TECHNICAL NOTE

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Validation of Short Tandem Repeats (STRs) for Forensic Usage: Performance Testing of Fluorescent Multiplex STR Systems and Analysis of Authentic and Simulated Forensic Samples*

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ABSTRACT: The amplification and typing conditions for the 13 core CODIS loci and their forensic applicability were evaluated. These loci are CSF1PO, FGA, TH01, TPOX, vWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, and D21S11. Results were obtained using the multiplex STR systems AmpFℓSTR® Profiler Plus™ and AmpFℓSTR COfiler™ (Applied Biosystems, Foster City, CA), GenePrint™ PowerPlex™ (Promega Corporation, Madison, WI), and subsets of these kits. For detection of fluorescently labeled amplified products, the ABI Prism® 310 Genetic Analyzer, the ABI Prism 377 DNA Sequencer, the FMBIO® II Fluorescent Imaging Device, and the FluorImager™ were utilized. The following studies were conducted: (*a*) evaluation of PCR parameter ranges required for adequate performance in multiplex amplification of STR loci, (*b*) determination of the sensitivity of detection of the systems, (*c*) characterization of non-allelic PCR products, (*d*) evaluation of heterozygous peak intensities, (*e*) determination of the relative level of stutter per locus, (*f*) determination of stochastic PCR thresholds, (*g*) analysis of previously typed case samples, environmentally insulted samples, and body fluid samples deposited on various substrates, and (*h*) detection of components of mixed DNA samples. The data demonstrate that the commercially available multiplex kits can be used to amplify and type STR loci successfully from DNA derived from human biological specimens. There was no evidence of false positive or false negative results and no substantial evidence of preferential amplification within a locus. Although at times general balance among loci labeled with the same fluorophore was not observed, the results obtained were still valid and robust. Suggested criteria are provided for determining whether a sample is derived from a single source or from more than one contributor. These criteria entail the following: (*a*) the number of peaks at a locus, (*b*) the relative height of stutter products, and (*c*) peak height ratios. Stochastic threshold levels and the efficiency of non-templated nucleotide addition should be considered when evaluating the presence of mixtures or low quantity DNA samples. Guidelines, not standards, for interpretation should be developed to interpret STR profiles in cases, because there will be instances in which the standards may not apply. These instances include (*a*) a primer binding site variant for one allele at a given locus, (*b*) unusually high stutter product, (*c*) gene duplication, and (*d*) translocation.

KEYWORDS: forensic science, short tandem repeats, core CODIS loci, CSF1PO, FGA, TH01, TPOX, vWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, polymerase chain reaction, multiplex amplification, fluorescence, DNA typing, validation

Multiplex amplification by the polymerase chain reaction (PCR) and typing of short tandem repeat (STR) loci provide a sensitive, potentially highly discriminating and rapid means of characterizing forensic specimens. Accordingly, 13 STR loci have been selected as core loci for the U.S. national database, the Combined DNA Indexing System (CODIS). These loci (CSF1PO, FGA, TH01, TPOX, vWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11) (1) can be typed using a combination of two or more of the following fluorescent multiplex amplification kits: AmpFℓSTR® Profiler™, Profiler Plus™, COfiler™, Blue, and Green 1 (Applied Biosystems, Foster City, CA); and GenePrint™ PowerPlex™ and CTTv (Promega Corp., Madison, WI). Additionally, the amelogenin locus for gender identification is included in some of the kits. For detection of single- and multi-fluorophore systems, the ABI Prism® 310 Genetic Analyzer, the ABI Prism 373 and 377 DNA Sequencers, the Automatic Laser Fluorescent (A.L.F.) DNA Sequencer™, the FMBIO® II Fluorescent Imaging Device, and the FluorImager™ have been utilized extensively.

The utility and validity of STR typing for forensic applications have been substantiated (1–9). The use of commercially available multiplex amplification kits for the typing of fluorescently labeled STR products using the ABI Prism 310, 373 and 377; the FMBIO II; and the FluorImager SI was therefore evaluated for forensic ap-

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plicability. This study presents an assessment of performance characteristics of the STR kits. Consistency and reproducibility of results were demonstrated with previously typed case samples, environmentally insulted samples, and body fluid samples deposited on various substrates. The ability to detect components of mixed DNA samples was also assessed. The characterization of non-allelic PCR products, the comparison of heterozygous peak intensities, and the extent of non-templated nucleotide addition are presented.

Materials and Methods

DNA Samples

DNA was extracted either by an organic phenol/chloroform method followed by Microcon®-100 (Amicon, Inc., Beverly, MA) filtration (10) or by using Chelex® 100 (Bio-Rad, Hercules, CA) (11,12). The quantity of DNA in each sample was estimated using a chemiluminescence-based, slot-blot procedure that entails hybridization of a human alphoid probe (13,14).

Samples used in the evaluation of PCR components and thermocycling parameters included: (*a*) DNA from bloodstains and semen stains extracted organically, (*b*) DNA from duplicate bloodstains from four donors, extracted organically and with Chelex for each donor, (*c*) DNA from both bloodstains and semen stains from each of five donors extracted organically, and (*d*) commercially prepared DNA from human cell lines 9947A (Applied Biosystems) and K562 (Promega Corp.).

Environmentally insulted samples and previously typed case samples were a subset of those analyzed previously for the loci D1S80 (15); LDLR, GYPA, HBGG, D7S8, Gc and DQA1 (16); and CSF1PO, TH01, TPOX (CTT) (2). These samples included the following: (*a*) DNA from blood and semen deposited on carpet, denim, leather, nylon, wallboard, and wood and extracted after 1 day and 20 weeks, (*b*) DNA from bloodstains and semen stains stored up to 20 weeks in sunlight, shade, and room temperature, (*c*) DNA from bloodstains, semen stains, and saliva stains treated with either oil, gas, dirt, bleach, detergent, 1 N NaOH, 10% acetic acid, or 1 N HCl, (*d*) DNA extracted from mixtures of blood/semen, blood/saliva, and semen/saliva, (*e*) mixtures of DNA samples from two donors prepared in ratios of 1:20, 1:10, 1:5, 1:2, 1:1, 2:1, 5:1, 10:1, and 20:1 (total DNA amount: 2 ng for ABI Prism detection; 5 ng for FMBIO II and FluorImager detection), and (*f*) reference and evidentiary DNA samples from 45 previously typed cases. All case samples and simulated forensic samples were organically extracted; samples that potentially contained semen were differentially extracted to separate sperm and non-sperm DNA (17).

Amplification

Amplification was conducted using AmpFℓSTR kits (Profiler, Profiler Plus, COfiler, Blue, Green 1, Green 2, and Yellow; Applied Biosystems) and/or GenePrint kits (PowerPlex 1.1 and 1.2, Promega Corp.) in 50 μL or 25 μL volumes, respectively, according to the manufacturers' suggested protocols (18–25; Table 1), except as noted [Note: Dye configurations in two kits that were used in earlier studies are different from the dye configurations in the currently available kits. Primers used for amplification of the current AmpFℓSTR Green 1 loci were initially labeled with a yellow dye (NED), and primers for the current AmpFℓSTR Yellow loci were initially labeled with a green dye (JOE). Changes in the dye configurations had no effect on amplification performance or typing ability. Kits are named herein according to the current designations (that is, Green 1 = CSF1PO, TPOX, TH01; and Yellow = D7S820, D13S317, D5S818).]

TABLE 1—*Manufacturers' recommended conditions (18–25) for amplification of STR loci using the Gene Amp PCR System 9600.*

	AmpFℓSTR Kits	GenePrint Kits
DNA amount	1.0–2.5 ng	1–2 ng
$MgCl_2$ concentration	1.25 mM	1.5 mM
DNA polymerase	5 units per reaction: AmpliTaq Gold (use AmpFℓSTR Buffer which contains 200 μM each dNTP and 0.16 μg/μL BSA)	2.25 units per reaction: Taq (use STR 10X Buffer which contains 200 μM each dNTP; add 0.16 μg/μL BSA) or AmpliTaq Gold (use Gene Amp PCR Buffer; add 200 μM each dNTP and 0.16 μg/μL BSA)
Reaction volume	50 μL	25 μL
PCR cycles	28 cycles of: 94°C for 60 s 59°C for 60 s 72°C for 60 s	10 cycles of: 94°C for 30 s 60°C for 30 s 70°C for 45 s followed by: 20 cycles of: 90°C for 30 s 60°C for 30 s 70°C for 45 s
Final extension	60°C for 45 min	60°C for 30 min

Standard amplification reactants in the STR kits included reaction mix (with buffer, $MgCl_2$, dNTPs, and BSA) and primer mix. Reactions typically contained 0.5–1 ng of genomic DNA for detection using the ABI Prism 310 (Applied Biosystems) or 1–2 ng of genomic DNA for detection using the ABI Prism 377 (Applied Biosystems); 5 ng of genomic DNA were used for detection with the FMBIO II (Hitachi Software Engineering America, Ltd., San Bruno, CA) or FluorImager SI (Molecular Dynamics, Sunnyvale, CA). The GeneAmp® 9600 PCR System (GAPS 9600; Applied Biosystems) or the GAPS 9700 (using GAPS 9600-simulation mode; Applied Biosystems) were used for amplification. AmpliTaq Gold® DNA Polymerase was included routinely in the PCR with an initial 11 min heat soak at 95°C (26). AmpliTaq® DNA Polymerase was used for certain experiments, as noted.

PCR Components

Single reaction components and thermocycling parameters were systematically varied, as described below, to assess the effects on STR amplification and to define "windows" of acceptable performance. All reactions were prepared immediately prior to amplification as master mixes that contained all reaction components except for the variable component.

DNA Template Amount—Duplicate amplifications (using AmpFℓSTR Profiler, Profiler Plus, COfiler, Green 1 and 2, Blue and Yellow; GenePrint PowerPlex 1.1) were assessed with the following amounts of serially diluted DNA template: 10, 5, 2.50, 1.25, 0.62, 0.31, and 0.16 ng (0.078 ng was also tested on the ABI Prism 310).

$MgCl_2$ Concentration—$MgCl_2$ (Applied Biosystems) was added to reactions containing GeneAmp PCR Buffer II (Applied Biosys-

tems) to yield the following final $MgCl_2$ concentrations: 0.75, 1.00, 1.25, 1.50, 1.75, 2.00 mM. Replicate reactions containing DNA from blood and semen samples were prepared with final concentrations of 1X primer mix (as provided by manufacturers), 200 μM each dNTP, 0.16 μg/μL BSA, and 0.1U/μL AmpliTaq Gold DNA polymerase. The initial $MgCl_2$ studies assessed amplification performance of AmpFℓSTR Green 1 and Yellow on the ABI Prism 373; and AmpFℓSTR Green 2 and Yellow on the FluorImager SI. Subsequently, using four DNA samples, 1.25 and 1.50 mM $MgCl_2$ (the concentrations used in the AmpFℓSTR and GenePrint systems, respectively) were both compared for each multiplex using a dilution series of genomic DNAs (10 to 0.15 ng).

Taq Polymerase—Replicate reactions containing (*a*) AmpliTaq or (*b*) AmpliTaq Gold DNA Polymerases (Applied Biosystems) were compared with (*c*) replicate manual hot start reactions containing AmpliTaq DNA Polymerase. The initial denaturation period at 95°C was 2 min for reactions containing AmpliTaq and was 11 min for reactions containing AmpliTaq Gold. Manual hot start was conducted by initially heating reaction mixtures without DNA polymerase for 8.5 min at 95°C and then adding AmpliTaq as heating continued for an additional 2.5 min (AmpliTaq was therefore heated for 2.0 to 2.5 min, as in the standard protocol without a hot start). The DNA samples were from (*a*) cell line 9947A, (*b*) blood, and (*c*) both blood and semen from each of three donors.

Bovine Serum Albumin (BSA)—To examine the effects of BSA usage on STR amplification in the presence of a PCR inhibitor, DNA (1.25 ng) extracted from semen (which lacks heme) stains was amplified using GenePrint PowerPlex 1.1 in the presence of hematin at the following final concentrations: 0, 0.25, 0.50, 1, 5, 10, and 20 μM, prepared from a stock of 1 mM hematin in 0.1 N NaOH. AmpliTaq and AmpliTaq Gold DNA Polymerases were used with GeneAmp PCR Buffer, to which 1.50 mM $MgCl_2$ and 0.16 μg/μL BSA (Sigma Chemicals, St. Louis, MO) (27) were added.

Thermocycling Parameters

Cycle Number—Using replicate reactions containing DNA extracted from one bloodstain and one semen stain (from different donors), the manufacturers' recommended number of amplification cycles was compared to PCRs with ±1 and ±2 cycles. Also, using four DNA samples (two organically extracted, one Chelex-extracted, and 9947A DNA), the manufacturers' recommended number of cycles and the recommended number +1 cycle were used to amplify serially diluted DNA templates (10 to 0.15 ng).

Annealing Temperature—Replicate reactions containing DNA template extracted from cell line K562 or 9947A, one or two bloodstains, and one semen stain (all from different donors) were amplified using the manufacturers' recommended annealing temperature and the recommended temperature ±1°C and ±3°C.

Annealing Time—Using replicate reactions containing DNA extracted from bloodstains from two donors and semen stains from a third donor, amplification was conducted with annealing times of 10, 30, 45, and 60 s. Also, using four DNA samples (two organically extracted, one Chelex-extracted, and 9947A DNA), the manufacturers' recommended 60-s annealing time was compared with a 90-s annealing time.

Final Extension—Amplification of replicate reactions containing DNA extracted from bloodstains and semen stains was conducted with final extension periods of 0, 15, and 30 min at 60°C. Additional experiments assessed the efficiency of non-templated nucleotide addition by comparing the omission of the final extension step to usage of the manufacturers' recommended time period and temperature.

Post-PCR Sample Filtration

Some PCR products were filtered using Microcon-100 devices prior to capillary electrophoresis. Filtration was conducted as follows: 100 μL dH_2O and 50 μL amplified product were added sequentially to the sample reservoir of a Microcon-100 unit. The device was centrifuged at 1000 g for 5 min. Two hundred μL dH_2O were added, and the device was centrifuged at 1000 g for 5 min. To retrieve the sample, 50 μL dH_2O were added, the filtrate cup was removed, and the device was inverted into a 1.5 mL tube. The inverted tube assembly was centrifuged at 1000 g for 5 min. A final volume approximately equivalent to that of the initial reaction volume was recovered. [Note: while Microcon-100 treatment resulted in a slight increase in peak heights, as compared with samples lacking Microcon-100 treatment, the relative heights of alleles within the multiplexes and the genotyping were not affected by the filtration and washing (28).]

Electrophoresis and Detection

Products from AmpFℓSTR COfiler amplification were assessed using the ABI Prism 310. Products from Profiler and Profiler Plus amplification were assessed using the ABI Prism 377. Products from AmpFℓSTR Blue, Green 1 and 2 and Yellow and GenePrint PowerPlex 1.1 amplification were assessed using the FMBIO II. Products from CTTv amplification were assessed using the FluorImager SI. [Exception: AmpFℓSTR Yellow products generated in the annealing temperature study and AmpFℓSTR Green 1 products generated in the final extension study were analyzed using the ABI Prism 377.]

ABI Prism 310 Genetic Analyzer—Some PCR products generated using the AmpFℓSTR Profiler Plus and COfiler systems were analyzed on the ABI Prism 310. To prepare the sample for capillary electrophoresis, 1 μL of the sample was added to either (*a*) 11.5 μL deionized formamide [Life Technologies, Inc., Gaithersburg, MD (initial studies); or Amresco, Solon, OH (latter studies; recommended)] and 0.5 μL GeneScan®-500 [ROX] Internal Lane Size Standard (Applied Biosystems) or (*b*) 23 μL deionized formamide and 1.0 μL GeneScan-500 [ROX]. The preparations were heated at 95°C for 3 min and snap-cooled at 4°C for 3 min. Following a 5 s injection at 15 kV, electrophoresis was conducted at 15 kV and 60°C with Performance Optimized Polymer 4 (POP™ 4, Applied Biosystems) as the separation medium. Data were collected using GeneScan Collection software (version 1.0.2) with virtual filter set A or F. (On the ABI Prism 310, filter sets A and F are identical.) Results were analyzed using GeneScan Analysis software (version 2.1) and Genotyper® software (version 2.0). Additional details are provided in (22,23).

ABI Prism 377 DNA Sequencer—Some PCR products generated using the AmpFℓSTR Green 1 and 2, Yellow, Profiler and Profiler Plus systems were analyzed on the ABI Prism 377. PCR product (1.5 μL) was added to 0.5 μL GeneScan-350 or -500 [ROX] (Applied Biosystems) and 4 μL loading dye (which contained 8M urea

and 5 mg/mL Blue Dextran; Applied Biosystems). The preparations were heated at 95°C for 2 min and snap-cooled at 4°C, and 1.5 μL were loaded into 5% Long Ranger™ gels (FMC Bioproducts, Rockland, ME) with a 36-cm well-to-read. Electrophoresis was conducted at 3000 V. Data were collected using GeneScan Collection software (version 1.1.0) with virtual filter set A. Results were analyzed using GeneScan Analysis software (version 2.0.2 or 2.1) and Genotyper software (version 1.1.1 or 2.0). Additional details are provided in (22,23).

FMBIO II and FluorImager SI—Some PCR products generated using the AmpFℓSTR Blue, Green 1 and 2, and Yellow systems and the GenePrint PowerPlex 1.1 system were analyzed on the FMBIO II and/or the FluorImager SI. Three μL of PCR product were added to 3 μL loading dye (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol). The preparations were heated at 95°C for 2 min and snap-cooled at 4°C. Electrophoresis of 5 μL of each sample was conducted initially at 80 W for 5 min, and then at 35 to 40 W, in continuous, denaturing polyacrylamide gels [6%T, 5%C; bisacrylamide cross-linker; 7 M urea; 1X TBE, pH 8.3; 31 cm long; 0.4 mm thick] at ambient temperature using a Model SA 32 Gel Electrophoresis System (Life Technologies, Inc.). PCR products were detected using the FMBIO II with either (*a*) a 505-nm filter for AmpFℓSTR Blue and GenePrint PowerPlex GammaSTR products (5-FAM- and fluorescein-labeled, respectively), (*b*) a 585-nm filter for AmpFℓSTR Green 1 and 2 products (JOE-labeled) and for AmpFℓSTR Yellow products (NED-labeled), or (*c*) a 605-nm filter for GenePrint PowerPlex CTTv products [tetramethylrhodamine (TMR)-labeled]. PCR products were detected using the FluorImager SI with either (*a*) a 530-nm filter for AmpFℓSTR Blue products or (*b*) a 570-nm filter for AmpFℓSTR Green 1 and 2 and Yellow.

Percent Stutter and Heterozygous Peak Ratios

The proportion of the stutter product relative to the true allele (e.g., presented as percent stutter values) and the proportion of the less intense allele relative to the more intense allele at a given heterozygous locus (e.g., heterozygous peak ratios, presented as percentages) were derived using the AmpFℓSTR Profiler Plus system (for the loci D3S1358, D5S818, D7S820, D8S1179, D13S317, D18S51, D21S11, vWA and FGA; analyzed on the ABI Prism 377) and the AmpFℓSTR COfiler system (for the loci D3S1358, D7S820, D16S539, CSF1PO, TPOX, and TH01; analyzed on the ABI Prism 310). Peak height values were used for the calculations. The manufacturer's recommended conditions were used for amplification.

Percent Stutter—Percent stutter was calculated (based on peak height) using single-source DNA samples that exhibited heterozygous alleles that were at least 8 bases (two tetranucleotide repeat units) apart and were resolved to baseline, and homozygous alleles. Alleles that differ in size by 4 bases were excluded from the analyses since (*a*) the smaller allele and the stutter product of the larger allele are the same size and therefore are detected as a single peak, and (*b*) the peak height of the smaller allele is augmented by the stutter peak of the larger allele. Also, only profiles with a relative fluorescence intensity of the alleles that did not exceed the instrument's threshold of detection were used in the analysis (that is, peaks were not "off-scale;" selected peak height values for the analyzed data were ≤4500 rfu on the ABI Prism 310 and ≤3000 rfu on the ABI Prism 377; these values are below those recommended by the manufacturer, such that raw data components are below the maximum detection threshold).

Heterozygous Peak Ratios—Heterozygous peak ratios were calculated using heterozygous alleles from single-source samples. Alleles included in the analyses were at least 8 bases apart, were resolved to baseline, and exhibited fluorescent intensities within the linear dynamic range of the detector (as noted above).

Results and Discussion

The purpose of the study was to define the utility and potential limitations of STR typing of forensic specimens using commercially available multiplex amplification kits. The responses to manipulation of experimental conditions and the trends observed in the analysis of case samples and simulated forensic samples were generally similar among all kits. The data demonstrate that the typing of biological materials using STR loci and the commercial kits described in these studies yield reliable results under various conditions. Observations are presented regarding the performance of the STR systems that may assist in interpreting STR profiles from case samples.

Performance Parameters

The analytical system and conditions (such as annealing temperature, reaction volume, primer sequences, fluorescent labels, etc.) associated with the AmpFℓSTR and GenePrint kits differ. However, reliable genotyping results were obtained with both the AmpFℓSTR and GenePrint systems. Furthermore, the PCR conditions (such as DNA template amount, $MgCl_2$ concentration, Taq polymerase type, inclusion of BSA, annealing temperature, and duration of the annealing and final extension steps) assessed in the current study were varied to assess kit performance and to define "windows" of acceptable performance. For example, the range of annealing temperatures within which the PCR results are relatively invariable delineates a window of acceptable performance (Fig. 1); if amplification is conducted with an annealing temperature well within this range, then minor fluctuations in temperature which may be encountered during amplification, either within or among laboratories, are inconsequential. The results of the various performance tests indicate that within a range of analytical conditions around those recommended/used by the manufacturers, successful typing of STR loci can be achieved. Some variation in the concentration of PCR components, DNA template amount, and thermocycling conditions can be tolerated and will not affect the reliability of typing (e.g., Fig. 1; other data not shown). Additionally, reliable STR typing results were obtained from DNA samples that were extracted by either phenol/chloroform- or Chelex 100-based extraction methods. Therefore, the manufacturers' recommended conditions are robust, and when the recommended protocols are followed, assays are conducted within safe windows of performance.

All of the commercial kits tested exhibited the expected responses to experimental manipulation of the PCR. For example:

- Increases of 3°C in the annealing temperature resulted in substantial reduction of PCR product at some loci, because as the temperature exceeds the melting temperature (T_m) of a primer(s), the ability of the primer(s) to anneal to the template generally decreases. Despite increases in annealing temperature, only three outcomes were evident: typable results were obtained, results were inconclusive, or no result

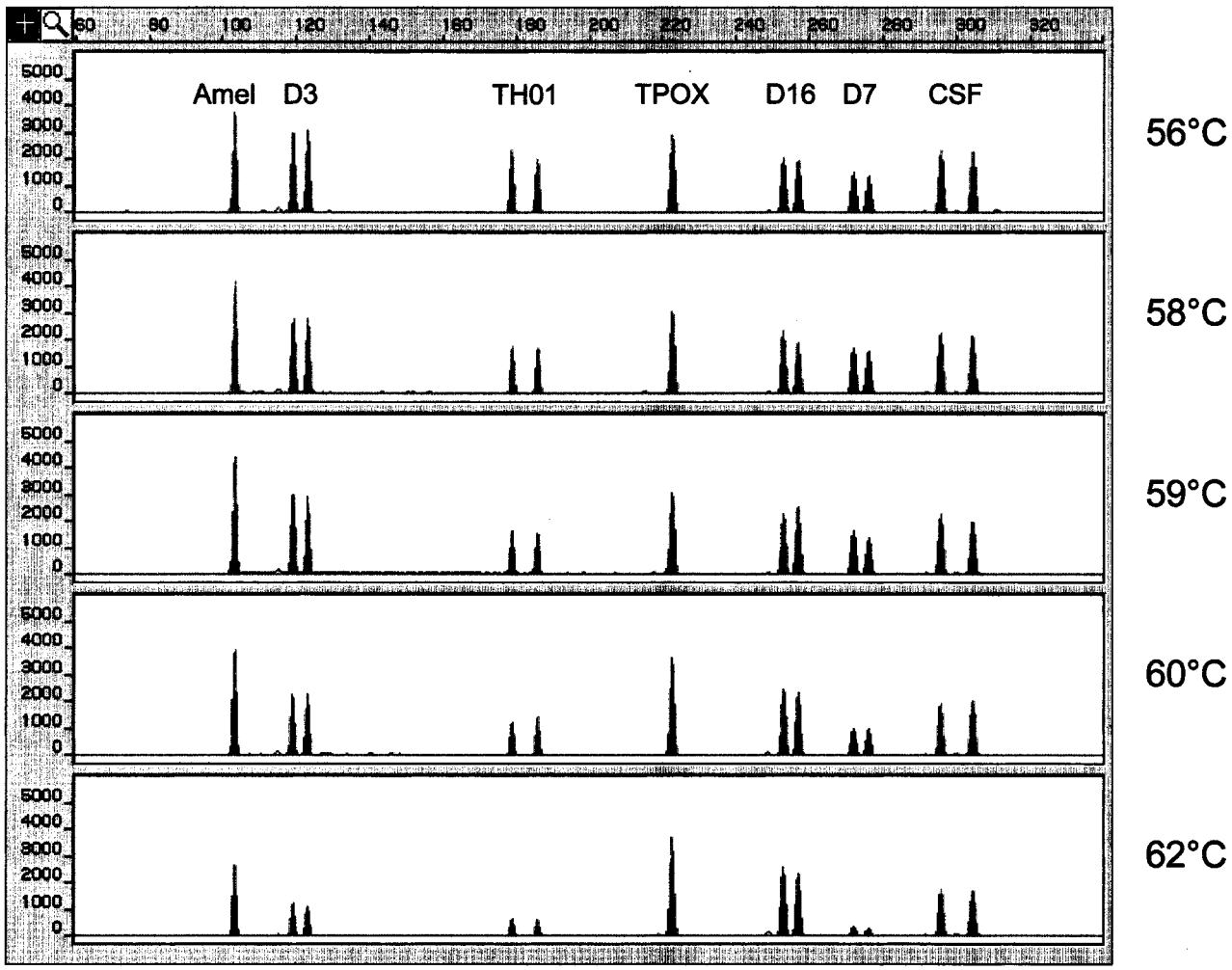

FIG. 1—*Annealing temperature study using AmpFℓSTR COfiler. Annealing temperatures of 56, 58, 59, 60 and 62°C were used in the amplification of a 1 ng DNA sample. The manufacturer's recommended annealing temperature is 59°C. PCR products were separated and detected on the ABI Prism 310 Genetic Analyzer. The loci shown are amelogenin (Amel), D3S1358 (D3), TH01, TPOX, D16S539 (D16), D7S820 (D7), and CSF1PO (CSF).*

was observed. There were no false positive results. As shown in Fig. 1, using AmpFℓSTR COfiler, all loci could be typed within the window of ±3°C that was tested (56-62°C) around the manufacturer's recommended annealing temperature of 59°C.

- For all of the systems tested, no substantial increase in product was observed with increased annealing time (from 60 to 90 s), and, as expected, shortened annealing times (<60 s) resulted in lower product yield at some loci (data not shown).
- Because Mg^{2+} functions as a cofactor of Taq polymerase, substantial reduction in the concentration of $MgCl_2$ resulted in decreased PCR product. However, STR typing could be achieved within a range of concentrations (generally, 1.0 to 2.0 mM $MgCl_2$ for all loci; 0.75 to 2.0 mM $MgCl_2$ was tested) around the manufacturers' suggested values. The two manufacturers employ slightly different concentrations of $MgCl_2$ (AmpFℓSTR reactions include 1.25 mM $MgCl_2$, and GenePrint reactions include 1.50 mM $MgCl_2$). Correct STR typing could be obtained using either concentration of $MgCl_2$ with both manufacturers' kits. Figure 2 compares the usage of 1.25 and 1.50 mM $MgCl_2$ with AmpFℓSTR Yellow. Generally, the use of 1.50 mM $MgCl_2$, compared with 1.25 mM, resulted in a slightly greater product yield (personal observation). In some instances, stutter was more apparent with the use of 1.50 mM $MgCl_2$ because of the overall increased yield, but the percent stutter was not appreciably different between samples amplified with 1.50 or 1.25 mM $MgCl_2$.
- DNA samples could be amplified using AmpliTaq or AmpliTaq Gold DNA Polymerases (Fig. 3). The use of AmpliTaq Gold with both the AmpFℓSTR and GenePrint systems reduces the potential for mispriming and thus minimizes the production of nonspecific amplification products (26). Accordingly, AmpliTaq Gold is well-suited for automated reaction preparation and amplification (26).
- The addition of one cycle to the PCR generally improved the ability to type weak (and, potentially, degraded) samples and gave reliable results (data not shown). However, amplification of DNA samples with a low copy number may be subject to stochastic effects, where two alleles at a heterozygous locus exhibit considerably different peak heights or, although less likely, one of the two alleles at a locus fails to amplify (discussed later). Therefore, the use of additional cycle(s) in the PCR should not be exercised without substantial validation (to include at least all studies described in this paper).

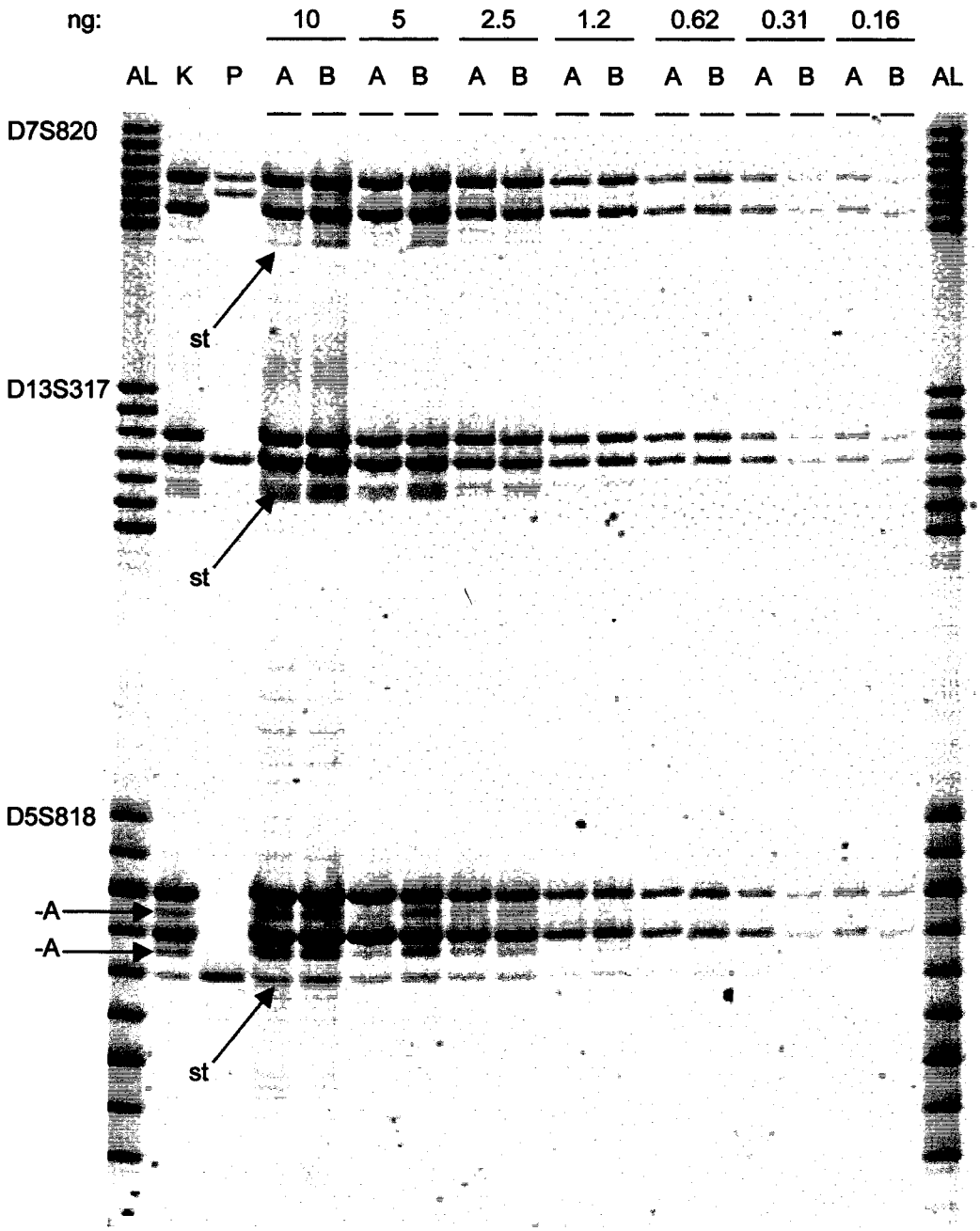

FIG. 2—*Comparison of 1.25 and 1.50 mM $MgCl_2$ with the use of AmpFℓSTR Yellow. A dilution series of DNA (10 to 0.16 ng) extracted from blood was amplified in reaction buffer containing 1.25 or 1.50 mM $MgCl_2$. Electrophoresis was conducted on a SA-32 Gel Electrophoresis Apparatus, and the gel image was visualized using an FMBIO II. A = 1.25 mM $MgCl_2$. B = 1.50 mM $MgCl_2$. AL = allelic ladder. K = AmpFℓSTR kit reaction buffer, with 1.25 mM $MgCl_2$, and 5 ng of DNA template. P = Positive control DNA 9947A (1.25 ng) in AmpFℓSTR kit reaction buffer. st = stutter. −A = lack of non-templated nucleotide addition.*

- As shown in Fig. 3, amplification using the GenePrint PowerPlex kit in the presence of hematin was inhibited as follows: without BSA in the amplification reaction and with use of AmpliTaq or AmpliTaq Gold and GeneAmp PCR Buffer, complete profiles were only obtained from 0 to 0.50 μM hematin. Partial profiles were obtained with 1.0 μM hematin in the absence of BSA (with use of both polymerases), and dropout at all loci occurred with use of ≥10 mM hematin. With the inclusion of 0.16 μg/μL BSA in the amplification reaction, complete profiles were obtained in the presence of ≤20 μM hematin (highest concentration tested).

The fluorescence detection instruments used in the present study demonstrated different sensitivities of detection for the fluorescent dyes. Generally, the best results were obtained with the following DNA template amounts: ~0.5-1 ng DNA detected on the ABI Prism 310; ~1-2 ng DNA on the ABI Prism 377; and ~5 ng on the FMBIO II. However, partial or complete profiles were attained on the ABI

Prism 310 from as little as 78 pg of DNA template, and on the ABI Prism 377 and FMBIO II from as little as 156 pg (data not shown). The use of excessive DNA template amounts should generally be avoided since: (*a*) non-allelic amplification products, such as stutter products and "minus A" products, may be more apparent and (*b*) the percent stutter may appear to be augmented if an allele exceeds the instrument's maximum detection limit. "Pull-up" (the observation of a single peak or band in more than one color, due to spectral overlap of the fluorescent dyes and inadequate color separation) was observed with some overamplified samples more so on the ABI Prism 377 and the FMBIO II than on the ABI Prism 310. On the ABI Prism 310, unanalyzed data typically have elevated baselines, and peaks in the raw data that exceed 8,191 rfu are off-scale (on the ABI Prism 377, data points used in 3-channel averaging that are >8,191 rfu are off-scale; the FBMIO II does not have a means of determining whether peaks are off-scale). During multicomponent analysis (the process of color separation), elevated baselines are normalized (or, brought down to zero rfu). Consequently, off-scale peaks (>8,191 rfu in the unanalyzed data) may appear to be on-scale in the analyzed data. As shown in Fig. 4*A*, the off-scale peaks at vWA (5,676 rfu, analyzed data) and D5S1358 (4,369 rfu, analyzed data) exhibit relatively high stutter percentages (15% and 29% respectively). The stutter products (Fig. 4*A*) are not off-scale and therefore exhibit true peak heights. In contrast, peak height values of the off-scale alleles are not quantitative, and the stutter percentages may therefore be falsely augmented. In our study, when the PCR products were diluted to be on-scale (Fig. 4*B*), the actual stutter percentages were found to be 5 and 6%, respectively.

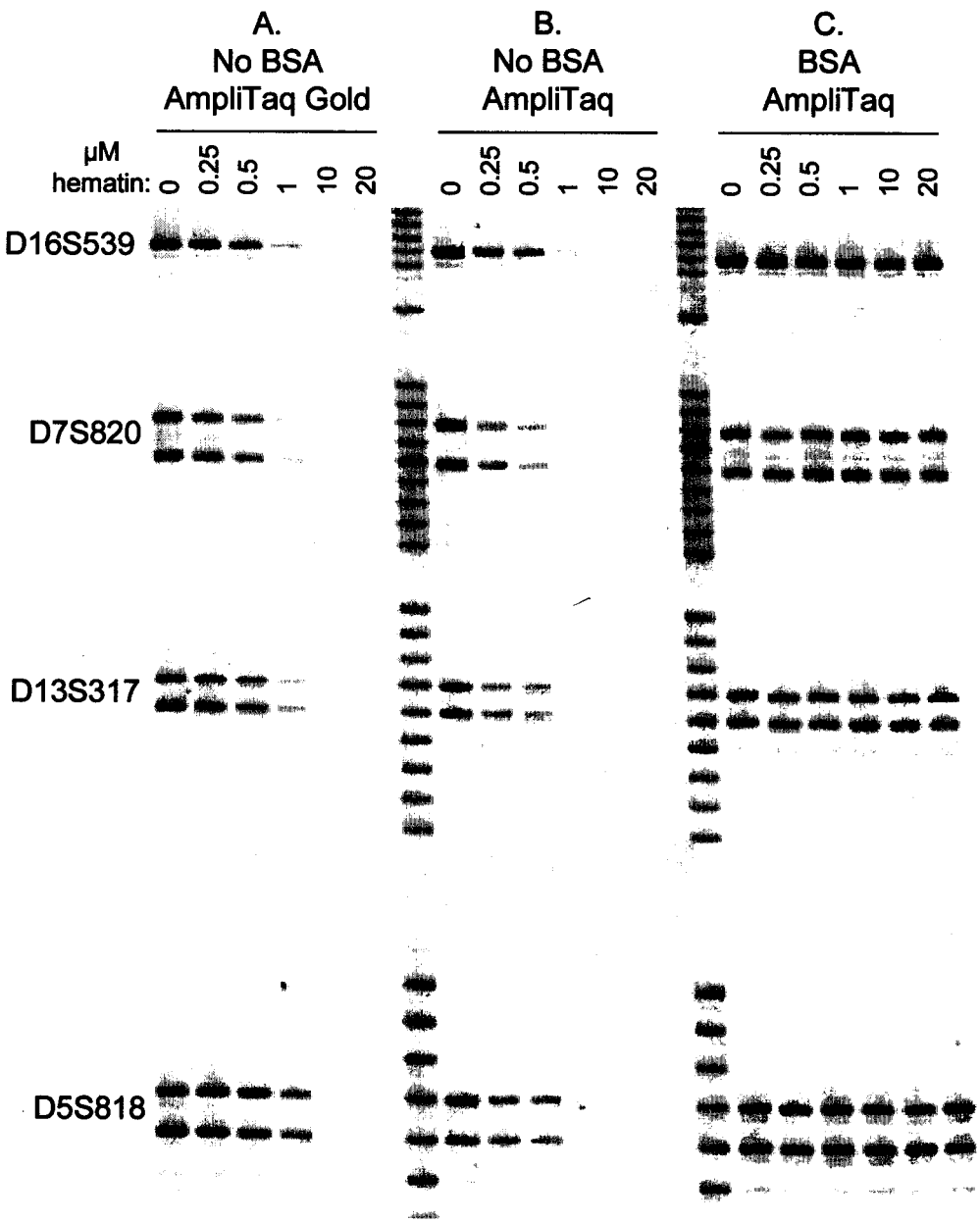

FIG. 3—*Amplification in the presence of a PCR inhibitor. DNA (5 ng) extracted from semen was amplified using GenePrint PowerPlex 1.1. Reactions containing 0–20 μM hematin were conducted with AmpliTaq Gold* (A) *and AmpliTaq* (B) *DNA polymerases in the absence of BSA, and with AmpliTaq in the presence of BSA (0.16 μg/μL)* (C). *The GammaSTR loci are shown, as detected on the FMBIO II.*

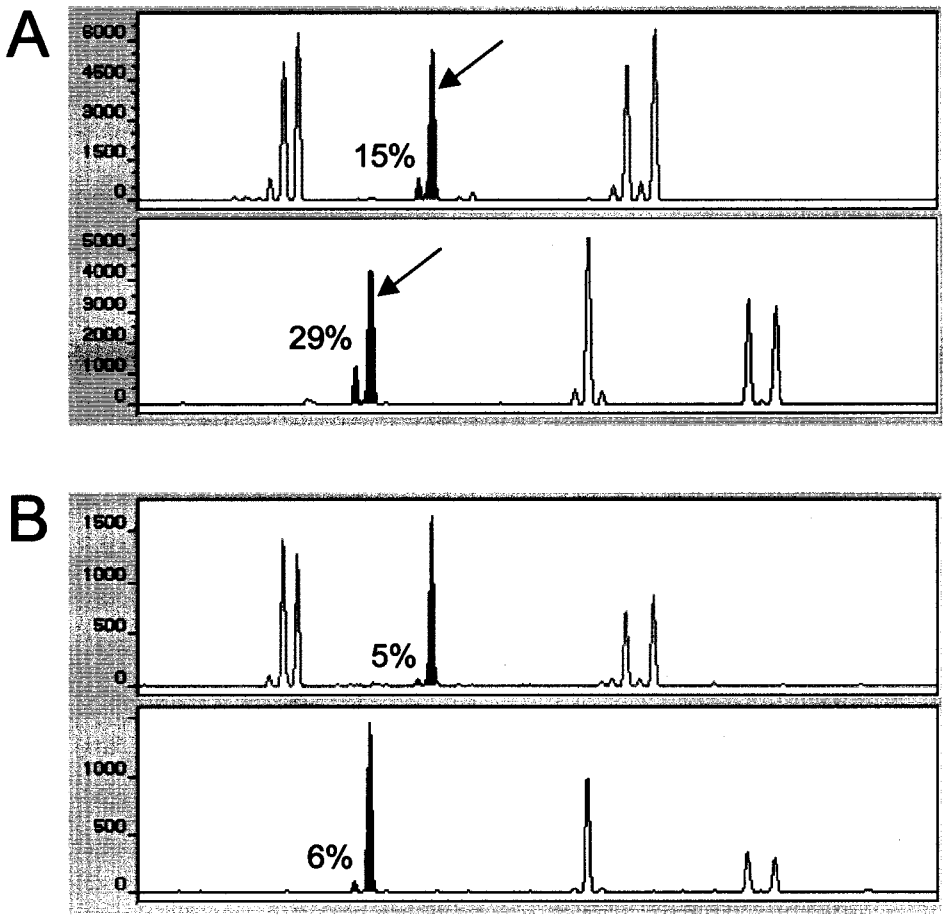

FIG. 4—*Apparent augmentation of stutter due to off-scale data. Runs A and B were conducted on the ABI Prism 310. The Blue (upper) and Yellow (lower) profiles of a DNA sample extracted from blood and amplified with Profiler Plus are shown in both* (A) *and* (B)*. Due to excessive DNA template amount, the sample shown in* (A) *exhibited peaks (arrows) that exceeded the instrument's threshold of detection; the stutter percentages (15 and 29%) are therefore falsely augmented. When the same PCR products were diluted and re-run* (B)*, the allelic peaks were not off-scale, and the actual stutter percentages (5 and 6%, respectively) were demonstrated.*

In all of the multiplex systems tested, peak heights at different STR loci that are labeled with the same fluorophore may or may not be balanced (excluding degradation). However, comparing the different fluorophores within each multi-color system, PCR products labeled with a yellow dye [NED (AmpFℓSTR); TMR (GenePrint)] typically exhibited lower peak heights than blue-labeled [FAM (AmpFℓSTR); fluorescein (GenePrint)] or green-labeled [JOE (AmpFℓSTR)] products. The following observations are important to consider when comparing profiles in casework: first, differential amplification across loci can occur at loci that are labeled with the same fluorophore. As anticipated when typing degraded DNA samples, decreased intensity or loss of larger loci was exhibited in some case samples and simulated forensic samples (Fig. 5). Second, when DNA samples were amplified multiple times and subsequently analyzed, allelic peak heights and the relative peak height ratios of heterozygous alleles varied slightly. For example, as shown in Fig. 6 with the D16S539 locus, in the initial analysis (Fig. 6*A*), the smaller allele had a lower relative fluorescence intensity than the larger allele; when the DNA sample was reamplified and analyzed (Fig. 6*B*), the smaller allele had a higher intensity than the larger allele. Slight differences in peak height between two heterozygous alleles may thus be attributed to variability in amplification and/or fluorescence detection. The accuracy of typing, though, is not affected by these minor fluctuations; the allelic designations did not change.

Interpretational Aids

For each STR locus, relative stutter percentages and heterozygous peak height can be used to assist in interpreting STR profiles and, in particular, determining whether a forensic specimen contains DNA from more than one contributor.

Stutter—The stutter product for an allele with "*n*" tetranucleotide repeats is four bases shorter ($n - 4$) than the true allele and is inherent to the amplification of STR loci (29). Stutter products were observed at $n - 8$, $n - 12$, and $n + 4$ positions only when excessive amounts of DNA template (e.g., >10 ng) were used in the PCR or an excessive number of cycles were employed in the PCR (data not shown). The percent of stutter product relative to the true allele for the STR loci analyzed using the AmpFℓSTR Profiler Plus and COfiler systems is presented in Table 2. The average percent stutter for all loci was less than 8.4%. When analyzing STR profiles, the average percent stutter plus three standard deviations for each locus may be used as a guide for whether a small peak at the $n - 4$ position is a stutter product or might also conceal minor component in a

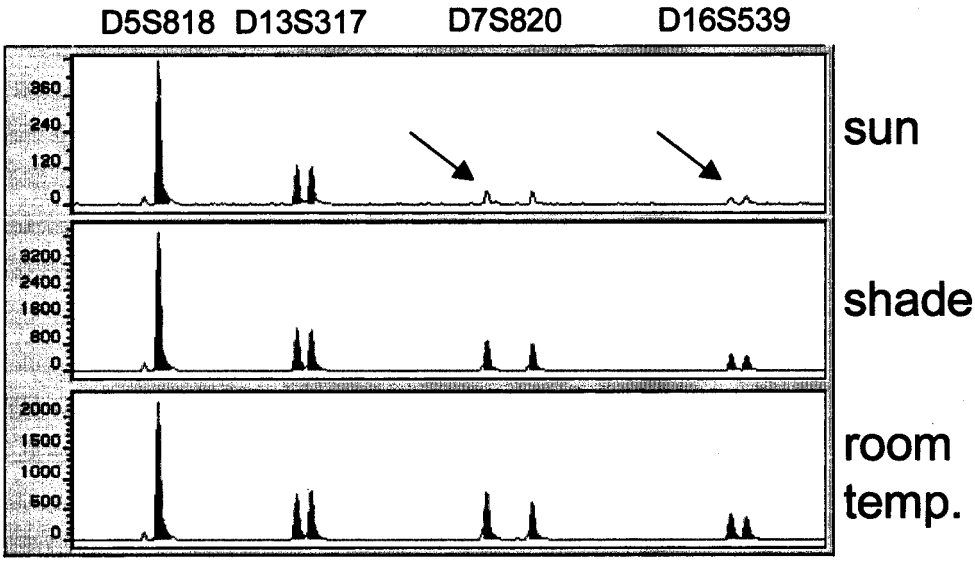

FIG. 5—*STR profile exhibiting DNA degradation. Semen stains were exposed to sunlight, shade, and room temperature for 6 weeks. Extracted DNA (1.25 ng) was amplified using GenePrint PowerPlex 1.2. Products were separated and detected using the ABI Prism 377 DNA Sequencer. DNA degradation is exhibited (arrow) at the larger loci in the sample exposed to sunlight.*

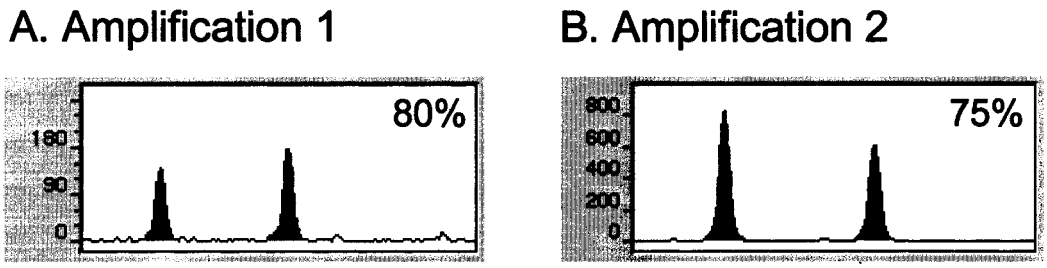

FIG. 6—*Variation in relative peak heights following initial and subsequent amplification. The D16S539 locus is shown following amplification of the same DNA extract in two reactions using AmpFℓSTR COfiler and separation and detection on the ABI Prism 310 Genetic Analyzer. Separate reactions may exhibit peaks with relative peak heights that differ (e.g.,* A*, the smaller-sized peak has a lower peak height than the larger-sized peak;* B*, the smaller-sized peak has a greater peak height than the larger-sized peak) and with peak height ratios that vary slightly (*A*, 80%;* B*, 75%).*

TABLE 2—*Stutter percentages of STR loci analyzed using the AmpFℓSTR Profiler Plus and COfiler systems.*

Locus	AmpFℓSTR System*	*n*	Mean Stutter %	S.D.	Upper Range Stutter %	% of Data <Upper Range Stutter %
CSF1PO	COfiler	228	4.56	1.18	8.10	99.12
FGA	Profiler Plus	177	6.78	1.61	11.61	99.40
TH01	COfiler	59	4.45	3.83	15.94	100.00
TPOX	COfiler	116	3.83	3.07	13.04	96.55
vWA	Profiler Plus	160	7.08	1.54	11.70	97.50
D3S1358	Profiler Plus	172	6.83	1.50	11.33	99.42
	COfiler	381	6.87	1.43	11.16	98.95
D5S818	Profiler Plus	64	5.89	1.45	10.24	100.00
D7S820	Profiler Plus	54	7.22	1.82	12.68	100.00
	COfiler	185	4.47	1.47	8.88	99.46
D8S1179	Profiler Plus	79	6.78	2.07	12.99	98.70
D13S317	Profiler Plus	51	5.75	1.73	10.94	98.04
D16S539	COfiler	306	5.16	1.52	9.72	99.35
D18S51	Profiler Plus	173	7.75	2.05	13.90	99.42
D21S11	Profiler Plus	108	6.22	1.64	11.14	98.15

* Profiler Plus samples were detected using the ABI Prism 377; COfiler samples were detected using the ABI Prism 310.
n = number of alleles analyzed at each locus.
S.D. = Standard Deviation.
Upper Range Stutter Percent = Mean + 3 S.D.

mixture. These upper range values, however, are not used as absolute thresholds, but rather as guides for interpreting STR profiles because: (*a*) some single-source samples (<4% of all samples analyzed at each locus) exhibited stutter peaks that exceeded the upper range and (*b*) minor peaks in a mixed sample can reside at the $n - 4$ position of a peak derived from a major contributor. In this study, alleles that differed by only one repeat were not included in the stutter calculations; however, it can be predicted that the percent stutter for the smaller of the two alleles would be slightly lower than has been described above. Evaluation of the other loci in a multi-locus STR profile can assist in determining whether an evidentiary sample contains DNA from more than one donor.

Peak Height Ratios—A mixed sample contains DNA from at least two individuals. The presence of three or more alleles at more than one locus may be indicative of a mixed sample. Also, the presence of two alleles, at a given locus, with substantially different peak heights may be indicative of a mixed sample. However, because two heterozygous peaks are seldom of equal heights, the lower range heterozygous peak height ratio (expressed as a percentage) (which equals the average minus three standard deviations; Table 3) at a locus can serve as a guide for determining whether two imbalanced peaks either (*a*) represent heterozygous alleles from a single donor or (*b*) are possibly derived from two individuals. Peak height ratios can also be useful, given a locus with three or more alleles, for assigning alleles to profiles of one or more of the contributors. The average peak height ratio for STR loci amplified from 1 ng of DNA template using the Profiler Plus and COfiler systems was >88% at each locus. The lower range heterozygous peak height ratio was >59% for all loci; however, <2% of single-source samples exhibited values below the lower range peak height ratio at each locus. There will be situations where the heterozygous peak height ratio at a locus is less than 59% (e.g., due to a variant in the primer binding site) when using the STR amplification systems tested. Therefore, a minimum threshold of approximately 59% should be used only as a guide, not as a rule, when evaluating whether imbalanced peaks at a given Profiler Plus or COfiler locus indicate a single source or a mixed sample. Again, profiles at all loci in the multiplex can be useful in determining if the sample is a mixture.

When few copies of the DNA template are present, stochastic amplification may occur (16), resulting in either a substantial imbalance of two alleles at a given heterozygous locus or allelic dropout. Therefore, the amount of DNA used in the PCR can have an impact on stochastic effects. The reverse dot blot systems (AmpliType® PM and DQA1+PM, Applied Biosystems) include a means (e.g., the "S" or "C" dots) of evaluating whether a DNA template used in the PCR is above the level at which stochastic effects may impact on the relative yield of two alleles at a given heterozygous locus (16). Similarly, peak height can serve as the equivalent of a stochastic control for STR typing. The quality control measure for an effective stochastic interpretation threshold should be developed based on a minimum peak height value. This minimum threshold should be determined in-house because of variation in DNA quantitation system efficiency and sensitivity of detection of analytical instruments. Peaks with heights below the threshold value should be interpreted with caution. Finally, because of the possibility of stochastic effects on amplification when analyzing low copy number DNA templates, caution should be used in modifying the thermocycling parameters (e.g., using additional cycles) and electrophoretic conditions (e.g., increasing the injection time during capillary electrophoresis) to enhance product intensity.

In contrast to stochastic amplification, preferential amplification refers to the difference in amplification efficiency that may occur between two alleles at a given locus, even when sufficient quantities of template DNA are used in the PCR. Preferential amplification typically occurs when heterozygous alleles differ substantially in size, such as has been observed at the locus D17S5, for which alleles may differ in size by as much as several hundred bases (30). Using both compromised and uncompromised DNA samples, no substantial evidence of preferential amplification was found at any of the STR loci analyzed with the various kits (data not shown). This observation is expected due to the relatively small range of allele sizes (typically <60 bases) at each locus.

TABLE 3—*Peak height ratios of heterozygous STR loci analyzed using the AmpFℓSTR Profiler Plus and COfiler systems.*

Locus	AmpFℓSTR System	*n*	Mean Peak Height Ratio	S.D.	Lower Range Peak Height Ratio	% of Data >Lower Range Peak Height Ratio
CSF1PO	COfiler	234	88.23	9.09	60.96	98.72
FGA	Profiler Plus	269	91.77	5.72	74.61	98.51
TH01	COfiler	318	90.57	7.98	66.63	99.98
TPOX	COfiler	298	88.38	8.56	62.70	98.66
vWA	Profiler Plus	180	92.09	5.59	75.35	99.44
D3S1358	Profiler Plus	167	92.94	5.20	77.34	98.80
	COfiler	193	89.28	9.08	62.04	99.98
D5S818	Profiler Plus	122	93.06	4.92	78.30	100.00
D7S820	Profiler Plus	159	92.02	5.91	74.29	98.74
	COfiler	261	88.71	9.42	60.45	100.00
D8S1179	Profiler Plus	180	93.36	4.83	78.87	98.33
D13S317	Profiler Plus	173	92.74	5.60	75.94	97.69
D16S539	COfiler	220	88.10	9.43	59.81	99.99
D18S51	Profiler Plus	271	89.33	6.65	69.38	99.63
D21S11	Profiler Plus	230	92.18	5.54	75.56	99.13

NOTE: Approximately 1 ng of DNA template was used in all amplifications. Profiler Plus samples were detected using the ABI Prism 377; COfiler samples were detected using the ABI Prism 310.

n = number of heterozygotes analyzed at each locus.

S.D. = Standard Deviation.

Lower Range Heterozygous Peak Height Ratio = Mean − 3 S.D.

Non-templated Nucleotide Addition—Taq polymerase has a propensity to add a single nucleotide (typically adenine, hence the name "plus/minus A") at the 3′ end of an amplicon in a template-independent fashion (31). Primer design and/or a final extension of 30 min or more at 60°C at the end of thermocycling promote non-templated nucleotide addition. To assess the efficiency of non-templated nucleotide addition at each locus, a study was performed in which the final extension was omitted. Even without the final extension step, some loci (in particular CSF1PO, FGA, TPOX, D7S820, D13S317, D16S539, D18S51, and D21S11) exhibited efficient non-templated nucleotide addition. Other loci required at least a 15 or 30 min final extension to convert all detectable "*n*" products ("minus A") to "*n*+1" products ("plus A"). These loci are vWA, TH01, D3S1358, D5S818, and D8S1179 (Fig. 7). Because the sequence of a primer can have an effect on the efficiency of non-templated nucleotide addition (32), and because the primers for a given locus contained within different commercial kits may differ, the data presented here should be applied only to the specific STR systems used in the adenylation assessment. Adenylation efficiency is dependent of primer design and is therefore kit-specific. Notably, even under conditions that promote non-templated nucleotide addition, minus A-product may be seen. Usually minus A-product is observed when excessive amounts of template are used in the PCR or when an inhibitor of Taq polymerase is present in the sample (data not shown). When present, "minus A" is typically observed at both alleles of a heterozygous locus (Fig. 7). If minus A-product is detected, depending on the profile, an interpretation could be made (for example, if both alleles at a heterozygous locus, or at multiple loci, demonstrate minus A-product). Alternatively, when minus A-product is demonstrated, one can (*a*) add additional Taq polymerase (not AmpliTaq Gold) and incubate at 60°C for at least 30 min to promote adenylation or (*b*) dilute the DNA template and re-amplify.

Simulated Forensic Samples

DNA isolated from various body fluids (blood, semen, and/or saliva) from single donors yielded identical STR profiles (data not shown). These observations are consistent with reports on somatic stability of microsatellite loci (33). In addition, the reliability of typing DNA derived from environmentally insulted samples and body fluids deposited on various substrates is also well-substantiated (2,5,7–9,16,34–39). The samples evaluated in the current study were previously typed using the loci D1S80 (35), PM+DQA1 (16,36) and CSF1PO-TH01-TPOX (CTT) (2). With use of the AmpFℓSTR and GenePrint kits in the current study, the general trends in STR typing results, in response to sunlight/temperature exposure, sample aging, and chemical treatments, were identical to each other and to typing results demonstrated with other loci. The collective data (not shown) indicate that the effects of substrate contact and of environmental and chemical insults on DNA, irrespective of the genetic locus or type of polymorphism, are: (*a*) sunlight can degrade DNA, (*b*) semen and saliva are more susceptible to degradation than blood, (*c*) some chemicals can degrade DNA, (*d*) degradation becomes more extensive over time, (*e*) partial profiles may result from the amplification of degraded DNA samples, (*f*) surfaces and objects such as clothing, household furnishings and structures, and environmental materials may contain substances that may co-extract with DNA and may inhibit the PCR, and (*g*) DNA deposited on various substrates may become degraded over time. When following recommended protocols, substrate contact, environmental and chemical insults, and DNA degradation will not produce false positive results. Using the AmpFℓSTR and GenePrint kits, identical types were obtained from replicate samples, from single donors, that were subjected to the various conditions; that is, mistyping did not occur, and non-specific or "extra" products that could affect typing were not evident. These conclusions were further corroborated by analyzing the same DNA samples using multiple kits and generating consistent results. Furthermore, typing results were concordant when generated on different detection instruments (e.g., ABI Prism 377 and 310, FMBIO II) Given the wealth of data from various studies (2,3,5,8,9,15,16,34–40), efforts to examine the potential consequences of environmental and chemical insults and of substrate contact provide no additional information that is useful for DNA typing.

Fundamental Validation Exercises

Additional validation studies for forensic application are: (*a*) assessment of species specificity, (*b*) examination of the ability of the system to distinguish the components of mixed samples, and (*c*) evaluation of adjudicated evidentiary samples.

Species Specificity—Studies on cross-species reactivity using the AmpFℓSTR and GenePrint systems were previously conducted using several non-primate and non-human primate (3,9,18–21,41; J. Schumm, Promega Corp., personal communication). These studies were therefore not repeated in the current work. The previous studies demonstrated that the STR primers used in the AmpFℓSTR systems and the GenePrint PowerPlex and CTTv systems failed to amplify non-primate genomic DNA, including that from bacteria and yeast. Using non-human primate genomic DNAs, as expected, some fragments in the same general size range as the human fragments were generated (9,19,20,41; J. Schumm, Personal Communication). The amelogenin primers in both the AmpFℓSTR and GenePrint systems generated detectable PCR product from genomic DNA of dog, pig, cow, and horse (19, 20; tested with AmpFℓSTR primers) and from dog, raccoon, fox, ferret, and lynx (J. Schumm, personal communication; tested with GenePrint primers). However, cross-reactivity of primers with DNA from other species does not invalidate the usage of a DNA typing system.

Mixtures—Body fluid mixtures that were evaluated in the current study were: blood/blood, blood/semen, blood/saliva, and semen/saliva. Additionally, DNA samples from two donors were mixed in ratios ranging from 1:20 to 1:1 to 20:1; one donor exhibited a TH01 9.3 allele, and the other donor exhibited a TH01 10 allele. Interpretation was facilitated by quantitative peak height or area data. On the ABI Prism 310 with the separation medium POP4, the minor component (e.g., TH01 allele 9.3 in a 9.3/10 mixture) could be detected and reliably typed when present at 10% of the major component in the mixed sample (2 ng total DNA template); at 5%, the minor component in some samples could be discerned (Fig. 8) but at times was not typable.

Case Studies—Forty-five previously typed [using RFLP, DQA1+PM, D1S80 and/or CTT] cases were analyzed with the AmpFℓSTR Profiler and Profiler Plus kits on the 377 DNA Sequencer, and a subset (35 cases) was typed using the GenePrint PowerPlex 1.2 kit on the 377 DNA Sequencer. A subset (10 cases) was also typed using the AmpFℓSTR Green 1 and Blue and GenePrint PowerPlex 1.1 kits on the FMBIO II. Due to sample de-

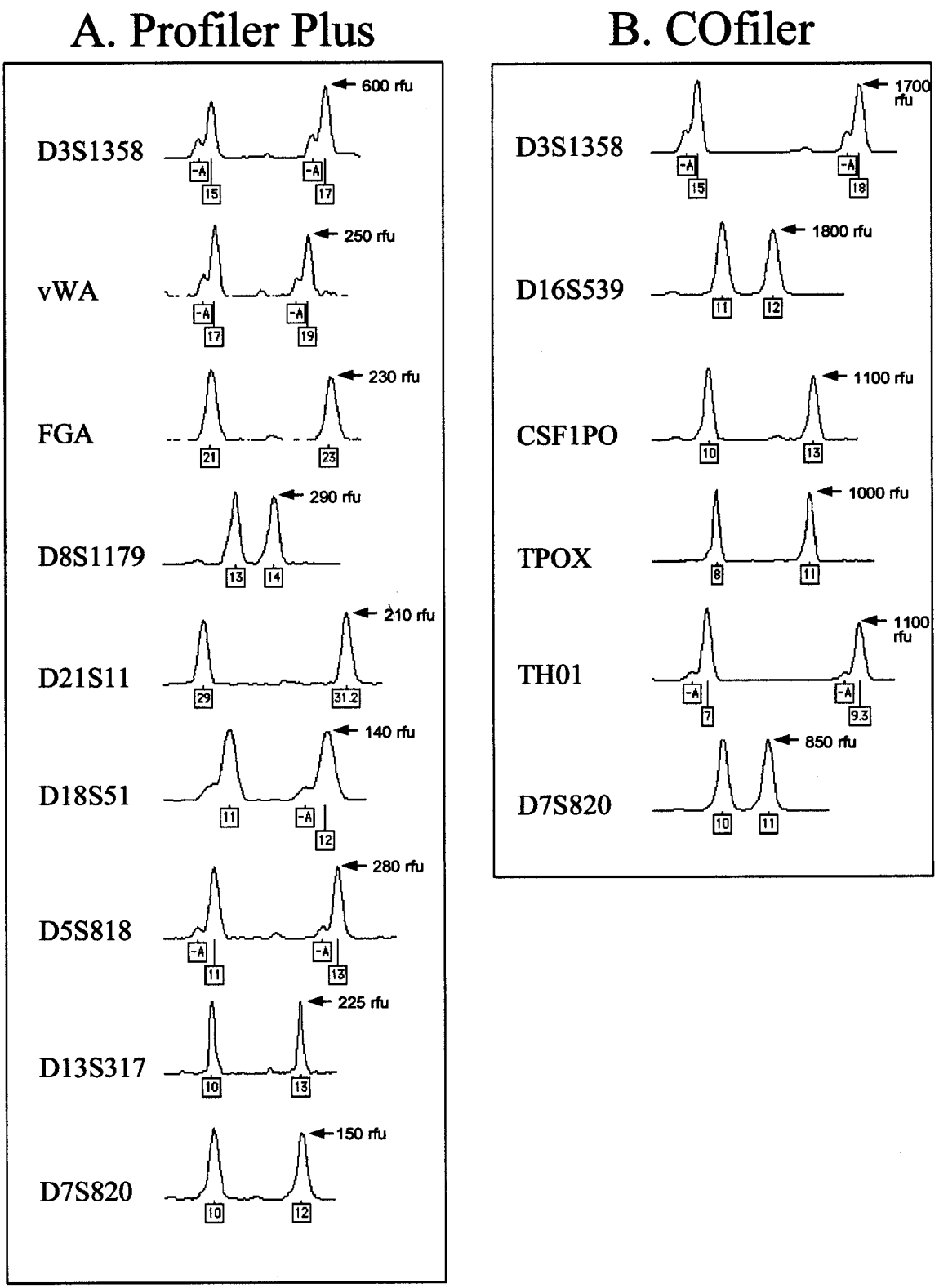

FIG. 7—*Efficiency of non-templated nucleotide addition. The final extension step (45 min at 60°C) was omitted in Profiler Plus* (A) *and COfiler* (B) *reactions conducted with 1 ng of DNA template extracted from blood. PCR products were separated and detected on the ABI Prism 310 Genetic Analyzer. Approximate peak height values (rfu) are indicated.*

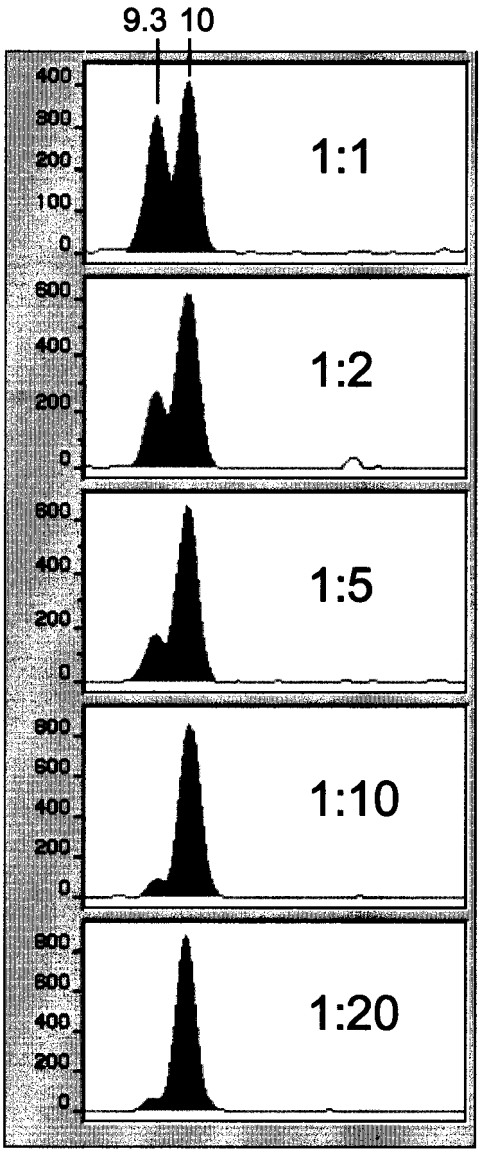

FIG. 8—*Mixtures of DNA samples in various ratios. Two DNA samples (one of which exhibited a TH01 9.3 allele, and the other of which exhibited a TH01 10 allele; extracted from blood) were prepared as mixtures with sample ratios ranging from 1:1 to 1:20. Using the separation medium POP4 and a 47-cm capillary on the ABI Prism 310 Genetic Analyzer, the alleles were resolved such that the minor component (e.g., TH01 9.3 allele) in mixtures ranging from 1:1 to 1:20 could be discerned and reliably typed.*

pletion, 10 additional cases were evaluated using both AmpFℓSTR COfiler and Profiler Plus kits on the 310 Genetic Analyzer. Case samples were derived predominantly from sexual assault cases and were comprised of a total of 112 known samples and 86 questioned samples. Most samples yielded typable results; a few generated partial profiles due to degradation of the DNA template (data not shown). When typed using different kits, the resultant profiles with redundant loci exhibited the same genotype (data not shown). Compared with the previous typing of different loci using the same case samples, there were no discrepancies as to the inclusion of suspects or victims with use of any of the STR systems (data not shown). These results support the reliability of STR typing and, in particular for the ABI Prism 310 and 377, the reliability of the Genotyper software for designating alleles.

Conclusions

The commercially available kits, AmpFℓSTR Profiler, Profiler Plus, COfiler, Blue, and Green 1 and GenePrint PowerPlex, can be used to amplify and type STR loci successfully from DNA derived from human biological specimens. The current study demonstrates that the procedures used to type STR loci using these commercial kits are robust and valid. Samples typed using multiple kits and/or different detection instruments generated the same typing results. There was no evidence of false positive or false negative results and no substantial evidence of preferential amplification within a locus. Although at times general balance among loci labeled with the same fluorophore was not observed, the results obtained were reliable. The study shows that some samples containing little DNA can be typed successfully. In addition, samples containing DNA that is somewhat degraded (such that the larger-sized STR loci in the multiplex are not typable) may be typed at the smaller-sized STR loci. The scientist should consider certain criteria for determining whether a sample is derived from a single source or from more than one contributor. These criteria entail the following: (*a*) the number of peaks at a locus, (*b*) the relative height of stutter products, and (*c*) peak height ratios. Stochastic threshold levels and the efficiency of non-templated nucleotide addition also aid in profile evaluation. While these guides should be applicable to the majority of samples for determining the presence or absence of multiple contributors, there will be instances in which the guides may not apply. These instances include: (*a*) a primer binding site variant for one allele at a given locus, (*b*) unusually high stutter product, (*c*) gene duplication, and (*d*) translocation. An evaluation of all the typable loci in the profile should therefore be made first for mixture interpretations. Thereafter, these criteria can be used when assessing the relevance of the data at each locus. This study reinforces previous findings that multiplex STR typing is sufficiently robust for implementation into forensic laboratories and will be effective for characterizing the vast majority of human biological samples encountered at crime scenes.

Acknowledgment

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