci 1998;43:117–33.
20. Sambrook J, Fritch EF, Maniatis T. Molecular Cloning: A Laboratory Manual, New York: Cold Spring Harbor Laboratory Press 1989.
21. Walsh PS, Metzger DA, Higuchi R. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques 1991;10:506–18.
22. Holt CL, Stauffer C, Wallin JM, Lazaruk KD, Nguyen T, Budowle B, et al. Practical applications of genotypic surveys for forensic STR testing. Forensic Sci Int 2000;112(2–3):91–109.
23. Walsh PS, Valaro J, Reynolds R. A rapid chemiluminescent method for quantitation of human DNA. Nucleic Acids Res 1992;20:5061–65.
24. PE Applied Biosystems. AmpFℓSTR Green I PCR Amplification Kit User's Manual, 1997 Version A.
25. PE Applied Biosystems. AmpFℓSTR Profiler PCR Amplification Kit User's Manual, 1997 Version A.
26. PE Applied Biosystems. AmpFℓSTR Profiler Plus PCR Amplication Kit User's Manual, 1997 Version A.
27. PE Applied Biosystems. AmpFℓSTR COfiler PCR Amplification Kit User Bulletin, 1998 Version A.
28. Moretti TR, Baumstark AL, Defenbaugh DA, Keys KM, Smerick JB, Budowle B. Validation of short tandem repeats (STRs) for forensic usage: performance testing of fluorescent multiplex STR systems and analysis of authentic and simulated forensic samples. J Forensic Sci 2001; 46(3):647–60.
29. Walsh PS. Commentary on Kline MC, Jenkins B, Rogers S. Non-amplification of a vWA allele. J Forensic Sci 1998;43:250. J Forensic Sci 1998;43:1103–4.
30. Walsh PS, Fildes NJ, Reynolds R. Sequence analysis and characterization of stutter products at the tetranucleotide repeat locus vWA. Nucleic Acids Res 1996;24:2807–12.
31. Lazaruk K, Wallin J, Holt C, Nguyen T, Walsh PS. Sequence variation in humans and other primates at six short tandem repeat loci used in forensic identity testing, Forensic Sci Int. 2001;119(1):1–10.
32. Filter thresholds in the Genotyper software Kazam macro stated in AmpFℓSTR User Manuals were based on [allele peak height/stutter peak height] × 100 (24,25,26,27). The macro defines stutter percentage as [(allele peak height − stutter peak height)/stutter peak height] × 100. Actual threshold values used by the software tool are shown in Figs. 6–9. 13% for each of the AmpFℓSTR Blue locus.
33. Gill P, Urquhart A, Millican E, Oldroyd N, Watson S, Sparkes R, et al. A new method of STR interpretation using inferential logic—development of a criminal intelligence database. Int J Legal Med. 1996;109: 14–22.
34. Lazaruk KD, Walsh PS, Oaks F, Gilbert D, Rosenblum B, Menchen S, et al. Genotyping of forensic STR systems based on excellent sizing precision in a capillary electrophoresis instrument. Electrophoresis 1998;19:86–93.

Additional information and reprint requests:
Cydne L. Holt
Senior Criminalist
San Francisco City and County Crime Laboratory
850 Bryant Street
San Francisco, CA 94103