J Forensic Sci, September 2003, Vol. 48, No. 5
Paper ID JFS2002188_485
Available online at: www.astm.org

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AmpFℓSTR® Profiler Plus™ Short Tandem Repeat DNA Analysis of Casework Samples, Mixture Samples, and Nonhuman DNA Samples Amplified under Reduced PCR Volume Conditions (25 μL)

ABSTRACT: As part of the validation of the AmpFℓSTR® Profiler Plus™ short tandem repeat (STR) system, under reduced polymerase chain reaction (PCR) volume conditions (i.e., 25 μL), a total of 275 casework samples were processed. Examples of profiles are presented along with amplification conditions to improve the odds of obtaining balanced and complete profiles for samples showing partial results or profiles with a descending slope. Data collected and used to develop our interpretation guidelines are included. From the mixture studies, full profiles were obtained for minor contributors, using 2 ng of DNA, with ratios of 10:1 or 1:20 and using 1 ng of DNA, with ratios of 10:1 and 1:8. The specificity of the Profiler Plus™ amplification reaction performed in 25 μL was examined and confirmed using a large spectrum of nonhuman DNAs. This report supports the use of the AmpFℓSTR® Profiler Plus™ STR system for casework DNA typing under reduced PCR volume conditions.

KEYWORDS: forensic science, DNA typing, D3S1358, HumvWA, HumFGA, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820, reduced volume, mixture

Two major goals in the development of new DNA typing technology for forensic use have always been: (1) to achieve the highest power of discrimination possible using a valid and reliable method, and (2) to preserve limited evidentiary samples by utilizing the smallest portion possible for analysis. The discovery of the STR markers (1–3) as well as the development of fluorescence detection instruments (4–9) provided forensic scientists with sophisticated means of achieving their original goals. The development of highly discriminatory megaplex STR systems used in a fluorescence-based detection mode significantly increased the capability for analyzing a larger number of samples that have a limited amount of biological material (10–16). This combined approach offered great sensitivity and accuracy as well as facilitated the interpretation of DNA mixtures by providing data, such as peak height and peak area, that can be integrated into formulas and subsequently analyzed to assist in profile interpretation (17–20).

Many validation studies have been published that indicate that STR systems, in multiplex formats, provide excellent typing results for samples subjected to a variety of environmental and experimental conditions (15,21–27). Despite this valuable information, newly developed STR multiplex systems must be subjected, by the practicing forensic community, to a thorough examination in order to define their limitations and establish their robustness and reliability under specific circumstances and/or experimental conditions. Only from such studies can scientifically sound interpretation guidelines be derived and a universal consensus for data sharing among different forensic laboratories be obtained.

This report presents some of the studies that were conducted during the validation of the AmpFℓSTR® Profiler Plus™ STR amplification system using reduced PCR volume conditions (i.e., 25 μL). Although the manufacturers of the AmpFℓSTR® commercial kits recommend the use of 50 μL as the PCR volume (15,28), previous experience gained using STR multiplexes under reduced PCR volume conditions (21,29) and our involvement in the FBI STR Standardization Project suggested the evaluation of the amplification conditions described in this report. Six different categories of Profiler Plus™ profiles were defined following the processing of 275 biological evidence in 25-μL PCR volume. Examples for each category are presented along with data collected and used to develop our interpretation guidelines. As a means to improve the odds of obtaining balanced and complete profiles for casework samples showing partial profiles or profiles with a slope, several amplification conditions are presented. In addition, a series of simulated mixtures representing ten different mixture scenarios was prepared to establish the limit of detection of a minor profile using the Profiler Plus™ multiplex system. Two different amounts of total DNA (1 and 2 ng) and different ratios ranging from 1:20 to 20:1 were selected to cover a wide range of potential casework mixtures. Finally, the specificity of the Profiler Plus™ multiplex amplification reaction was also examined under reduced PCR volume conditions using a large spectrum of nonhuman DNAs, varying the amounts of template DNA (2.5 and 50 ng). The results obtained from these studies are described below.

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Received 29 May 2002; and in revised form 2 Nov. 2002; accepted 30 March 2003; published 4 Aug. 2003.

Methods

DNA Extraction and Quantitation

Casework specimens from the RCMP and the Bureau of Criminal Apprehension of the State of Minnesota, as well as blood from animals, were subjected to standard DNA extraction protocols using organic solvents and ethanol precipitation (30). Differential extractions were performed on specimens potentially containing semen in order to separate the female cell component (referred to as the F2 fraction) from the male cell component (F3 fraction) (30). All DNA extracts were further purified using Microcon-100 size-exclusion columns (Amicon Inc., Beverly, MA) following the recommendations from the manufacturer and stored at −20°C in filtered, autoclaved, and deionized (FAD) water.

Quantitation of human genomic DNA extracted from the RCMP casework samples was determined using a slot blot hybridization procedure with chemiluminescence-based detection (31). A biotinylated primate-specific D17Z1 α-satellite probe was used to hybridize to the casework and reference samples, i.e., two-fold serial dilutions of the control cell line GM9947A (32; NIST Standard Reference Material No. 2391 PCR-based DNA Profiling Kit) immobilized on a membrane. Nonhuman DNA samples were quantified by UV spectrophotometry and by spectrofluorometry using a PicoGreen-based assay (Molecular Probes, Inc., Eugene, OR).

Amplification Conditions

Simultaneous amplification of the nine STR systems comprised in the AmpFℓSTR® Profiler Plus™ amplification system (28), as well as the gender determination marker, amelogenin, was conducted in a 25-μL final reaction volume containing 2.5 ng of genomic DNA (or as specified in the text or in the figure legends; in a total sample volume of 10 μL, with FAD water completing the volume), 9.5 μL of the AmpFℓSTR PCR Reaction Mix, 5 μL of the AmpFℓSTR *Profiler Plus*™ Primer Set Solution and 0.5 μL of AmpliTaq Gold™ DNA Polymerase (5 U/μL stock). The reagents were kept in the same proportions for DNA samples amplified in final volumes of 5 and 10 μL. In these cases, the amounts of input DNA tested were 0.5, 1, and 2 ng; the DNA aliquots were dried out in the amplification tubes under vacuum prior to the addition of the cocktail mix and oil to prevent evaporation.

The reaction mixtures were subjected to a hot start at 95°C for 11 min in order to activate the AmpliTaq Gold™ DNA Polymerase. Amplifications were carried out for 28 cycles using the following parameters: denaturation for 60 s at 94°C, annealing of primers for 90 s at 59°C, and extension for 90 s at 72°C. These cycling parameters differ from those recommended by the manufacturer of the AmpFℓSTR® Profiler Plus™ kit. An increase in the annealing step and the extension step by 30 s (i.e., using 90 s instead of 60 s for each cycle) was found to enhance the yield of amplicons by a factor of 2 to 3 in reduced amplification volumes (33). A final extension at 60°C for 45 min, followed by an overnight incubation at room temperature, were also included as these conditions were required to promote the 3′ terminal transferase activity of the AmpliTaq Gold™ DNA Polymerase. All amplifications were conducted using a Perkin Elmer GeneAmp™PCR System 9600 thermal cycler and thin-walled 0.2-mL MicroAmp™Reaction Tubes.

Analysis of Profiler Plus™ Fluorescent Amplification Products

An aliquot of 1 to 1.5 μL of each PCR reaction was mixed with 0.5 μL of ABI GeneScan-500 Internal Lane Size Standard (labeled with 6-carboxy-X-rhodamine [ROX, a fluorescent dye from ABI]) and 4 μL of denaturing loading buffer (20 mg/mL blue dextran, 7.3 *M* urea, 2X TBE, 20 m*M* EDTA). Following denaturation at 95°C for 2 to 3 min, samples were snap-cooled in ice-cold water and 1.5-μL aliquots were loaded on a 4% (19:1) acrylamide:bisacrylamide gel containing 6 *M* urea (36-cm well-to-read glass plate format; prerun at constant voltage (1000 V) for 30 min and equilibrated to 51°C). Electrophoresis was conducted for 2.5 h at constant voltage (3000 V) in 1X TBE using an ABI PRISM® 377 DNA Sequencer with the laser power set at 40 mW. Allele sizes were determined using the GeneScan® Analysis v.2.1 software and the Local Southern size calling method. Automatic allele designation was achieved using the Genotyper® v.2.0 software (Applied Biosystems Division of Perkin Elmer).

Validation Experiments

Casework Study

In total, 275 miscellaneous samples (111 knowns, 164 questioned sources) representing 48 cases (22 homicides, 19 sexual assaults, 7 other crimes such as incest, impaired driving causing death, and break and enter) were examined. These samples were provided as DNA extracts remaining from concluded RFLP and PCR cases by the Royal Canadian Mounted Police (RCMP) Biology Operations Section (38 cases dated 1990–1994) and by the Bureau of Criminal Apprehension of the State of Minnesota (ten cases dated 1994–1996). The known samples consisted of 99 bloodstains, seven scalp hair samples, three pubic hair samples, one semen sample, and one saliva sample. The questioned specimens consisted of 94 bloodstains, four hair samples, 25 semen stains, 28 vaginal swabs, three pubic hair samples in cervix (three separate and distinct samples), seven anal/rectal swabs, one sample of fingernail scrapings, one sample of human tissue, and one cigarette butt.

Following DNA quantitation, 33 of the 275 samples showed very light chemiluminescence signals (<0.2 ng of total DNA) or no signals at all. Only specimens with larger amounts of DNA (i.e., ≥0.2 ng of total DNA) were processed further. For some samples (those < 2.5 ng of total DNA), the entire DNA extract was consumed during PCR amplification, but, in the majority of cases, 2.5 ng of DNA were used in a 25-μL PCR reaction volume with 28 cycles of amplification.

Mixture Study

DNA extracts from blood of 14 individuals (males and females), selected from one of the RCMP Caucasian databases, were used to prepare ten different mixture scenarios (Table 1). The amount of total template DNA used was set at both 1 and 2 ng in ratios ranging from 20:1 to 1:20 (i.e., 20:1, 10:1, 8:1, 6:1, 4:1, 2:1, 1:1, 1:2, 1:4, 1:6, 1:8, 1:10, 1:20).

NonHuman DNA Study

The specificity of the primer sets included in AmpFℓSTR® Profiler Plus™ was evaluated under reduced PCR volume conditions using different amounts of DNA from a large survey of animals and microorganisms from the vaginal flora and intestinal tract. Many representatives from the higher primates were evaluated, and results for those samples will be presented in another paper. The 26 nonprimate species and number of specimens tested included: dog [6], cat [3], pig [4], cow [5], horse [5], mouse [5], hamster [1], sheep [6], goat [4], deer [11], moose [2], bison [2], elk [3], caribou [2], antelope [2], lemming [2], kangaroo [1], ostrich [2], goose [1], whale [2], dolphin [1], penguin [3], shark [2], fish [14], turtle [2],

TABLE 1—*Simulated mixture scenarios.*

Mixture	Amelogenin Profile*	STR Locus Types†
1	3X. 1Y	Type A—6 loci Type B—3 loci
2	4X	Type A—4 loci Type B—2 loci Type C—2 loci Type D—1 locus
3	2X, 2Y	Type A—4 loci Type B—2 loci Type F—3 loci
4	3X, 1Y	Type A—2 loci Type B—7 loci
5	3X, 1Y	Type A—3 loci Type B—3 loci Type C—2 loci Type E—1 locus
6	2X, 2Y	Type B—4 loci Type C—2 loci Type D—1 locus Type E—1 locus Type G—1 locus
7	4X	Type A—2 loci Type B—4 loci Type C—1 locus Type D—1 locus Type E—1 locus
8	3X, 1Y	Type A—2 loci Type B—3 loci Type C—2 loci Type F—1 locus Type G—1 locus
9	3X, 1Y	Type A—2 loci Type B—4 loci Type C—1 locus Type D—1 locus Type E—1 locus
10	2X, 2Y	Type B—3 loci Type C—3 loci Type D—2 loci Type G—1 locus

* Equivalent copy number of X and Y chromosomes in the mixture.

† Type A = 4 peaks, 2 heterozygous, no shared peaks. Type B = 3 peaks, 2 heterozygous profiles, 1 shared peak. Type C = 3 peaks, 1 heterozygous profile, 1 homozygous profile, no shared peaks. Type D = 2 peaks, 1 heterozygous profile, 1 homozygous profile, 1 shared peak. Type E = 2 peaks, 2 homozygous profiles, no shared peaks. Type F = 2 peaks, 2 heterozygous profiles, 2 shared peaks. Type G = 1 peak, 2 homozygous profiles, 1 shared peak.

TABLE 2—*Profiler Plus™ typing results from casework samples* and associated controls.*

Known/Questioned	Number of Samples	Full Profiles	Partial Profiles	No Profiles
Bloodstains from Cases	70	62	2	6
Bloodstains (Blood Internal Standard)†	22	20	2	…
Scalp Hair	6	4	2	…
Pubic Hair	3	3	…	…
Semen	1	1	…	…
Saliva	1	1	…	…
Questioned Bloodstains	78	61	4	13
Hair	1	1	…	…
Vaginal Swabs F1	9	7	…	2
Vaginal Swabs F2	12	10	1	1
Vaginal Swabs F3	6	4	…	2
Semen Stains F1	6	2	…	4
Semen Stains F2	10	6	…	4
Semen Stains F3	7	5	1	1
Pubic Hair in cervix F1	1	1	…	…
Pubic Hair in cervix F2	1	…	1	…
Anal/Rectal Swabs F1	2	1	…	1
Anal/Rectal Swabs F2	1	1	…	…
Anal/Rectal Swabs F3	2	…	…	2
Cigarette butts	1	…	1	…
Fingernail scrapings	1	1	…	…
Human tissue	1	1	…	…

* Number of cases examined = 48.

Number of exhibits processed for PCR = 103 known samples and 139 questioned samples including F1, F2, and F3 fractions.

All DNA extracts were subjected to Microcon-100 purification prior to amplification. Between 0.2 and 2.5 ng of template DNA were used for PCR.

† A blood internal standard is used to monitor the performance of the DNA extraction reagents.

sea lamprey [1]. The 13 bacterial and yeast strains examined included: *Bacteroides vulgatis, Bacteroides fragilis, Bacteroides thetaiotaomicon, Streptococcus agalactiae, Streptococcus intermedius, Clostridium perfringens, Gardnerella vaginalis, Lactobacillus acidophilus, Lactobacillus fermentum, Peptostreptococcus asaccharolyticus, Escherichia coli HB101, Candida albicans,* and *Saccharomyces cerevisiae.* The amount of DNA amplified was equivalent to that used in standard casework assays (i.e., 2 to 2.5 ng). To mimic extreme situations of contamination, 50 ng of template DNA was also amplified.

Results and Discussion

Casework Study

As detailed in Table 2, 88% of the known samples (91 of 103, blood internal standards included) produced a complete AmpF*ℓ*STR® Profiler Plus™ profile. Ten bloodstains and two scalp hair samples presented some challenges and produced partial or no results. Approximately 73% of the questioned DNA extracts (101 of 139) generated full profiles that were consistent with those of the corresponding known samples. Another 21% of the ques-

tioned specimens failed to amplify, whereas 6% showed partial results. In total, full profiles were generated for 79% of the samples (192 of 242). Partial profiles were observed in 6% of samples (14 of 242), and failure to obtain a profile was apparent in 15% of the extracts examined (36 of 242).

Table 3 presents the AmpFℓSTR® Profiler Plus™ results obtained according to the amount of target DNA used for amplification. The majority of specimens had sufficient DNA to permit the use of the recommended amount of DNA (i.e., 2.5 ng). Even so, there were twelve samples (6%) in that group that failed to produce any typing results. Other exhibits had enough DNA remaining from previous typing analyses for only one round of PCR (0.5 to 2 ng). The majority of samples in this category gave results, but a significant number of them (34%) produced no amplicons. Another subset of exhibits had suboptimal amounts of DNA for amplification (0.2 ng) but were nonetheless processed with AmpFℓSTR® Profiler Plus™. Interestingly, two samples of the 13 specimens in this group (15%) gave full profiles. However, the majority of these exhibits (69%) provided no genetic information.

The recommended optimal range of template DNA for amplification using AmpFℓSTR® Profiler Plus™ in 25-μL PCR reaction volume was established between 0.5 to 2.5 ng, although limited typing results were obtained using as low as 0.2 ng of template DNA. The success rate for amplification of samples using 0.5 to 2.5 ng of input DNA was 83%.

TABLE 3—*Profiler Plus™ typing results according to amounts of template DNA used in 25 μL PCR volume.*

Amount of Template DNA Used for Amplification ng	Number of Samples	Full Profiles	Partial Profiles	No Profiles
2.5	188	171	5	12
2	23	17	5	1
1.5	5	0	0	5
1	6	1	2	3
0.5	7	1	1	5
0.2	13	2	2	9

All AmpFℓSTR® Profiler Plus™ profiles generated from casework specimens could be grouped into six different categories.

Category 1—Full Profiles with Balanced Allele Signals

For the majority of casework samples examined (159 of 242, i.e., 66%), the profiles displayed fluorescent signals that were very balanced across the nine STR loci surveyed (Fig. 1).

Category 2—Profiles with Peak Heights <150 Relative Fluorescence Units (RFU)

Seven samples (3%) of the 242 samples processed for PCR showed full profiles with fluorescence intensity below 150 RFU. This value of 150 RFU is recommended by ABD as the threshold limit for true allele recognition (28) and was established using very specific amplification conditions (i.e., 50-μL PCR reaction volume with a 60-s annealing step and a 60-s extension step). As shown in Fig. 2, under the amplification conditions described in this paper, samples such as a semen stain on a quilt (female F2 fraction; Panel A), a control bloodstain from a complainant (Panel C) and blood swabbed off a telephone jack (Panel F) provided complete profiles despite low intensity signals. The profile obtained from a semen stain on blue denim shorts (Panel I) exhibited locus dropout at D18S51. In these examples, heterozygote peak height ratios calculated for most STR loci were between 73 to 100%, indicating that most alleles were amplified equally well (Table 4). However, for each profile, one or two STR loci displayed significant differences in peak heights (differences ≥30%, ratios ≤70%). Those are shown with an asterisk in Fig. 2. Interestingly, although weak profiles often displayed strong heterozygous peak height imbalances as shown in Table 4, this was not observed in every amplification that resulted in profiles < 150 RFU. Heterozygous peak height ratios as high as

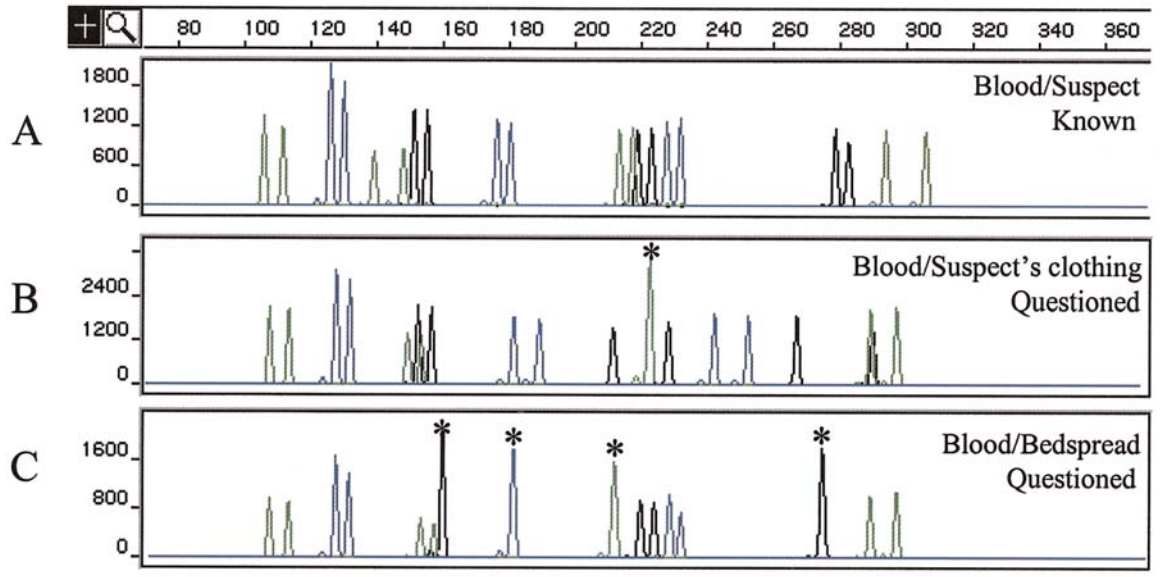

FIG. 1—*Profiler Plus™ profiles from casework specimens included in Category 1: "Full profiles with balanced signals." PCR amplifications were performed using 2.5 ng of template DNA in a 25-μL PCR reaction volume as detailed in the Methods section. Each panel depicts the relative fluorescence intensity (RFU, Y-axis) and the size estimate in bases (X-axis) derived from the internal lane standard GeneScan-500 [ROX] using the ABI GeneScan® Analysis version 2.1 software. The genetic markers observed from left to right, in order of size, are: Amelogenin, D3S1358, D8S1179, D5S818, HumvWA, D21S11, D13S317, HumFGA, D7S820, and D18S51.*

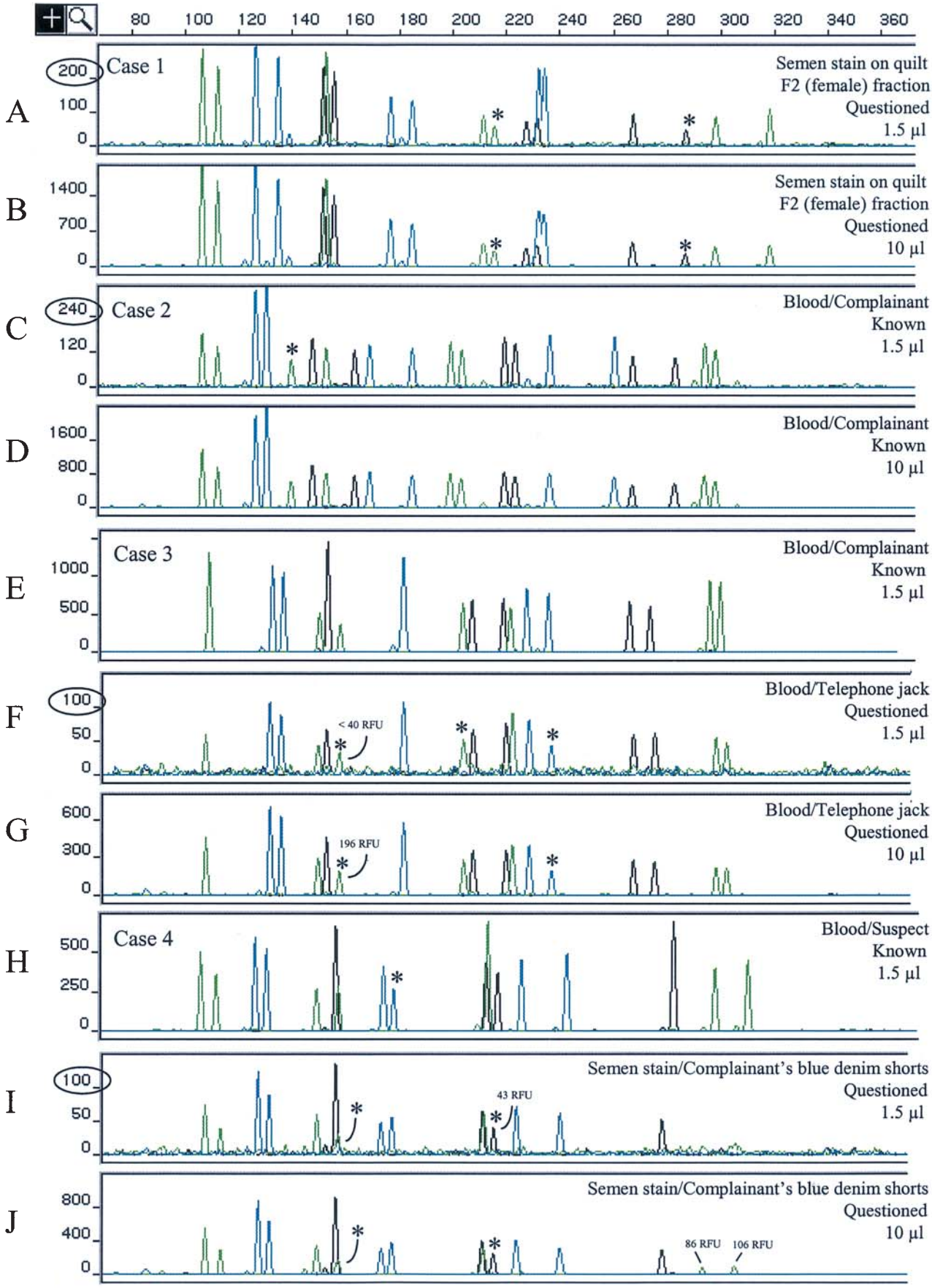

FIG. 2—*Profiler Plus™ profiles from casework specimens included in Category 2: "Profiles with peak heights < 150 RFU." PCR amplifications were performed using 0.2 ng of template DNA for the sample shown in Panel A, or 2.5 ng or DNA for the remaining samples, in a 25-µL PCR reaction volume as detailed in the Methods section. The volume indicated in each panel legend refers to the PCR aliquot used for analysis. For a description of the panel X- and Y-axis and the genetic markers included in Profiler Plus™, refer to the legend of Fig. 1. The STR loci that displayed significant imbalances in peak heights for both alleles comprised in a heterozygous profile (ratios < 70%) are marked with an asterisk (refer to Table 4 for actual peak height ratio percentages). Peaks that are close or below the threshold limit of 40 RFU are shown with their respective fluorescence intensity.*

TABLE 4—*Heterozygous peak height ratio (%) for Profiler Plus profiles < 150 RFU*.*

		Case 1		Case 2		Case 3			Case 4		
STR Locus	Dye Label†	Panel A LIP‡	Panel B Boosted§	Panel C LIP	Panel D Boosted	Panel E Known	Panel F LIP	Panel G Boosted	Panel H Known	Panel I LIP	Panel J Boosted
D3S1358	B	83	81	83	84	91	**83** (90/109)	90	88	**73** (91/125)	73
HumvWA	B	**94** (138/147)	91	**91** (132/145)	89	n/a	n/a	n/a	65	**84** (49/58)	78
HumFGA	B	100	96	96	90	92	**54** (45/83)	51	92	**86** (63/73)	77
D8S1179	G	n/a‖	n/a	**70** (95/135)	77	75	**82** (36/44)	66	92	**62** (38/61)	52
D21S11	G	**64** (58/91)	66	**83** (129/156)	85	92	**59** (54/92)	74	n/a	n/a	n/a
D18S51	G	**78** (86/110)	90	**83** (125/151)	81	97	**88** (49/56)	96	89	LDO¶	**81** (86/106)
D5S818	Y	93	90	**79** (128/163)	76	n/a	n/a	n/a	n/a	n/a	n/a
D13S317	Y	**83** (72/87)	88	87	86	97	**88** (68/77)	99	85	**65** (43/66)	61
D7S820	Y	**51** (49/96)	53	**93** (98/105)	93	90	**95** (61/64)	96	n/a	n/a	n/a

* Heterozygous peak height ratio = lower intensity allele/higher intensity allele.
† G refers to Green (JOE), B is for Blue (FAM), and Y is for Yellow (NED).
‡ LIP = low intensity profile; bold values indicate percentages calculated with one or two peak heights <150 RFU. The intensity of the peaks (in RFU) are indicated in parenthesis.
§ Boosted = genetic profile generated using 10-μL amplicons instead of 1.5 μL of the PCR amplification reaction.
‖ n/a = not applicable as the profile at that locus was homozygous.
¶ LDO = locus dropout, both expected alleles missing.

95% were calculated for D7S820 with peaks as low as 61 and 64 RFU (Table 4, results for Panel F). Percentages such as 88% were noted at D18S51 and D13S317 with combinations of peaks as low as 49/56 and 68/77 RFU, respectively. Table 4 indicates that heterozygous peak height ratios > 90% were observed four times out of 24 heterozygous STR profiles showing peaks < 150 RFU, heterozygous peak height ratios between 80 and 90% were observed ten times, heterozygous peak height ratios between 70 and 80% were observed four times, heterozygous peak height ratios between 60 and 70% were observed three times, and heterozygous peak height ratios between 50 and 60% were observed three times.

The overall fluorescent signals were enhanced when seven-fold aliquots of the PCR were used for analysis (10 μL instead of 1.5 μL; see Fig. 2 Panels B, D, G, and J). This allowed the detection of alleles that were below the threshold limit of 40 RFU (see below) before enhancement. Examples are shown in Panel G at D8S1179, where the allele peak height detected below 40 RFU was increased to a value of 196 RFU using the "boosting" strategy and Panel J at D18S51, where both alleles were barely visible before boosting but were clearly detected following enhancement. Although fluorescent signals were enhanced, the heterozygote peak height ratios observed remained essentially the same with an overall range of 73 to 99% for all boosted profiles examined (Table 4). The STR loci that were identified as presenting significant differences in peak heights in the original (unboosted) profiles still showed major differences following enhancement. In general, variations between 1 to 12% were noted when peak height ratios were compared between low intensity profiles and boosted profiles for each individual STR locus. In one specimen, however, peak height variations as high as 15 and 16% were noted at D8S1179 and D21S11 (Panel G).

Under the experimental conditions described herein, the 150 RFU lower signal threshold, recommended by ABD (28) for the recognition of a true allele, represents an overly conservative lower limit for casework samples. Results provided in the present section indicated that profiles with peak height values less than 150 RFU could provide crucial information about a complainant's or a suspect's genetic profile. This genetic information, either complete or partial, is very important in an investigation and could expedite the exclusion of suspects and assist in the inclusion of others. In order to effectively use the important genetic information provided by degraded or highly compromised evidentiary samples, the lower threshold of 40 RFU was chosen for the recognition of potential alleles (30). Any fluorescent signals below this limit could potentially represent background noise and may be difficult to interpret. It is important to keep in mind that potential alleles (from a single source sample or a minor component in a mixture) detected with marginal peak heights (i.e., just below threshold) can be boosted to bring their peak heights above the threshold of detection to allow them to be assigned an allele number by Genotyper. In such instances, efforts are made to keep the intensity of the alleles comprised in the single source profile or the major component alleles below the 3500 RFU ceiling established for Profiler Plus profile analysis, as above this value the presence of PCR artefacts is more prevalent and could interfere with the interpretation of mixtures. As shown in Fig. 2, adopting the boosting strategy does not impact significantly on the differential peak heights observed. All peaks are kept essentially in the same proportions as noted in the initial profiles, before enhancement, with less than 10% variation.

Category 3—Full Profiles with a Descending Signal Gradient from Amelogenin to D18S51

Descending signal gradients were observed in 14% of casework samples tested (33 of 242). Examples are shown in Fig. 3*A*, and the overall gradient values expressed as percentages for each profile

generated using 2 to 2.5 ng of DNA in a 25-μL PCR volume are provided in Table 5. Gradients were calculated using the lowest allele peak height value over the highest allele peak height value in the profile for each colored set of STR loci and using half the peak height value for homozygous loci. The average of the three gradient values calculated is provided for comparison. As depicted in Table 5, under the amplification conditions selected for the AmpFℓSTR® Profiler Plus validation, different specimens produced different profile gradients.

This pattern observed could be the result of progressive DNA degradation or inhibition by unknown factors still present in the DNA extracts even after performing the Microcon-100 purification step following DNA extraction. Such factors may have an adverse effect on amplification by preventing effective polymerization of the larger STR loci products. If the latter possibility was true, then reducing the volume of DNA extract used for amplification should decrease the concentration of potential PCR inhibitors and improve the quality of the profiles. The Profiler Plus™ typing results generated using 1 ng of DNA, instead of the initial 2 to 2.5 ng DNA for each sample shown in Fig. 3*A*, are exhibited in Fig. 3*B*. The reduction in the volume of DNA extract resulted in the production of very balanced profiles for all samples retested (11 out of 33 samples as some were used up during validation), suggesting that the original pattern observed was indeed due to PCR inhibition. This overall improvement in the fluorescent signal balance was reflected in the average gradient values calculated for all profiles generated using 1 ng of DNA (see Table 5). The signal balance was improved by approximately 9 to 27% and by as much as 53% in the case of human tissue (19% versus 72%) using less DNA. When the volume of DNA extract was further reduced by a factor of two, i.e., using 0.5 ng of input DNA in 25 μL, the profiles were essentially the same as those obtained using 1 ng of DNA, i.e., very balanced across all nine STR loci (see Table 5), with the exception of the fluorescence signal strength, which was proportionately reduced compared to the one noted for the profiles generated using 1 and 2 to 2.5 ng (data not shown).

PCR inhibition appeared to be overcome by reducing the amount of DNA extract used for amplification in a 25-μL reaction. However, using a limited quantity of DNA in a large PCR volume reduces the overall allele peak heights. In order to enhance the fluorescence signal of samples, we examined the effect of a simultaneous reduction of the volume of DNA extract and PCR reaction. We were interested in finding out if the inhibition seen using 2 to 2.5 ng in 25 μL, which disappeared using 1 or 0.5 ng in 25 μL, be observed again using 1 or 0.5 ng in 5 or 10 μL. Figures 4*A* and 4*B* present the results of the samples that showed the most significant slopes in Fig. 3*A* (Panels B, D, E, and G), suggesting the most inhibition under the amplification conditions used. They should be the most affected when amplified under reduced conditions of input DNA and reaction volume. Inhibition was indeed noted in the case of 1 ng of DNA in 5 μL for all samples shown (Figs. 4*A* and 4*B*, Panels B and F). All four profiles displayed the descending slope observed initially when 2 to 2.5 ng of DNA were used in 25-μL PCR volume. The average gradient values for the three colored sets of STRs were very similar to those calculated for profiles generated using 2 to 2.5 ng in 25 μL (see Table 5). In profiles generated using 1 ng in 5 μL, $n/n + 1$ products were even detected, which is indicative of the suboptimal amplification conditions. Using 1 ng of DNA in 10-μL PCR reaction provided very good typing results in all four samples (Figs. 4*A* and 4*B*, Panels D and H), and so did the use of 0.5 ng of DNA in 10-μL PCR reaction (Figs. 4*A* and 4*B*, Panels C and G) and 0.5 ng of DNA in 5-μL

TABLE 5—*Descending signal gradients observed in casework samples.**

	Case 1		Case 2	
PCR Conditions	Blood Complainant A, %	Human Tissue K, %	Blood Pillowsham 1, %	Blood Pillowcase 1, %
2.5 ng in 25 μL	63	19	58	34
1 ng in 25 μL	72	72	70	49
0.5 ng in 25 μL	58	62	56	53
0.5 ng in 5 μL	ND†	26	ND	57
1 ng in 5 μL	ND	20	ND	35
0.5 ng in 10 μL	ND	34	ND	59
1 ng in 10 μL	ND	31	ND	58
	Case 3		Case 4	
	Blood Complainant B, %	Anal Swab with Semen F1 Fraction, %	Blood Complainant C, %	Blood White Underwear, %
2.5 ng in 25 μL	16	60	27	40
1 ng in 25 μL	43	71	46	38
0.5 ng in 25 μL	80	70	42	42
0.5 ng in 5 μL	57	ND	45	ND
1 ng in 5 μL	42	ND	12	ND
0.5 ng in 10 μL	65	ND	66	ND
1 ng in 10 μL	63	ND	55	ND

* Gradients were calculated using the lowest over the highest allele peak height value for each colored set of STRs. The average of the three gradient values is provided for each profile.

† ND = not determined.

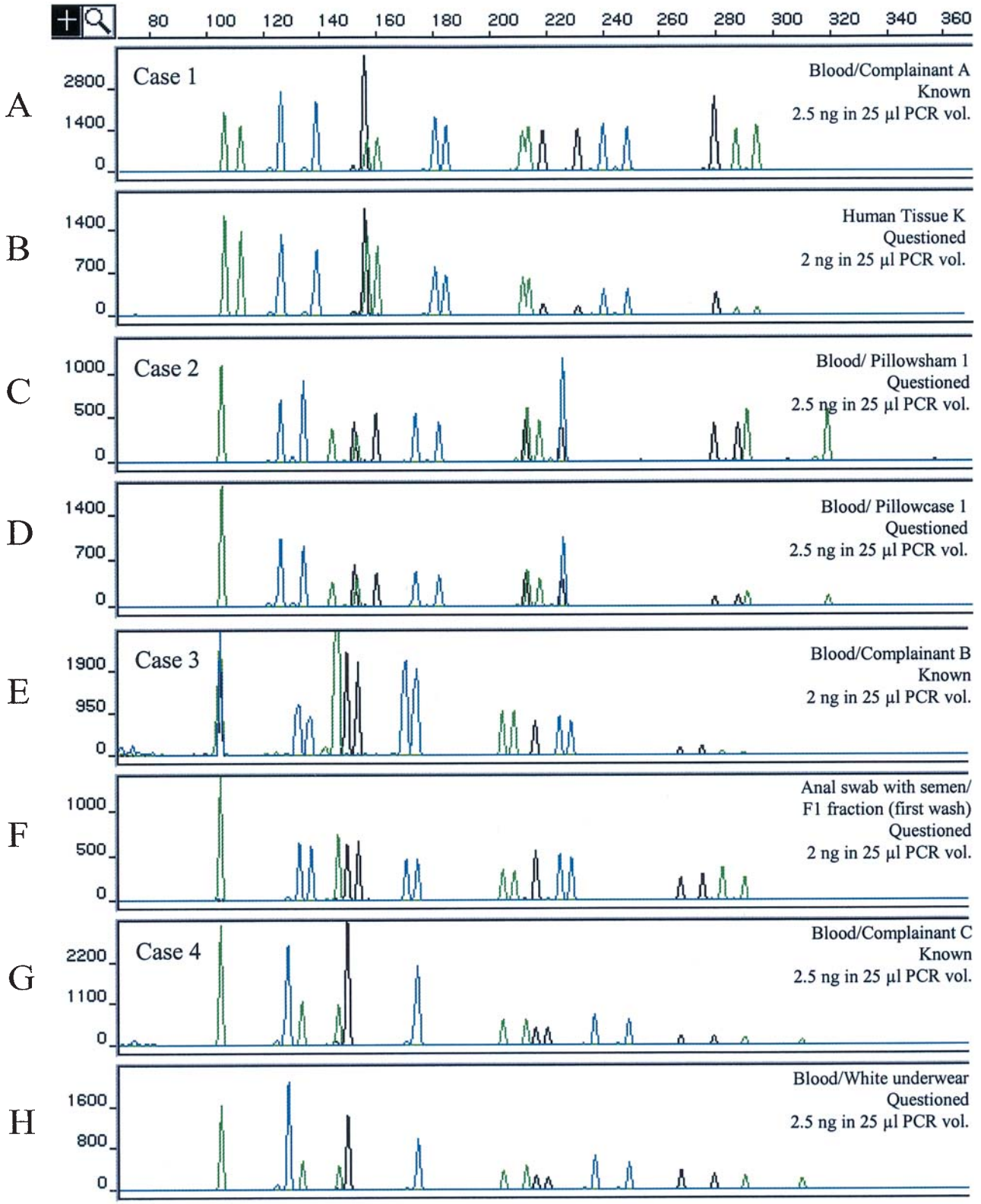

FIG. 3*A—Profiler Plus™ profiles from casework specimens included in Category 3: "Full profiles with a descending signal gradient from Amelogenin to D18S51," PCR amplifications were performed using 2 to 2.5 ng of template DNA in a 25-µL PCR reaction volume as detailed in the Methods section. For a description of the Panel X- and Y-axis and the genetic markers included in Profiler Plus™, refer to the legend of Fig. 1.*

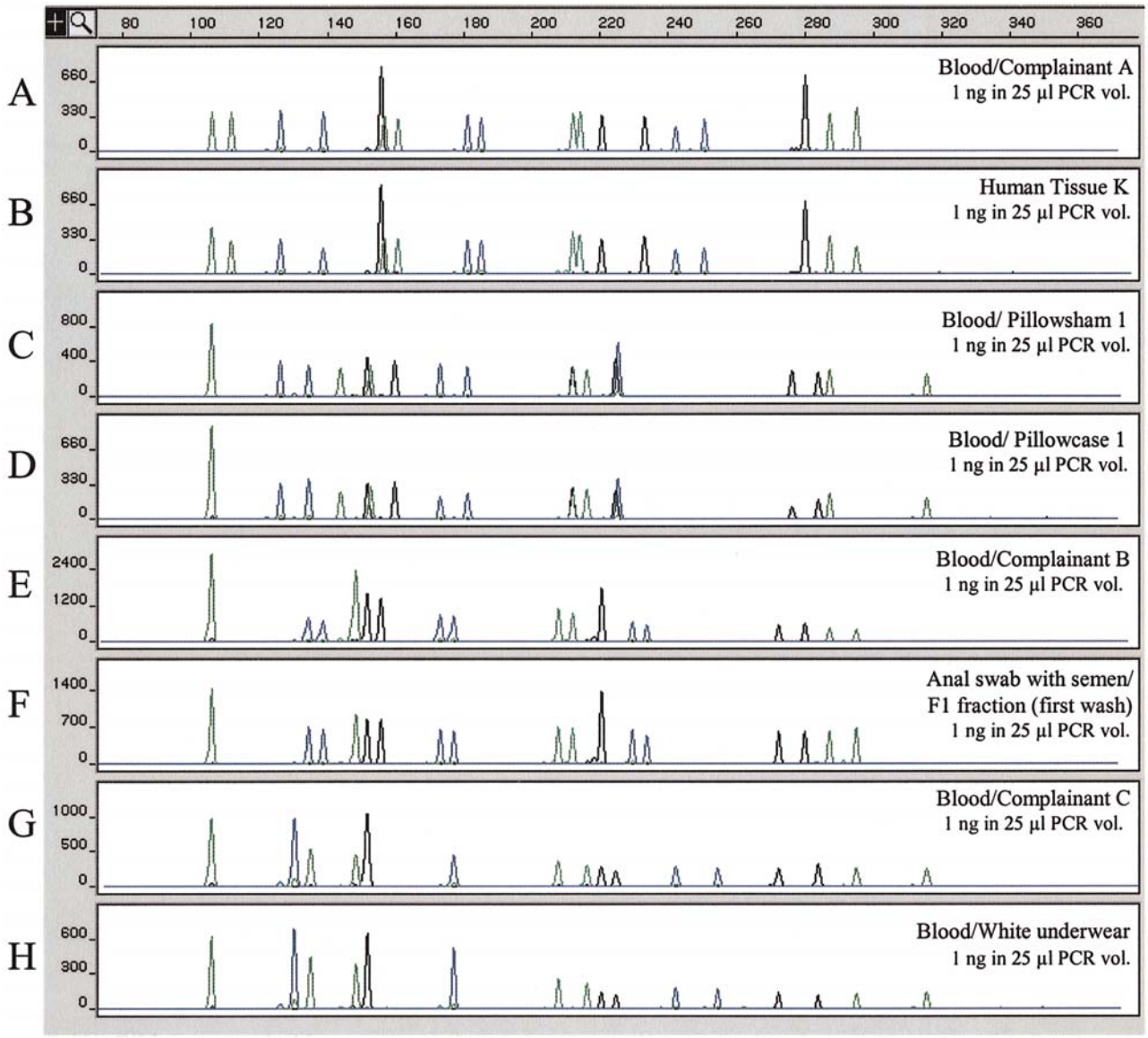

FIG. 3*B—Profiler Plus™ profiles from casework specimens included in Category 3: "Full profiles with a descending signal gradient from Amelogenin to D18S51" amplified under reduced template DNA conditions (1 ng). PCR amplifications were performed using 1 ng of template DNA in a 25-µL PCR reaction volume as detailed in the Methods section. For a description of the panel X- and Y-axis and the genetic markers included in Profiler Plus™, refer to the legend of Fig. 1.*

PCR reaction (Figs. 4*A* and 4*B*, Panels A and E). The overall signal balance was reflected in the average gradient values calculated for profiles generated under the specified PCR amplification conditions. The latter scenario (0.5 ng of DNA in 5-µL PCR reaction) allowed amplification to occur effectively, enhancing the fluorescence signals without affecting significantly the differential allele peak heights noted within a heterozygous profile.

Category 4—Partial Profiles

Some of the samples that failed to produce complete Profiler Plus™ profiles are shown in Fig. 5, Panels A, D, and G. Overall, 14 samples of the 242 specimens examined (6%) generated partial Profiler Plus™ profiles. In the majority of the partial profiles, a gradual loss of the largest STR amplicons was noted. However, there were three instances where STR loci with mid-size range alleles failed to amplify while larger STR alleles were efficiently amplified (data not shown).

Samples in this category were examined further to determine if other amplification conditions could improve the quality of their profiles or their typing outcome as failure to provide full profiles could be the result of DNA degradation or PCR inhibition by some factors present in the DNA extracts. The complete and balanced profiles generated using 1 or 0.5 ng of DNA in 25-µL PCR volume for three samples that initially produced partial results are shown in Fig. 5, Panels B, C, E, F, H, and I. Reducing the volume of DNA extract by a factor of two or four provided excellent results. The profiles obtained using 0.5 ng of DNA were complete, but the fluorescence intensity was half that noted when 1 ng of DNA was used. These results suggest that inhibition by unknown factors was preventing efficient amplification when 2 ng of DNA were used in 25 µL. Under reduced conditions of input DNA and PCR reaction volume, results were identical to those obtained for samples of Category 3. Excellent typing results were seen for 0.5 ng of DNA in 5 or 10-µL reaction and 1 ng in 10 µL, but obvious inhibition was noted using 1

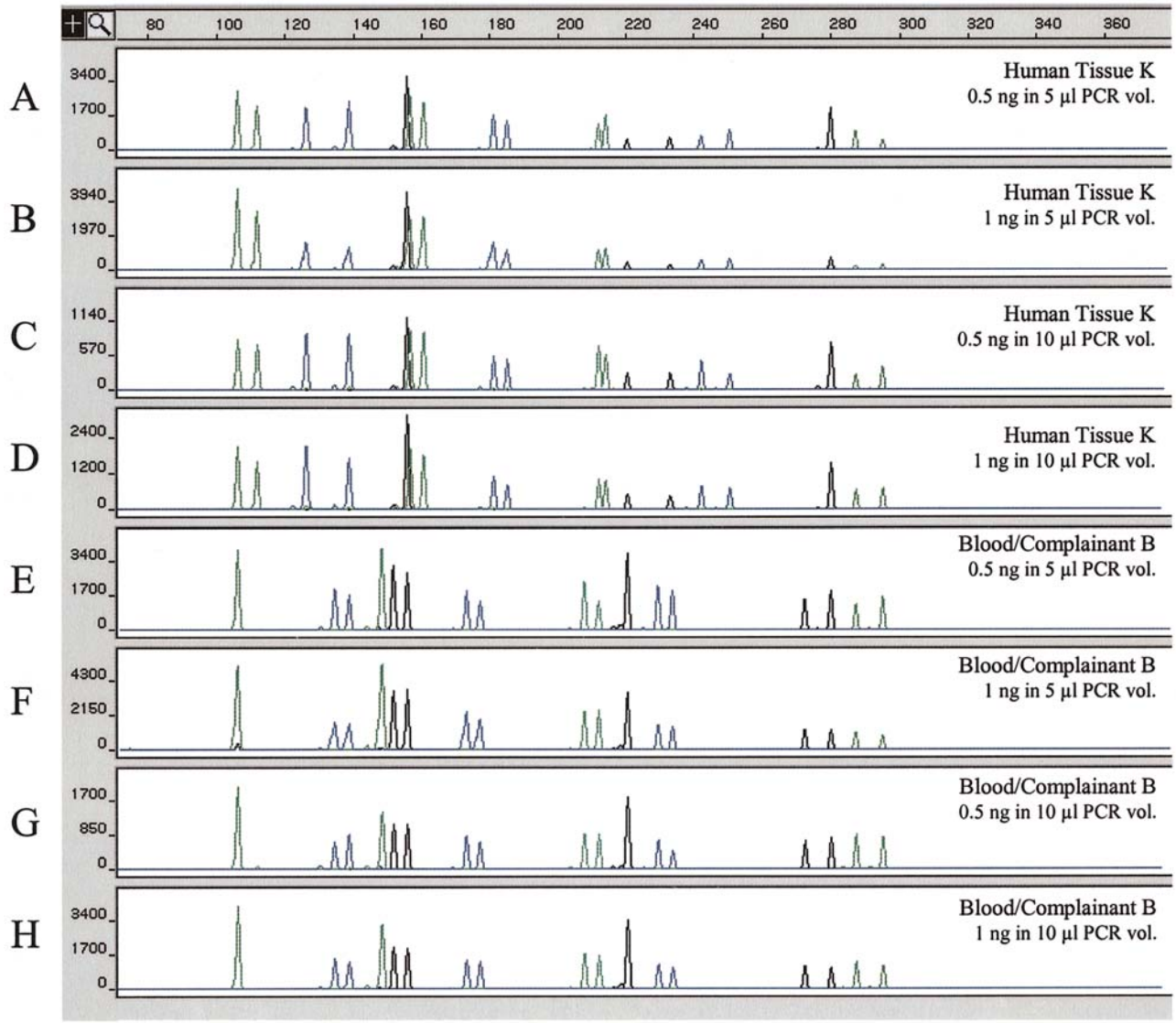

FIG. 4*A—Profiler Plus™ profiles from a human tissue specimen and a known blood from a complainant included in Category 3: "Full profiles with a descending signal gradient from Amelogenin to D18S51," amplified under reduced template DNA (0.5, 1 ng) and reduced PCR volume (5 µL, 10 µL) conditions. For details on PCR amplifications, refer to the Methods section. For a description of the panel X- and Y-axis and the genetic markers included in Profiler Plus™, refer to the legend of Fig. 1.*

ng in 5 µL (Table 5 and data not shown). In addition, under such reduced amplification conditions (1 ng in 5 µL), $n/n + 1$ products were detected as previously shown in Figs. 4*A* and 4*B*, Panels B and F.

As observed for profiles that exhibited the peak height pattern typical of partially degraded DNA (i.e., showed a slope), partial profiles could be converted into balanced and complete profiles simply by reamplifying the samples under reduced conditions of input DNA. There was no need for further purification of the DNA extracts using another Microcon-100 column as a second amplification using half or a quarter of the original amount of DNA (i.e., 1 or 0.5 ng) provided excellent results. Although limited to a few casework samples, a simultaneous reduction in the input DNA (0.5 ng instead of 2 ng) and PCR reaction volume (5 or 10 µL instead of 25 µL) provided excellent typing results for all samples retested. Such conditions allow the reduction of the cost of the amplification assay even further by a factor of five (5 µL used instead of 25 µL). A more detailed validation study using casework samples and mixtures was carried out under reduced PCR reaction volumes and similar conclusions were obtained (33). Under these amplification strategies, the AmpF*ℓ*STR® Profiler Plus™ amplification system provided excellent results in more than 85% of the samples. This percentage represents an underestimation because many samples showing partial or no profiles could not be retested under reduced conditions because they had been used up during validation.

Category 5—Profiles with $n/n + 1$ Products

Profiles with split peaks ($n/n + 1$) were noted in 35 samples of the 242 examined (14%). These split peaks were observed despite the additional extension period of 45 min at 60°C and the overnight incubation at room temperature included in the routine amplification protocol in order to promote the AmpliTaq DNA polymerase 3′-terminal transferase activity. As indicated in Table 6, of the nine STR loci included in Profiler Plus™, D3S1358, D8S1179, and HumvWA were the most resistant to the nontemplated nucleotide

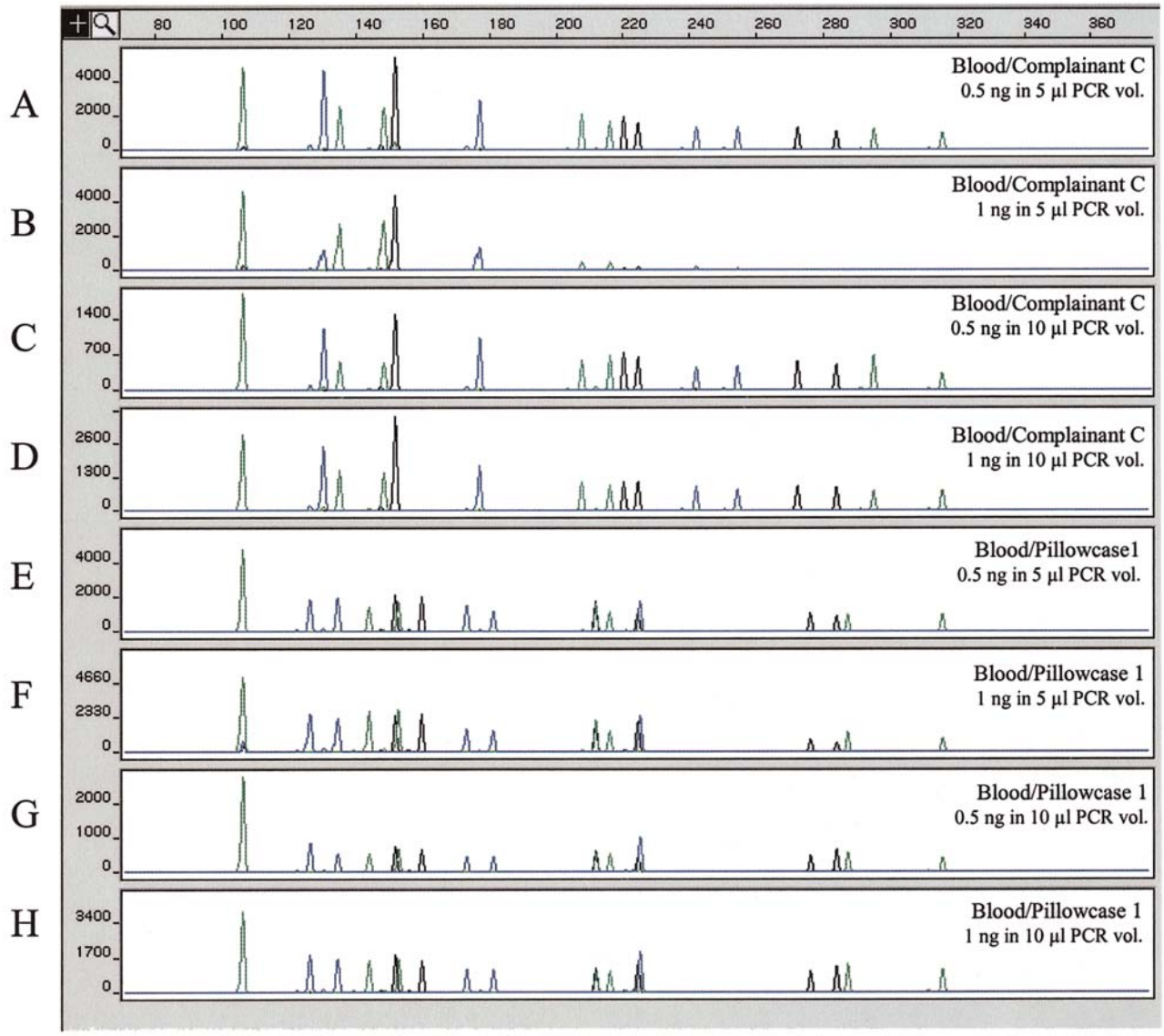

FIG. 4*B—Profiler Plus™ profiles from a known blood from a complainant and a bloodstain on a pillowcase included in Category 3: "Full profiles with a descending signal gradient from Amelogenin to D18S51," amplified under reduced template DNA (0.5, 1 ng) and reduced PCR volume (5, 10 µL) conditions. For details on PCR amplifications, refer to the Methods section. For a description of the panel X- and Y-axis and the genetic markers included in Profiler Plus™, refer to the legend of Fig. 1.*

addition, with the highest incidence of $n/n + 1$ products noted with D3S1358 (97% of the total samples exhibiting $n/n + 1$ products). In contrast, none of the 242 profiles produced from casework specimens during the validation study presented split peaks at D21S11, D13S317, and HumFGA. The remaining three STR loci (D5S818, D7S820, and D18S51) and amelogenin showed moderate resistance to the nontemplated nucleotide addition.

It has been shown that the AmpliTaq DNA polymerase 3′ terminal transferase activity is influenced by the nucleotide sequence at the 5′ end of the reverse primer (34). Magnuson's study also indicated that dATP or dGTP promote the addition of a nucleotide (preferentially dATP) at the 3′ end of the duplex molecule, while dCTP or dTTP are bad substrates for the addition. As the Profiler Plus primer sequences remain proprietary to ABD, it is not possible to verify that the D3S1358 reverse primer has indeed a dCTP or a dTTP at its 5′ end. This would explain the high propensity of D3S1358 to show $n/n + 1$ split peaks. To minimize the production of split peaks, amplification conditions described herein have been designed to drive the reaction towards the addition of a nucleotide at the 3′ end of all blunt-ended amplicons.

Category 6—Mixed Profiles

Twenty samples (8%) of the 242 exhibits tested presented mixed profiles. Mixed profiles were further dissected using data collected during validation of the Profiler Plus system regarding stuttering and heterozygous allele peak height differentials. Table 7 summarizes both the stutter percentages and the heterozygote peak height ratios noted for each of the STR loci included in Profiler Plus. The stutter percentage values were established from all nonmixture casework samples examined during the validation (35). The FAM- and JOE-labeled STR markers were more prone to stuttering as reflected in the higher stutter percentage values calculated for these markers (14–16%) versus values obtained for the NED-labeled STR loci (9 to 11%). As a conservative approach, stutter percentages for

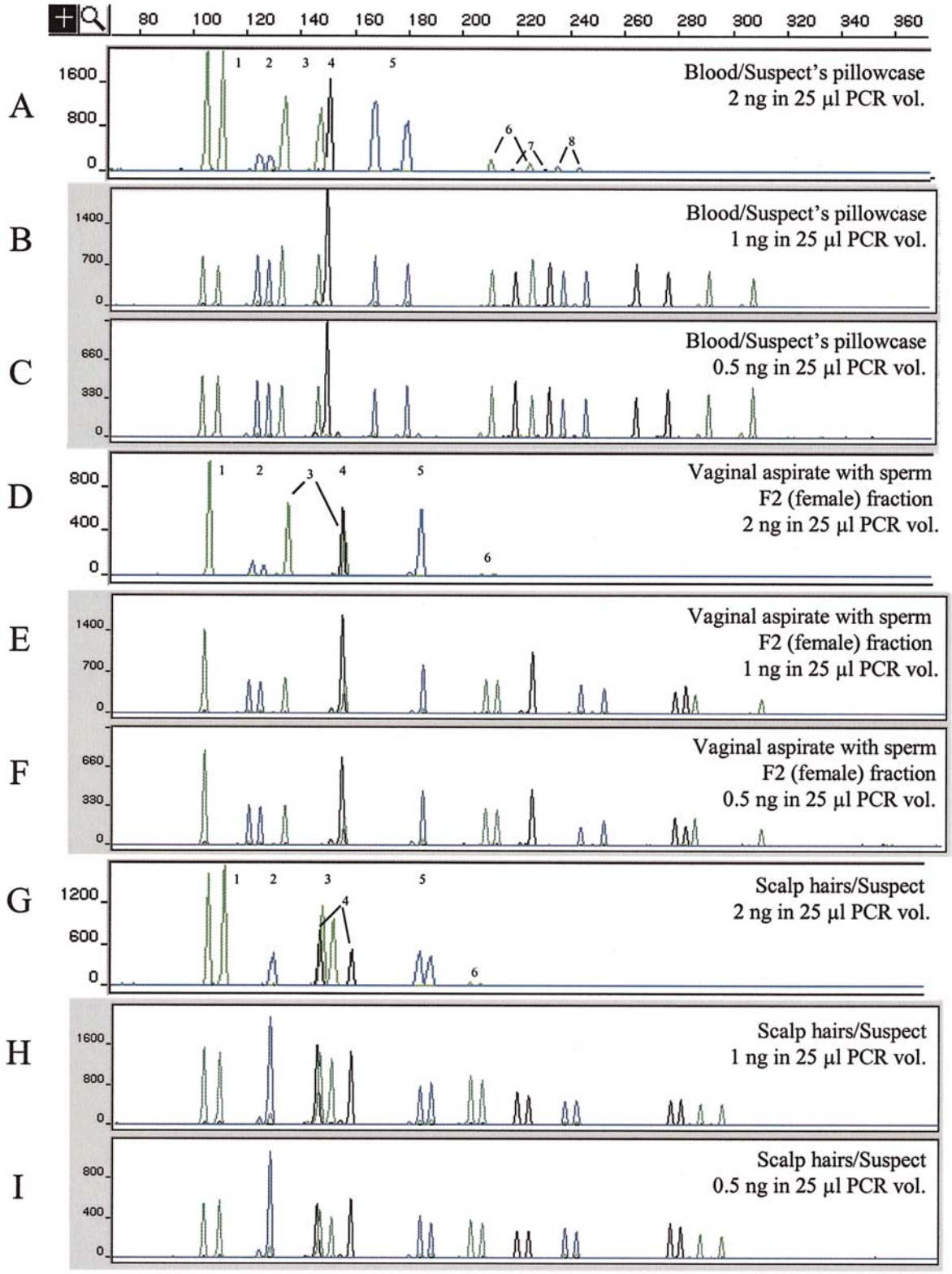

FIG. 5—*Profiler Plus™ profiles from casework specimens included in Category 4: "Partial profiles" amplified under normal and reduced template DNA and PCR volume conditions. PCR amplifications were performed using 2 ng of DNA in a 25-µL PCR reaction volume (Panels A, D, and G), 1 ng of DNA in a 25-µL PCR reaction volume (Panels B, E, and H), 0.5 ng of DNA in a 25-µL PCR reaction volume (Panels C, F, and I) as detailed in the Methods section. For a description of the panel X- and Y-axis, refer to the legend of Fig. 1. The labeling scheme for the genetic markers is: Amelogenin (1), D3S1358 (2), D8S1179 (3), D5S818 (4), HumvWA (5), D21S11 (6), D13S317 (7), HumFGA (8), D7S820 (9), and D18S51 (10).*

TABLE 6—*Propensity of the Profiler Plus™ megaplex to resist the nontemplated nucleotide addition.*

Genetic Marker*	Number of Samples with $n/n + 1$ Products†	Percentage of Total Samples Examined ($N = 242$)	Percentage of Total Samples with $n/n + 1$ Products ($N = 35$)
Amelogenin	3	1.2	8.6
D3S1358	34	14	97
D8S1179	11	4.5	31
D5S818	4	1.7	11
HumvWA	9	3.7	26
D21S11	0	…	…
D13S317	0	…	…
HumFGA	0	…	…
D7S820	1	0.4	2.9
D18S51	2	0.8	5.7

* Genetic markers are listed by size, i.e., from the smallest to the largest size range.
† Some samples had $n/n + 1$ products at more than one locus.

TABLE 7—*Percentage stutter and heterozygous peak height ratios for casework samples.*

STR Locus	Stutter Median + 3 SD*, %	*N*†	Heterozygote Ratio Median ± SD‡, %	*N*†
D3S1358	13.5	295	88 ± 12	174
HumvWA	14.4	321	85 ± 12	160
HumFGA	14.3	328	88 ± 12	163
D8S1179	13.9	313	89 ± 13	161
D21S11	15	306	90 ± 11	173
D18S51	15.9	343	84 ± 12	177
D5S818	10.7	293	89 ± 12	154
D13S317	9.4	301	90 ± 10	150
D7S820	9.7	313	88 ± 13	157

* For heterozygous profiles constituted of alleles differing by one core repeat (four bases), the larger allele was excluded from the stutter calculations. Stutter peaks for parent alleles < 100 RFU were excluded from the calculations. The median values plus 3 standard deviations (SD) are used for profile interpretation as these values include the most extreme stutter expected in 99.7% of all nonmixture sample measurements.
† N represents the number of "scorable" alleles for stutter calculations and the number of allele pairs used for heterozygous peak height ratio calculations.
‡ Heterozygous peak height ratio = lower intensity allele over higher intensity allele X100.

the blue and green STR systems in Profiler Plus have been set at ≤ 16% and at ≤ 12% for the yellow STR loci. In other words, a spike-shaped peak located in a stutter position (*n*-4) is considered a true allele when the ratio of that "stutter" peak to the sister peak is >16% for the blue and green STR loci and >12% for the yellow STR loci.

Optimization of the primers at each locus included in the Profiler Plus™ megaplex system was attempted by the manufacturer in order to balance the peaks (i.e., obtain the same peak height) in heterozygous samples. As shown in Fig. 1, the Profiler Plus profiles generated from forensic specimens during validation were generally well balanced. For nonmixture casework samples, the median values obtained for the peak height ratios of the lower peak (in RFU) to the higher peak (in RFU) was determined to be ≥ 84% at all loci (84% being the lowest observed median value) with a standard deviation of ≤ 13% (13% being the highest value); see Table 7. This means that heterozygous peak height ratios as low as 45% (84%–3SDs [13%]) could be observed when 99.7% of all nonmixture casework sample measurements are considered. Although rare, this strong signal imbalance could potentially be seen in casework samples.

Blood Mixture—Mixture Composed of Two Males; No Major/Minor Component Identified—Mixed profiles generated from bloodstains swabbed off a steering wheel are shown in Fig. 6. With blood samples, differential extraction is not performed, and the number of potential donors and the major and minor components are established over multiple loci. The identification of major and minor components at each STR locus is accomplished, first, by considering all possible genotypes that may have contributed the profile observed and, second, by calculating the ratios of the highest peak of any possible minor component to the lowest peak of any putative major component (in RFU). The four-peak profiles are usually examined first followed by the three-peak profiles. Based on the most extreme heterozygous allele peak height ratio calculated for nonmixture casework samples (i.e., 84%—3SDs [13%] = 45%) and results of the studies for both pristine mixtures and casework mixtures, it was initially and arbitrarily established that when the ratio of the highest peak (in RFU) of the putative minor component to the lowest peak (in RFU) of the most probable major component is ≤ 20%, at the majority of STR loci where calculations can be performed, a major component can be identified in a mixture comprised of two individuals. Subsequently, as casework experience was gained using the AmpF*ℓ*STR® Profiler Plus™ amplification system, that value was reassessed, deemed to be too conservative and was eventually changed to ≤ 30%. When more than one genotype combination may have contributed the profile observed at any one STR locus, as noted for D3S1358 and HumFGA (Fig. 6, Panel A), D8S1179 (Fig. 6, Panel B), D5S820 and D13S317 (Fig. 6, Panel C), the most conservative option is used and the most probable combination is said to be the one that presents a ratio > 30%. This approach favors combinations where no major component can be identified. Using this very conservative approach, no major component was identified at any of the STR loci evaluated in Profiler Plus™ for the mixed blood sample profiles shown in Panels A, B, and C. In this instance, no frequency estimates can be calculated for a major component, as it could not be unambiguously dissected out and attributed to a single source. No match to a major component is declared. Instead, an inclusion probability for the contributors can be derived using the program "STRmix," which enumerates all possible genotypes that could have contributed to the mixed profile. "STRmix" has been developed by Dr. George Carmody (Dept. of Biology, Carleton University, Ottawa) in collaboration with the RCMP (30). "STRmix" can be employed when the minor component has peak heights ≥100 RFU and is present at three or more loci. Although the minimum

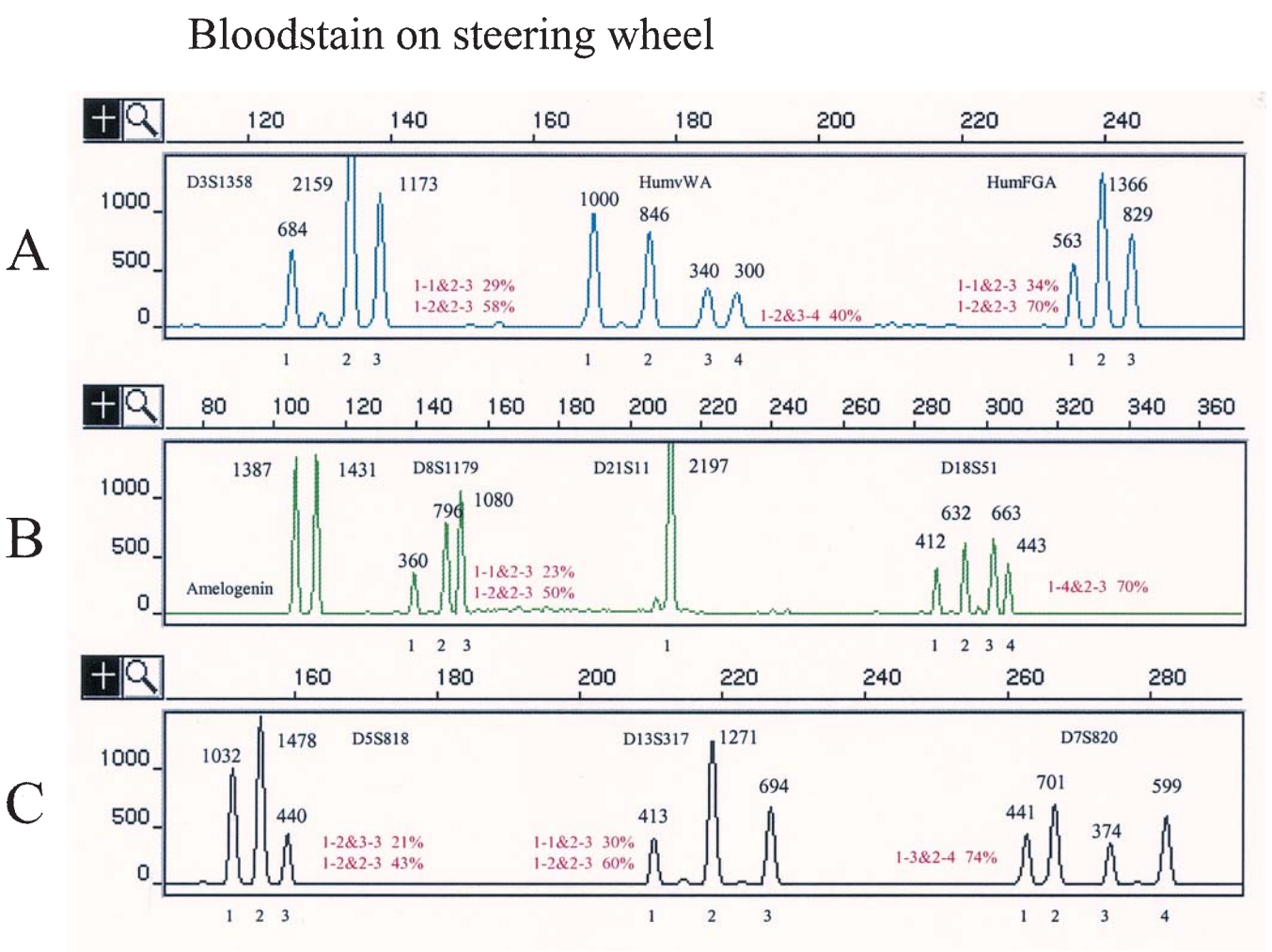

FIG. 6—*Profiler Plus™ profiles generated from a mixed blood sample. PCR amplifications were performed using 2.5 ng of DNA in a 25-μL PCR reaction volume as detailed in the Methods section. For a description of the panel X- and Y-axis, refer to the legend of Fig. 1. The fluorescence intensity (in RFU) for each of the peak observed is provided. All possible genotype combinations were considered for each locus. The peak height ratio of the highest peak of the putative minor component to the lowest peak of the putative major component were calculated for all possible combinations. The percentages for the most probable genotype combinations are shown. All other combinations were >100%. Example of calculations performed: At D3S1358, possible genotype combination homozygote 1-1 as minor component and heterozygote 2-3 as major component (342 RFU [half homozygous peak height 684 RFU] ÷ 1173 RFU = 29%) or heterozygote 1-2 as minor component and heterozygote 2-3 as major component (2159 − 684 = 1475 RFU; 684 ÷ 1173 RFU = 58%). At HumvWA, possible genotype combination heterozygote 3-4 as minor component and heterozygote 1-2 as major component (340 ÷ 846 RFU = 40%). At HumFGA, possible genotype combination homozygote 1-1 as minor component and heterozygote 2-3 as major component (282 RFU [half homozygous peak height 563 RFU] ÷ 829 RFU = 34%) or heterozygote 1-2 as minor component and heterozygote 2-3 as major component (1366 − 563 = 803 RFU; 563 ÷ 803 RFU = 70%). Following calculations, no minor or major component could be identified at any of the STR loci.*

threshold for a true allele has been set at 40 RFU, peak heights ≥ 100 RFU are used in "STRmix" to ensure that any sister peak accompanying a minor peak of 100 RFU would be detected. According to the heterozygous peak height ratios mentioned earlier, this sister peak could be as small as 45 RFU (median 84%—3SDs [13%] = 45%). When minor peaks are detected below the 100 RFU cut-off for "STRmix," the amplified samples are run again using larger aliquots of the PCR reaction in order to enhance the fluorescence signals and ensure that all minor peaks to be detected are indeed being detected. Only those loci where one is confident that all peaks in the mixture have been detected would be included in the estimation of the inclusion probability.

Differential Extraction—F3 Fraction is a Mixture of Male and Female—Mixed profiles derived from the differential extraction of an oral swab with semen are shown in Fig. 7. The FAM-labeled and JOE-labeled STR amplicons are presented. In this particular example, the F3 fraction presented a mixture consistent with having originated from two individuals. In this sexual assault case, the endogenous female profile was successfully subtracted out of the mixture in the F3 fraction and a single additional contributor was unambiguously dissected out at each locus (i.e., there was only one possibility for that profile at each locus). As this profile matched the known sample from the suspect, a random match probability was calculated using the specialized software "STRQuest" (30) at all STR loci where the male contributor was unambiguous.

In the majority of sexual assault cases, the F1 fraction (first wash of the differential extraction process) is not normally examined in the interpretation of the profiles generated from the F2 and F3 fractions as a result of the successful separation of the male and female components. However, there are instances where the F1 fraction will facilitate profile interpretation by providing a clearer view of the male profile. For example, in the case of vasectomized individuals whose profiles would most likely be detected in the F1 fraction

or in the case where the contribution of a male in the F3 fraction is minor and his profile is buried underneath the female component. During the validation of the Profiler Plus™ megaplex, 19 sexual assaults were processed. In total, eleven F1 fractions provided full profiles. Of those eleven profiles, seven corresponded to the complainant's profile, one was consistent with the suspect's profile, and three were consistent with a mixture of both the suspect's and complainant's profiles.

All typing results generated for casework samples using the megaplex Profiler Plus™, under the specific amplification conditions described in this report, corroborated the results obtained previously using other PCR-based STR multiplexes (21,29). Samples that failed to produce a profile using Profiler Plus™ in 25 μL also showed no results using other STR multiplexes under different amplification conditions (data not shown). The same conclusions were reached in most cases where all pertinent samples were still available for analysis. However, the wide range of heterozygous allele peak height ratios noted for nonmixture casework samples using Profiler Plus™ (Table 7) reduced the percentage ratio at which a minor component could be unambiguously identified in a mixture.

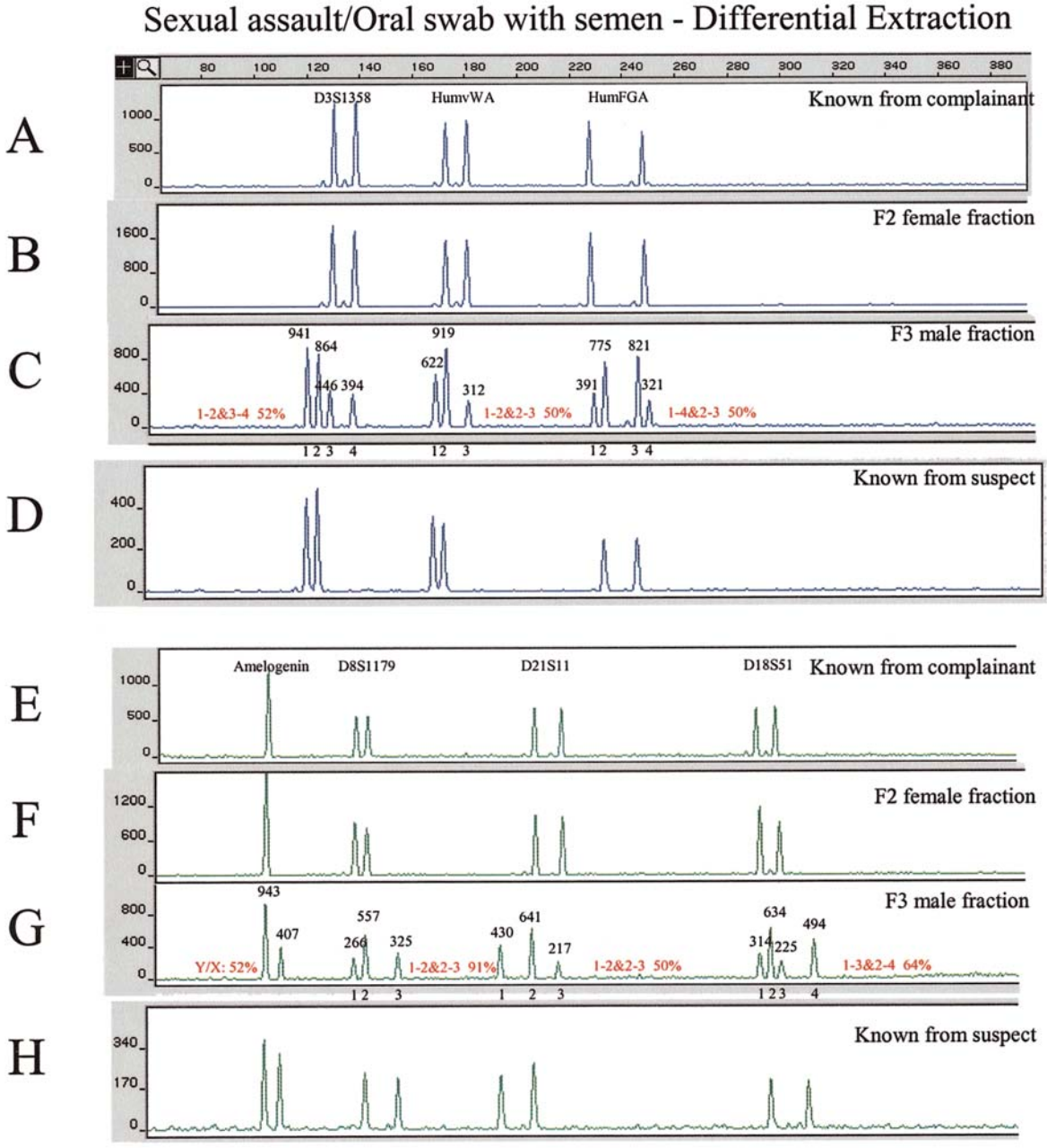

FIG. 7—*Profiler Plus™ profiles generated from a sexual assault specimen processed using a differential extraction. The FAM-labeled STR loci are shown in Panels A to D and the JOE-labeled STR loci are shown in panels E to H. PCR amplifications were performed using 2.5 ng of DNA in a 25-μL PCR reaction volume as detailed in the Methods section. For a description of the Panel X- and Y-axis, refer to the legend of Fig. 1. The fluorescence intensity (in RFU) for each of the peak observed in the F3 fraction is provided. The endogenous female profile was successfully subtracted out of the mixture in the F3 fraction, and a single additional contributor was unambiguously dissected out at each locus. Example of calculations performed in Panel C: At D3S1358, genotype combination heterozygote 1-2 as additional component and heterozygote 3-4 as endogenous component (446 ÷ 864 RFU = 52%), at HumvWA, genotype combination heterozygote 1-2 as additional component and heterozygote 2-3 as endogenous component (919-622 = 297 RFU; 312 ÷ 622 RFU = 50%), at HumFGA, genotype combination heterozygote 2-3 as additional component and heterozygote 1-4 as endogenous component (391 ÷ 775 RFU = 50%).*

Consequently, some cases for which a minor component was identified using previous STR multiplex systems (21,29) were assigned an inclusion probability to support the contribution of the donors of known samples to the mixture.

Mixture Study

Ten different mixture scenarios were set up, using total DNA amounts of 1 and 2 ng, in order to evaluate the ability of the Profiler Plus™ amplification system to detect minor profiles in mixtures. As detailed in Table 8, depending on the mixture scenario (i.e., if the minor component shared alleles with the major component or possessed alleles corresponding in size to *n*-4 stutter peaks of the major alleles), the limit of detection of a complete Profiler Plus™ profile at all loci for a minor component ranged from 10:1 (when contributor X1 was the major component; Mixtures 3, 4, and 5) to 1:20 (when Contributor X2 was the major component; Mixtures 7 and 10), using 2 ng of DNA for amplification. Very similar results were recently reported by Holt et al. (15) using 50 μL of PCR volume. Reducing the amount of template DNA from 2 to 1 ng also diminished the ability to detect minor components, as reflected by the lower detection limit of 1:8 obtained when contributor X2 was the major component (Mixtures 7 and 10). Detection of a mixture at a minimum of one STR locus was possible with ratios $< 20:1$ and $> 1:20$. Examples of electropherograms produced in this study and used to generate data presented in Table 8 are shown in Figs. 8*A* to 8*C*. The Profiler Plus™ profiles from Mixture 10 are shown for DNA ratios 10:1 and 8:1 in Fig. 8*A*. A comparison of the top panel

TABLE 8—*Limit of detection of full Profiler Plus™ profiles for a minor component in mixtures.*

Mixture	Total Amount of Target DNA, ng	Limit of Detection of Full Profiler Plus Profiles at All Loci DNA ratio of two contributors (Actual amounts of DNA in mixtures)	
1 Type A—6 loci* Type B—3 loci	1 2	8:1 and 1:4 6:1 and 1:6	(0.89 ng : 0.11 ng and 0.2 ng : 0.8 ng) (1.7 ng : 0.3 ng and 0.3 ng : 1.7 ng)
2 Type A—4 loci Type B—2 loci Type C—2 loci Type D—1 locus	1 2	8:1 and 1:4 6:1 and 1:10	(0.89 ng : 0.11 ng and 0.2 ng : 0.8 ng) (1.7 ng : 0.3 ng and 0.18 ng : 1.8 ng)
3 Type A—4 loci Type B—2 loci Type F—3 loci	1 2	10:1 and 1:4 10:1 and 1:6	(0.91 ng : 0.09 ng and 0.2 ng : 0.8 ng) (1.8 ng : 0.18 ng and 0.3 ng : 1.7 ng)
4 Type A—2 loci Type B—7 loci	1 2	8:1 and 1:2 10:1 and 1:2	(0.89 ng : 0.11 ng and 0.33 ng : 0.66 ng) (1.8 ng : 0.18 ng and 0.7 ng : 1.3 ng)
5 Type A—3 loci Type B—3 loci Type C—2 loci Type E—1 locus	1 2	6:1 and 1:4 10:1 and 1:6	(0.86 ng : 0.14 ng and 0.2 ng : 0.8 ng) (1.8 ng : 0.18 ng and 0.3 ng : 1.7 ng)
6 Type B—4 loci Type C—2 loci Type D—1 locus Type E—1 locus Type G—1 locus	1 2	2:1 and 1:2 2:1 and 1:6	(0.66 ng : 0.33 ng and 0.33 ng : 0.66 ng) (1.3 ng : 0.7 ng and 0.3 ng : 1.7 ng)
7 Type A—2 loci Type B—4 loci Type C—1 locus Type D—1 locus Type E—1 locus	1 2	1:1 and 1:8 1:1 and 1:20	(0.5 ng : 0.5 ng and 0.11 ng : 0.89 ng) (1 ng : 1 ng and 0.095 ng : 1.9 ng)
8 Type A—2 loci Type B—3 loci Type C—2 loci Type F—1 locus Type G—1 locus	1 2	8:1 and 1:8 8:1 and 1:10	(0.89 ng : 0.11 ng and 0.11 ng : 0.89 ng) (1.76 ng : 0.22 ng and 0.18 ng : 1.8 ng)
9 Type A—2 loci Type B—4 loci Type C—1 locus Type D—1 locus Type E—1 locus	1 2	2:1 and 1:4 2:1 and 1:6	(0.66 ng : 0.33 ng and 0.2 ng : 0.8 ng) (1.3 ng : 0.7 ng and 0.3 ng : 1.7 ng)
10 Type B—3 loci Type C—3 loci Type D—2 loci Type G—1 locus	1 2	8:1 and 1:8 8:1 and 1:20	(0.89 ng : 0.11 ng and 0.11 ng : 0.89 ng) (1.76 ng : 0.22 ng and 0.095 ng : 1.9 ng)

* For a definition of the locus types, see Table 1 footnotes.

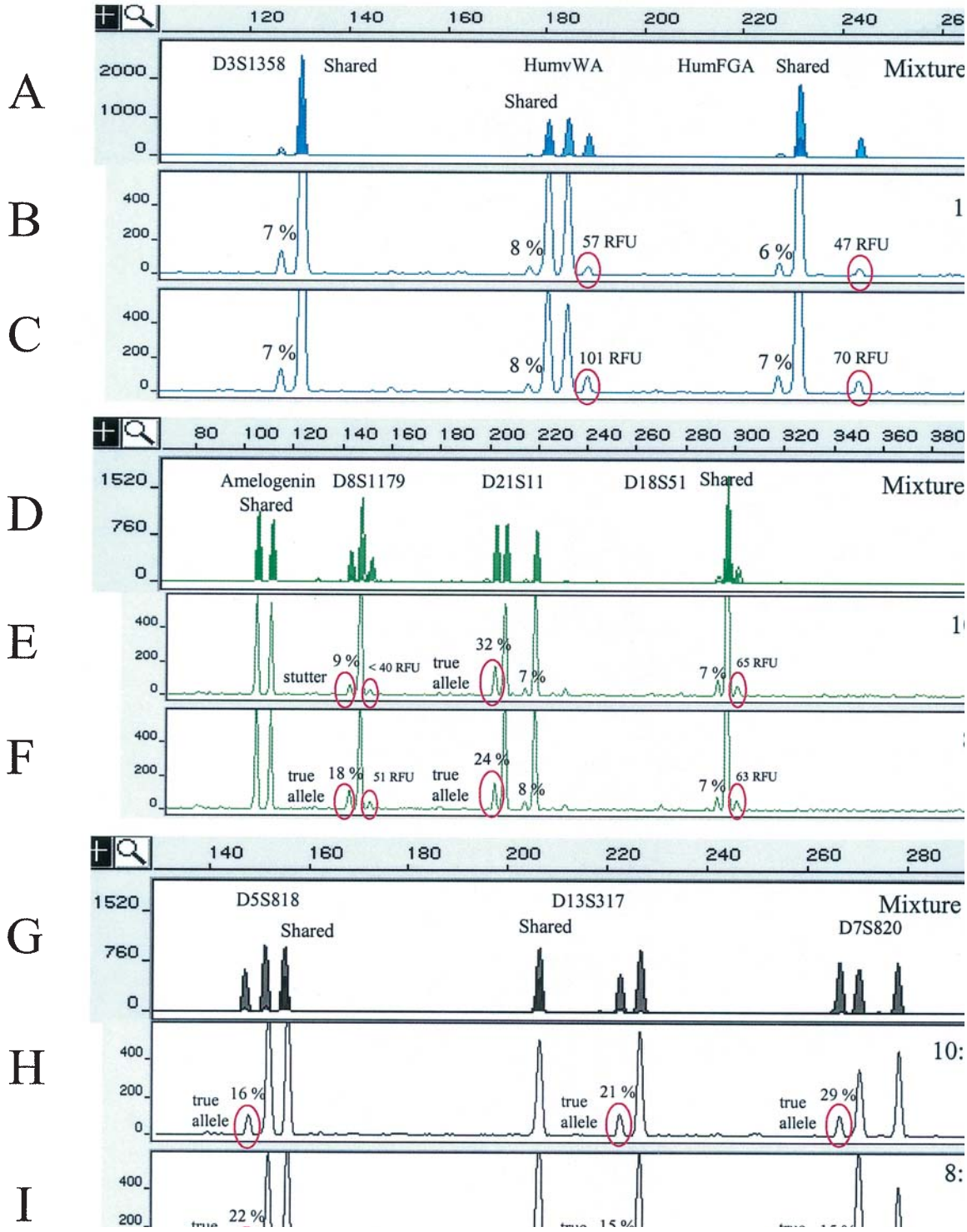

FIG. 8*A—Profiler Plus™ profiles generated from simulated mixture No. 10. PCR amplifications were performed using 2 ng of DNA in ratios of 10:1 and 8:1 in a 25-μL PCR reaction volume as detailed in the Methods section. For a description of the panel X- and Y-axis, refer to the legend of Fig. 1. The fluorescence intensity scale has been adjusted in Panels B, C, E, F, H, and I to facilitate the detection of the minor alleles. The pertinent information for the interpretation of mixture No. 10 is shown in each panel. This includes percentage stutter and minor allele peak height intensities (in RFU). The alleles in positions of stutter peaks that are recognized as true alleles following calculations are marked as such.*

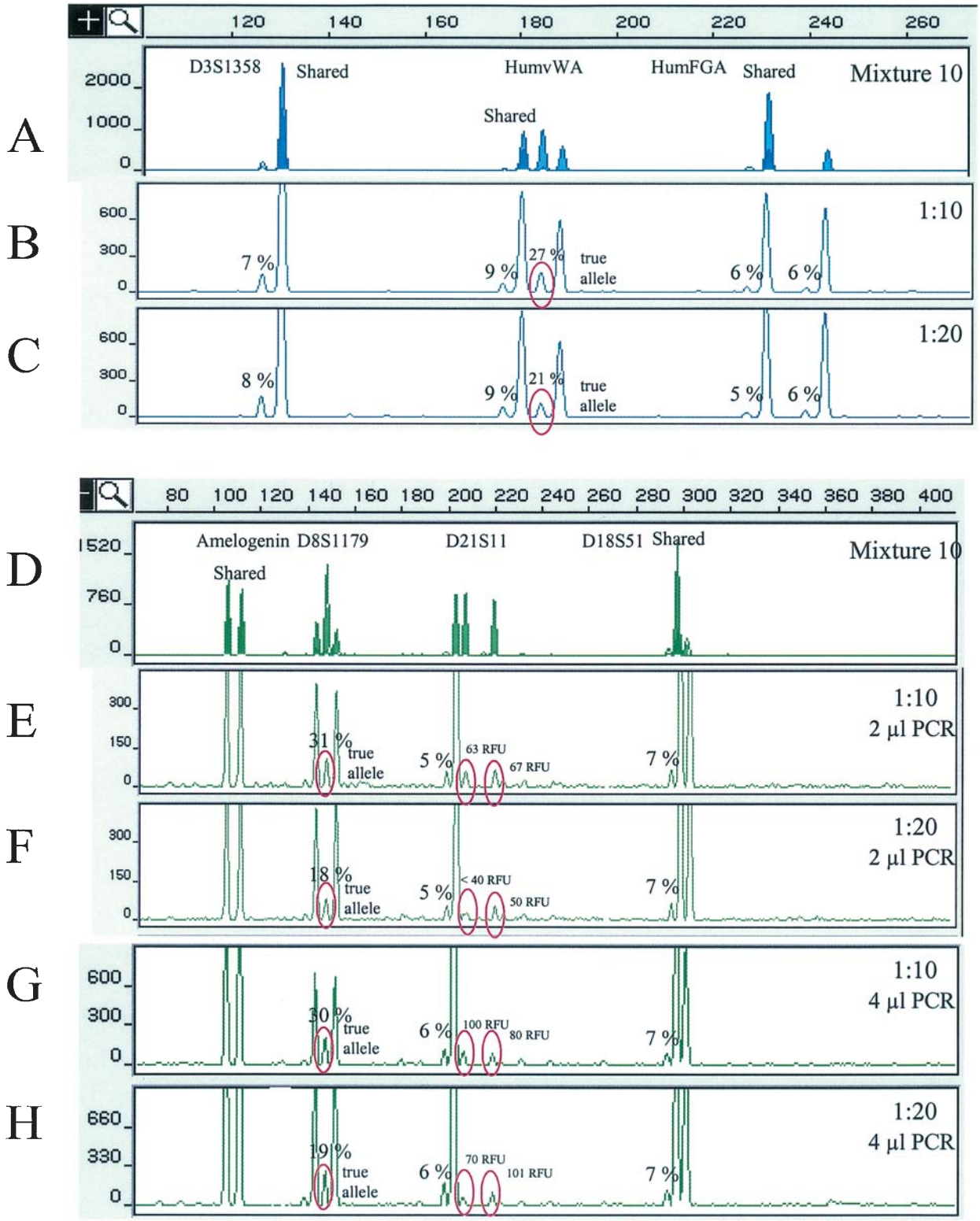

FIG. 8*B—Profiler Plus™ profiles generated from simulated mixture No. 10. PCR amplifications were performed using 2 ng of DNA in ratios of 1:10 and 1:20 in a 25-µL PCR reaction volume as detailed in the Methods section. FAM-labeled and JOE-labeled STR loci are shown. Panels B, C, E, and F: 2 µL of PCR used for analysis; Panels G and H: 4 µL of PCR used for analysis. For a description of the panel X- and Y-axis, refer to the legend of Fig. 1. The fluorescence intensity scale has been adjusted in Panels B, C, E, F, G, and H to facilitate the detection of the minor alleles and to demonstrate peak height sensitivity difference when larger PCR aliquots were used for analysis. The pertinent information for the interpretation of mixture No. 10 is shown in each panel. This includes percentage stutter and minor allele peak height intensities (in RFU). The alleles in positions of stutter peaks that are recognized as true alleles following calculations are marked as such.*

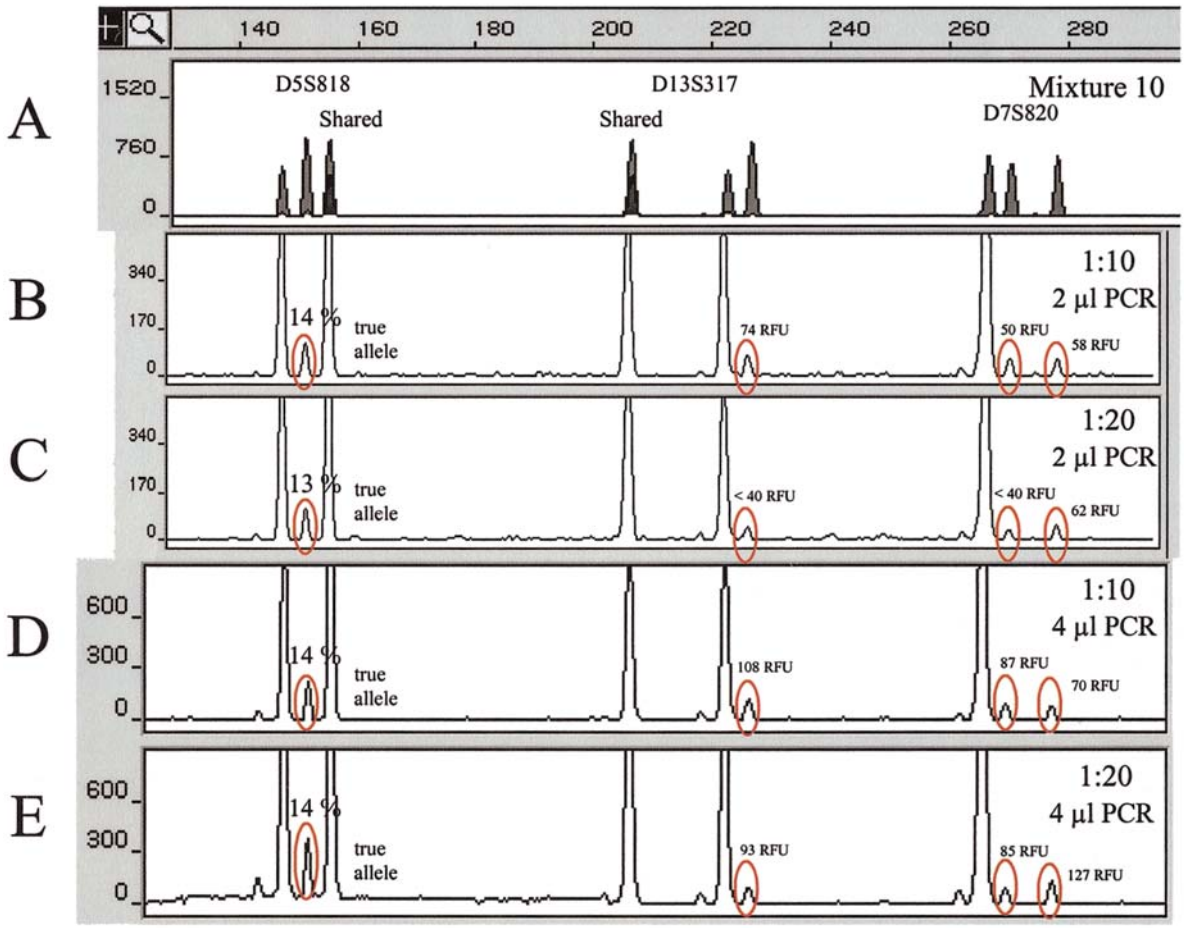

FIG. 8*C*—*Profiler Plus™ profiles generated from simulated mixture No. 10. PCR amplifications were performed using 2 ng of DNA in ratios of 1:10 and 1:20 in a 25-µL PCR reaction volume as detailed in the Methods section. NED-labeled STR loci are shown. Panels B and C: 2 µL of PCR used for analysis; Panels D and E: 4 µL of PCR used for analysis. For a description of the panel X- and Y-axis and other information, refer to the legend of Fig. 8B.*

to the bottom two panels for each set of dye-labeled STR systems revealed that, using 2 ng of DNA for amplification, all expected peaks for the minor component were detected at a ratio of 10:1 for each STR locus with the exception of two peaks at D8S1179 (see Panel E). The first allele was buried under a stutter peak and the second fell below the 40 RFU threshold limit of detection. For this combination of genotypes with contributor X1 as the major component, the limit of detection of full profiles at all STR loci was determined to be at a DNA ratio of 8:1. With Contributor X2 as the major component, the limit of detection of full profiles at all STR loci was determined to be at a DNA ratio of 1:20 (Figs. 8*B* and 8*C*). In these figures, all alleles in position of stutter peaks were easily identified since the stutter percentages were all above the normal range of stutter values for the STR loci examined (i.e., ≤12% for the NED-labeled STR loci and ≤16% for the FAM- and JOE-labeled STR loci). However, one allele at D21S11, one at D13S317, and one at D7S820 fell below the 40-RFU threshold limit of detection when 2-µL aliquots of the PCR were used for analysis (Fig. 8*B*, Panel F and Fig. 8*C*, Panel C). Increasing the PCR aliquot by a factor of two enhanced the overall signal intensity of the profile and allowed detection of these three alleles at 1:20 (compare Panels F with H in Fig. 8*B* as well as Panels C with E in Fig. 8*C*).

As the proportion of target DNA from contributor X1 reached that of contributor X2 in mixtures with DNA ratios of 2:1, 1:1, or 1:2, the detection of multiple alleles at any one STR locus was facilitated. However, the assignment of any one of these alleles to a specific donor became more challenging due to equal band intensities (data not shown). A systematic approach as detailed in the previous section is then required to interpret those mixtures.

NonHuman DNA Study

The human specificity of the STR loci included in the AmpF*ℓ*STR® Profiler Plus™ amplification system was tested on a limited number of animals and reported previously (28). Under reduced PCR volume conditions (i.e., 25 µL), all nine STR primer sets failed to yield amplified products from DNAs of the domestic and wild game animals tested (see list in Methods/Validation Experiments—*Nonhuman DNA Study*). The use of 2.5 or 50 ng of DNA for amplification did not produce any artefact peaks for any of the animals examined (data not shown).

None of the 13 bacterial or yeast strains examined showed peaks when amplified with the Profiler Plus™ system using 2.5 ng of DNA. However, two bacterial strains, i.e., *Bacteroides thetaiotaomicon* and *Streptococcus agalactiae,* produced minor peaks (85 and 97 RFU, respectively) using 50 ng of DNA. The green peak in *Bacteroides thetaiotaomicon,* sized at 231.74 bases, corresponds to a position within the allele size range for D21S11. The green peak in *Streptococcus agalactiae,* sized at 197.90 bases, also fell within the human D21S11 allele size range (data not shown).

Amelogenin results were obtained for the majority of animals tested. Gender determination was not possible in domestic and wild game animals as only one band sized at 102 bases was detected. No amplification products were detected in fish or any of the marine animals tested, with the exception of the whales, which displayed a 102-base fragment.

These results indicate that samples that may have come in contact with nonhuman biological sources, and more specifically with microorganisms, should provide clean and interpretable Profiler Plus™ profiles. This is especially true considering the excess target to contaminant ratio of DNA required before the monosignal is attained.

The ultimate evaluation of any DNA typing system resides in its performance with casework and mixture samples. The results presented in this report all point to the robustness and reliability of the AmpFℓSTR® Profiler Plus™ system when used under the experimental conditions described herein. Reducing the PCR volume to 25 μL allows major cost savings per sample analysis while maintaining high quality profiles. With this system, very high discrimination can be achieved with minimal analytical consumption of crucial evidentiary samples. These represent major advantages for forensic laboratories contemplating ways to reduce cost without compromising high quality service.

Acknowledgments

The authors would like to thank members of the RCMP Forensic Laboratory in Ottawa and in Halifax, as well as members of the Bureau of Criminal Apprehension of the State of Minnesota, for providing DNA extracts from some of their completed RFLP and PCR criminal cases for use in this validation study. We also thank Dr. John Bowen from the RCMP Forensic Laboratory in Ottawa for his many helpful comments.

References

1. Weber JL, May PE. Abundant class of human polymorphisms which can be typed using the polymerase chain reaction. Am J Hum Genet 1989;44:388–96.
2. Edwards A, Civitello A, Hammond HA, Caskey CT. DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. Am J Hum Genet 1991;49:746–56.
3. 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.
4. McBride LJ, O'Neill MD. Automated analysis of mutations responsible for genetic diseases in humans. Am Lab November 1991;52–9.
5. Ishino Y, Mineno J, Inoue T, Fujimiya H, Yamamoto K, Tamura T, et al. Practical applications in molecular biology of sensitive fluorescence detection by a laser-excited fluorescence image analyzer. BioTechniques 1992;13:936–43.
6. Gill P, Kimpton CP, Sullivan K. A rapid polymerase chain reaction method for identifying fixed specimens. Electrophoresis 1992;13:173–5.
7. Sullivan KM, Walton A, Kimpton C, Tully G, Gill P. Fluorescence-based DNA segment analysis in forensic science. Biochem Society Trans 1993;21:116–20.
8. Frégeau CJ, Fourney RM. DNA typing with fluorescently tagged short tandem repeats: a sensitive and accurate approach to human identification. BioTechniques 1993;15:100–19.
9. Frégeau CJ, Bowen KL, Elliott JC, Robertson JM, Fourney RM. PCR-based DNA identification: a transition in forensic science. In: Proceedings of the Fourth International Symposium on Human Identification; 1993 Sept; Scottsdale (AZ). Madison (WI): Promega Corporation, 1993; 107–18.
10. Urquhart A, Oldroyd NJ, Kimpton CP, Gill P. Highly discriminating heptaplex short tandem repeat PCR system for forensic identification. BioTechniques 1995;18:116–21.
11. Watson S, Kelsey Z, Webb R, Evans J, Gill P. The development of a third generation STR multiplex system (TGM). In: Olaisen B, Brinkmann B, Lincoln PJ, editors. Progress in forensic genetics. Proceedings of the Seventeenth Congress of the International Society for Forensic Haemogenetics; 1997 Sept 2–6; Oslo (Norway). Elsevier Science BV, 1998;192–4.
12. Schumm JW, Lins AM, Micka KA, Sprecher CJ, Rabbach DR, Bacher JW. Automated fluorescent detection of STR multiplexes—development of the GenePrint™ PowerPlex™ and FFFL multiplexes for forensic and paternity applications. In: Proceedings from the Seventh International Symposium on Human Identification, 1996 Oct 9–11, Scottsdale (AZ). Madison (WI): Promega Corporation, 1997; 70–88.
13. Lins AM, Micka KA, Sprecher CJ, Taylor JA, Bacher JW, Rabbach DR, et al. Development and population study of an eight-locus short tandem repeat (STR) multiplex system. J Forensic Sci 1998;43:1–13.
14. Lazaruk K, Holt C, Wallin J, Walsh PS. Optimization of the D8S1179, D21S11 and D18S51 STR loci for incorporation into AmpFℓSTR™ Green II and AmpFℓSTR Profiler II™. In: Proceedings from the Eighth International Symposium on Human Identification; 1997 Oct 9–11; Scottsdale (AZ). Madison (WI): Promega Corporation, 1998;86.
15. 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;47:66–96.
16. Wallin JM, Holt CL, Lazaruk KD, Nguyen TH, Walsh PS. Constructing universal multiplex PCR systems for comparative genotyping. J Forensic Sci 2002;47:52–65.
17. Gill P, Kimpton CP, Urquhart A, Oldroyd N, Millican ES, Watson SK, et al. Automated short tandem repeat (STR) analysis in forensic casework—a strategy for the future. Electrophoresis 1995;16:1543–52.
18. Evett IW, Gill PD, Lambert JA. Taking account of peak areas when interpreting mixed DNA profiles. J Forensic Sci 1998;43:62–9.
19. Clayton TM, Whitaker JP, Sparkes R, Gill P. Analysis and interpretation of mixed forensic stains using DNA STR profiling. Forensic Sci Int 1998;91(1):55–70.
20. Gill P, Sparkes B, Buckleton JS. Interpretation of simple mixtures when artefacts such as stutters are present—with special reference to multiplex STRs used by the Forensic Science Service. Forensic Sci Int 1998;95: 213–24.
21. Frégeau CJ, Bowen KL, Fourney RM. Validation of highly polymorphic fluorescent multiplex short tandem repeat systems using two generations of DNA sequencers. J Forensic Sci 1999;44:133–66.
22. Lygo JE, Johnson PE, Holdaway DJ, Woodroffe S, Whitaker JP, Clayton TM, et al. The validation of short tandem repeat (STR) loci for use in forensic casework. Int J Leg Med 1994;107:77–89.
23. Micka KA, Sprecher CJ, Lins AM, Comey CT, Koons BW, Crouse C, et al. Validation of multiplex polymorphic STR amplification sets developed for personal identification applications. J Forensic Sci 1996;41: 582–90.
24. Sparkes R, Kimpton C, Watson S, Oldroyd N, Clayton T, Barnett L, et al. The validation of a 7-locus multiplex STR test for use in forensic casework. I. Mixtures, ageing, degradation and species studies. Int J Leg Med 1996;109:186–94.
25. Sparkes R, Kimpton C, Gilbard S, Carne P, Andersen J, Oldroyd N, et al. The validation of a 7-locus multiplex STR test for use in forensic casework. II. Artefacts, casework studies and success rates. Int J Leg Med 1996;109:195–204.
26. 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;43:1–17.
27. 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: 647–60.
28. AmpFℓSTR Profiler Plus™ PCR Amplification Kit User's Manual 1997. Perkin Elmer Applied Biosystems, Human Identification Department, San Jose, CA.
29. Frégeau CJ, Bowen KL, Bowen JH, Gaudette BD, Fourney RM. National casework and national DNA database: The Royal Canadian Mounted Police perspective. In: Olaisen B, Brinkmann B, Lincoln PJ, editors. Progress in forensic genetics. Proceedings of the Seventeenth Congress of the International Society for Forensic Haemogenetics; 1997 Sept 2–6; Oslo (Norway). Elsevier Science BV 1998;541–3.
30. Royal Canadian Mounted Police, Forensic Laboratory Services Directorate. Biology Section Methods Guide. Rev. ed. Ottawa, ON, RCMP, 1998.

31. Waye JS, Presley LA, Budowle B, Shutler GG, Fourney RM. A simple and sensitive method for quantifying human genomic DNA in forensic specimen extracts. BioTechniques 1989;7:852–5.
32. Frégeau CJ, Aubin RA, Elliott JC, Gill SS, Fourney RM. Characterization of human lymphoid cell lines GM9947 and GM9948 as intra- and interlaboratory reference standards for DNA typing. Genomics 1995;28: 184–97.
33. Leclair B, Sgueglia JB, Wojtowicz PC, Juston AC, Frégeau CJ, Fourney RM. STR DNA typing: Increased sensitivity and efficient sample consumption using reduced PCR reaction volumes. J Forensic Sci 2003; 48(5):1–13.
34. Magnuson VL, Ally DS, Nylund SJ, Karanjawala ZE, Rayman JB, Knapp JI, et al. Substrate nucleotide-determined nontemplated addition of adenine by *Taq* DNA polymerase: implications for PCR-based genotyping and cloning. BioTechniques 1996;21:700–9.
35. Leclair B, Frégeau CJ, Bowen KL, Fourney RM. Systematic analysis of stutter percentages and allele peak height and area ratios at heterozygous loci for casework and database samples. (submitted to J Forensic Sci).

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