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TECHNICAL NOTE

CRIMINALISTICS

Dennis Y. Wang,[1] *Ph.D.; Chien-Wei Chang,*[1] *Ph.D.; Robert E. Lagacé,*[1] *B.A.; Lisa M. Calandro,*[1] *M.P.H.; and Lori K. Hennessy,*[1] *Ph.D.*

Developmental Validation of the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit: An Established Multiplex Assay with Improved Performance

ABSTRACT: Analysis of length polymorphism at short tandem repeat (STR) loci utilizing multiplex polymerase chain reaction (PCR) remains the primary method for genotyping forensic samples. The AmpFℓSTR® Identifiler® Plus PCR Amplification Kit is an improved version of the AmpFℓSTR® Identifiler® PCR Amplification Kit and amplifies the core CODIS loci: D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, CSF1PO, FGA, TH01, TPOX, and vWA. Additional loci amplified in the multiplex reaction are the sex-determinant, amelogenin, and two internationally accepted loci, D2S1338 and D19S433. While the primer sequences and dye configurations were unchanged, the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit features an enhanced buffer formulation and an optimized PCR cycling protocol that increases sensitivity, provides better tolerance to PCR inhibitors, and improves performance on mixture samples. The AmpFℓSTR® Identifiler® Plus PCR Amplification Kit has been validated according to the FBI/National Standards and Scientific Working Group on DNA Analysis Methods (SWGDAM) guidelines. The validation results support the use of the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit for human identity and parentage testing.

KEYWORDS: forensic science, DNA typing, short tandem repeat, CSF1PO, D2S1338, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D19S433, D21S11, FGA, TH01, TPOX, vWA, amelogenin

Short tandem repeat (STR) markers are the primary means of genetic analysis used today in the field of forensic DNA testing (1–3). STRs are highly polymorphic and capable of generating typing results from very little material through multiplex amplification using the polymerase chain reaction (PCR). Since its introduction in 2001, the AmpFℓSTR® Identifiler® PCR Amplification Kit (Life Technologies, Foster City, CA) has been used widely by both forensic databasing and casework laboratories to genotype STRs (4,5). This has resulted in the addition of millions of STR profiles to DNA databases leading to the resolution of countless cases by the criminal justice system. The proven success of leveraging DNA during the investigative process, along with the "CSI Effect" phenomenon (6), has driven the passage of new legislation to collect more DNA samples from offenders and/or arrestees (7) and resulted in the use of DNA evidence for an expanded number and wider variety of criminal cases.

In response to the increased demand for DNA analysis, laboratories are continuously seeking to implement enhanced technologies that allow them to process databasing and casework samples more efficiently and effectively. Examples of these recent advances include a mini-STR Kit that increases amplification performance particularly for compromised samples (8), dual quantitation assays that allow assessment of the potential probative value and performance of a sample in downstream analysis (9–11), and superior DNA extraction methodologies (12,13). The second generation of the Identifiler® Kits was developed to address the varying needs of the forensic databasing and casework laboratories. For databasing laboratories, the AmpFℓSTR® Identifiler® Direct PCR Amplification Kit was developed to alleviate the burden of expanded offender/arrestee samples (14). The Identifiler® Direct Kit was optimized to allow direct amplification of single-source blood and buccal samples on FTA® cards without the need for sample purification. The reduction in the number of steps required to obtain a result simplifies the integration of automated instrumentation and minimizes the potential for sample contamination or other processing errors. For casework laboratories, the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit was developed to address the desire for greater sensitivity, better tolerance to PCR inhibitors, and improved performance on mixture samples (15). The successful integration of the above-mentioned features in the Identifiler® Plus Kit enables forensic analysts to recover more interpretable results from increasingly challenging casework samples with greater confidence.

Both of the second-generation Identifiler® Kits utilize the same primers as the widely used Identifiler® Kit and harness new PCR amplification technology to provide a higher level of performance, data quality, and efficiency. The developmental validation of the AmpFℓSTR® Identifiler® Direct PCR Amplification Kit is described elsewhere (14). This article describes the developmental validation of the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit. The experiments were performed according to the guidelines issued by the Director of the FBI (16) and the revised guidelines published by the Scientific Working Group on DNA Analysis Methods (SWGDAM) (17). The results confirm the reliability of

[1]Life Technologies, 850 Lincoln Centre Drive, Foster City, CA 94404.

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the Identifiler® Plus Kit as required for forensic sample genotyping.

Materials and Methods

DNA Samples

Anonymous whole blood samples of known ethnicities were purchased from Seracare Life Sciences (Oceanside, CA) and Interstate Blood Bank, Inc. (Memphis, TN). DNA extraction and purification were performed using the ABI PRISM® 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA). Control DNA 9947A and 9948 were purchased from Marligen Biosciences (Ijamsville, MD) and Coriell Cell Repositories (Camden, NJ), respectively. The AmpFℓSTR® Control DNA 007 was obtained from Applied Biosystems, and the Raji DNA was purchased from Biochain Institute (Hayward, CA). Prior to PCR amplification, the DNA quantity of the samples was determined using either the Quantifiler® Human DNA Quantification Kit or the Quantifiler® Duo DNA Quantification Kit (Applied Biosystems) on the ABI PRISM® 7000 or 7500 Sequence Detection Systems (Applied Biosystems), respectively.

Purified primate DNA samples from chimpanzee, gorilla, orangutan, and macaque were purchased from BIOS Laboratories (New Haven, CT). Nonprimate whole blood samples were obtained from Pel-Freez Biologicals (Rogers, AK) and genomic DNA was extracted and purified using the ABI PRISM® 6100 Nucleic Acid PrepStation (Applied Biosystems). A pool of microbial DNA was created by combining the genomic DNA from the various human-associated microbial species. Individual microbial genomic DNA was prepared from samples grown and purified in-house with the Iso-Quick Nucleic Acid Extraction Kit (Orca Research, Inc., Bothell, WA). DNA concentration of nonhuman DNA was determined by measuring the absorbance of the sample at 260 nm.

PCR Amplification

Unless stated otherwise, the protocols in the Identifiler® Plus Kit User Guide were followed (15). The PCR amplification reaction mixture was prepared in a volume of 25 μL containing 10 μL of AmpFℓSTR® Identifiler® Plus Master Mix, 5 μL of AmpFℓSTR® Identifiler® Plus Primer Set, and a maximum volume of 10 μL of target DNA. Samples were amplified in MicroAmp® reaction tubes or MicroAmp® Optical 96-well reaction plate (Applied Biosystems) in the GeneAmp® PCR system 9700 with a gold-plated silver or silver block (Applied Biosystems). A two-step PCR cycling protocol was also optimized for the Identifiler® Plus Kit. The standard thermal cycling conditions in the 9600 emulation mode consisted of enzyme activation at 95°C for 11 min, followed by 28 or 29 cycles of denaturation at 94°C for 20 sec and annealing/extension at 59°C for 3 min. A final extension step was performed at 60°C for 10 min, followed by a final hold at 4°C if the PCR products were to remain in the thermal cycler for an extended time.

Sample Electrophoresis and Data Analysis

PCR products were separated and detected on the Applied Biosystems 3130 and 3130*xl*, ABI PRISM® 3100 and 3100-*Avant*, or ABI PRISM® 310 Genetic Analyzers using the specified G5 variable binning modules as described in the AmpFℓSTR® Identifiler® Plus Kit User Guide (15). As an example, the following protocol was used for sample preparation and electrophoresis on the Applied Biosystems 3130*xl* Genetic Analyzer: samples were prepared by adding 1 μL of the PCR product or allelic ladder to 9 μL of formamide/LIZ® solution (0.3 μL of GeneScan™ 500 LIZ® size standard and 8.7 μL of deionized Hi-Di™ Formamide; Applied Biosystems). Prior to electrophoresis, the samples were denatured at 95°C for 3 min then chilled on ice for 3 min. Samples were injected at 3 kV for 10 sec and electrophoresed at 15 kV for 1500 sec in Performance Optimized Polymer-4 (POP-4™ polymer; Applied Biosystems) with a run temperature of 60°C. Following data collection, electrophoresis results were analyzed using GeneMapper® *ID* Software v3.2.1 or GeneMapper® *ID-X* Software v1.0.1 (Applied Biosystems). Unless stated otherwise, allele peaks were interpreted when the peak heights were ≥50 relative fluorescence units (RFU).

Primer Set Concentration

The primer sequences of the Identifiler® Plus Kit are unchanged from the previously validated Identifiler® (4,5) and Identifiler® Direct (14) Kits. Furthermore, the primer concentrations remain unchanged between the Identifiler® and the Identifiler® Plus Kits. For the evaluation of primer mix performance, three genomic DNA samples (Control DNA 9947A, 9948, and 007) were amplified in triplicate at the standard primer mix concentration and at 10% intervals up to ±30% levels (v/v).

PCR Master Mix Components

The PCR master mix components of the AmpFℓSTR® Identifiler® Plus Master Mix include AmpliTaq Gold® DNA polymerase, buffer, salts, dNTPs, carrier protein, detergents, and 0.05% sodium azide. The inclusion of the carrier protein in the master mix was found to be beneficial in overcoming PCR inhibitors. It is possible that the carrier protein can sequester the inhibitors or enhance DNA polymerase activity (18). The individual master mix components were tested at increments of ±10%, ±20%, and ±30% (v/v) from the standard formulation to test for reliability and robustness of the master mix configuration. Three genomic DNA samples (Control DNA 9947A, 9948, and 007) were evaluated in triplicate for each PCR master mix component at each concentration.

Thermal Cycling Parameters

As with PCR master mix component optimization, thermal cycling parameters were evaluated to verify the optimized performance of amplification for the Identifiler® Plus Kit. Cycling parameters around the standard set of conditions were tested. Three genomic DNA samples (Control DNA 9947A, 9948, and 007) were evaluated in triplicate, along with nontemplate controls (NTCs), to test each thermal cycling parameter.

The following thermal cycler parameters were examined, with the standard conditions indicated in bold:

- Cycle number: 26, 27, **28**, **29**, 30, and 31 cycles.
- Denaturing temperature: 92.5, **94**, and 95.5°C.
- Annealing temperature: 55, 57, **59**, 61, and 63°C.
- Final extension time: 0, 5, **10**, 15, and 20 min.

Accuracy, Precision, and Stutter Studies

Two hundred genomic DNA samples purified from whole blood were used to measure the deviation of each sample allele size from the corresponding allele size in the allelic ladder. The genomic

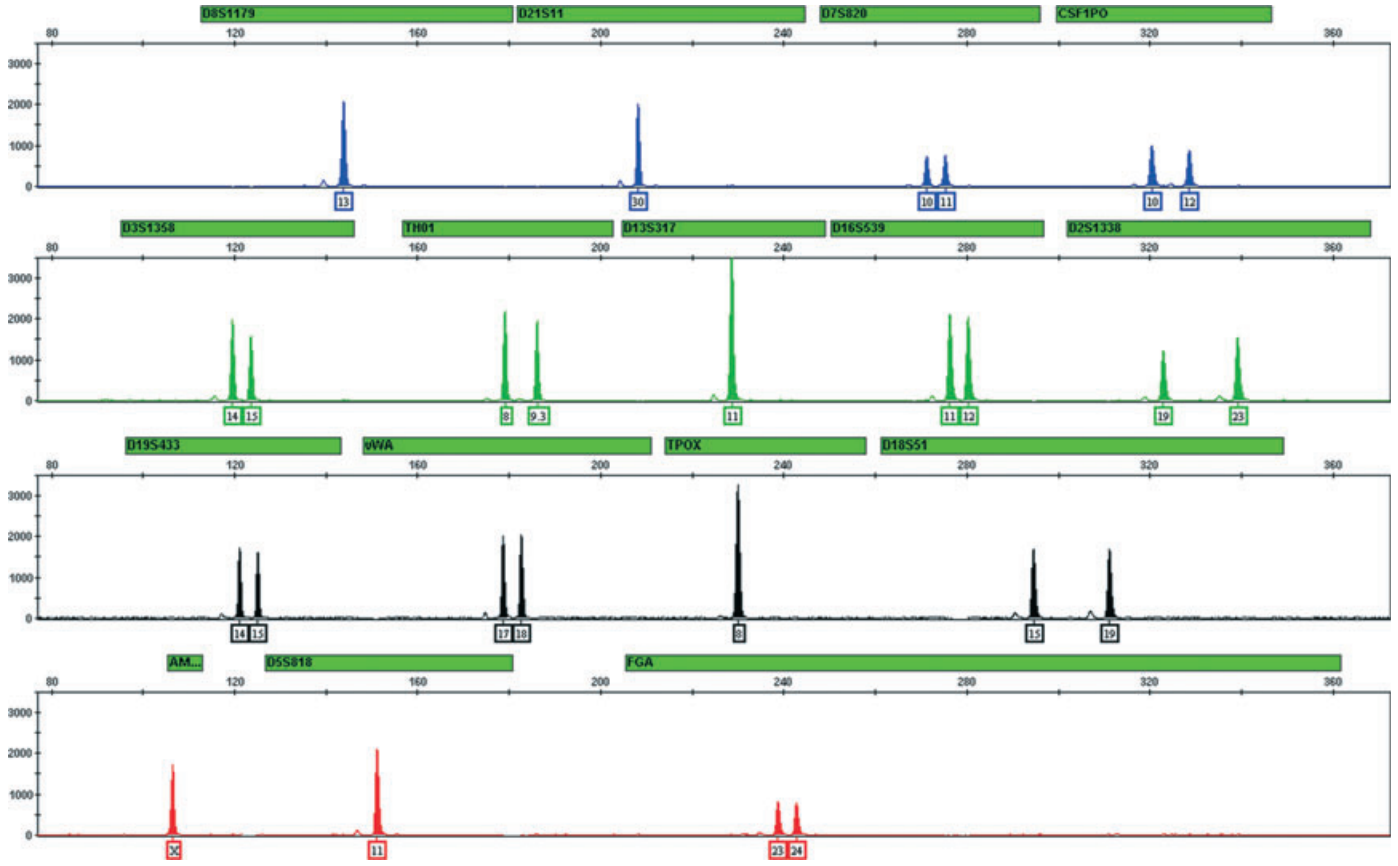

FIG. 1—*Representative electropherogram showing the profile of 1 ng of Control DNA 9947A amplified with the Identifiler® Plus Kit for 28 PCR cycles. The four panels correspond to (from top to bottom) 6-FAM™, VIC®, NED™, and PET® dye-labeled peaks. The genotype is shown with the allele number displayed underneath each peak.*

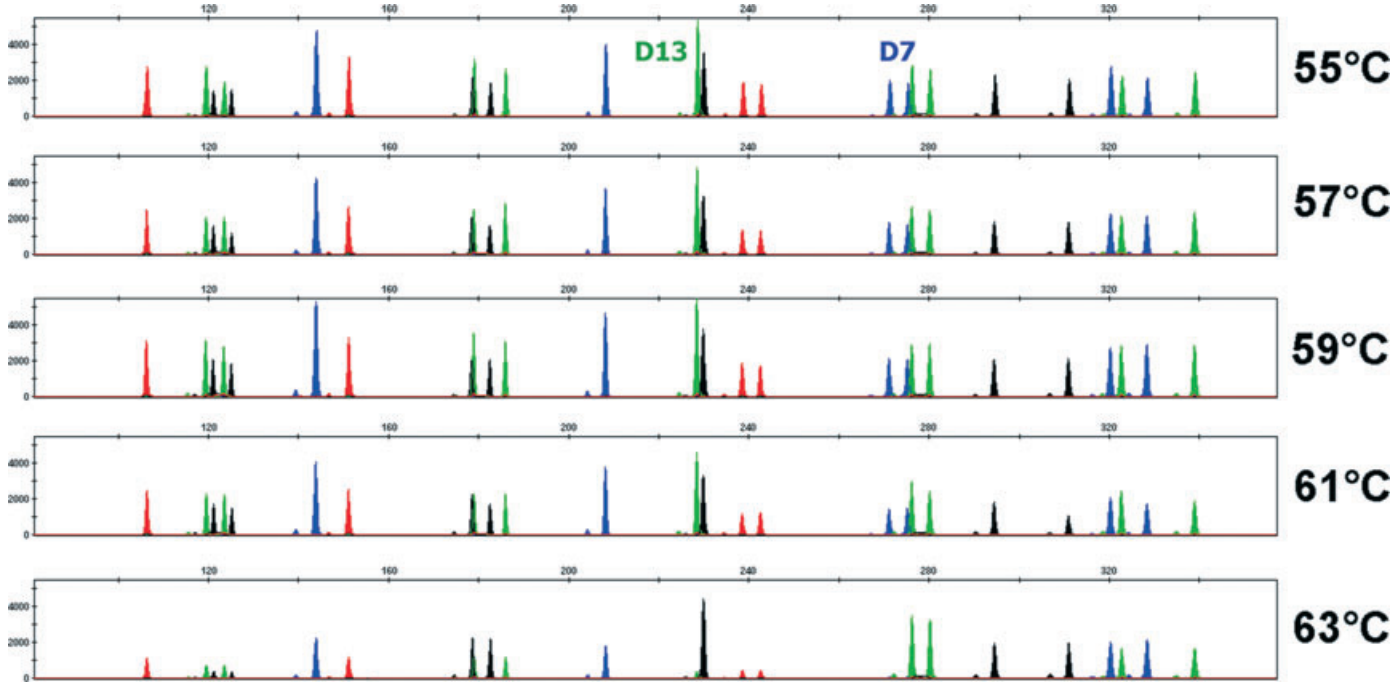

FIG. 2—*Representative electropherograms from the annealing temperature study using the Control DNA 9947A. One nanogram of DNA was amplified using the Identifiler® Plus Kit at the indicated annealing temperatures. At high annealing temperature, the peak heights of D13S317 and D7S820 loci were mostly affected. Peak heights were measured in relative fluorescent units.*

DNA samples were amplified with the Identifiler® Plus Kit at 1-ng input using the standard 28-cycle PCR protocol and subjected to electrophoresis. Allelic ladder sizing precision was calculated from multiple injections of the Identifiler® Plus Allelic Ladder. The proportion of the stutter product relative to the main allele (percent stutter) was measured by dividing the height of the stutter peak by the height of the associated allele peak. Peak heights were measured on 500 population samples at the loci detected in the Identifiler® Plus Kit. All samples were electrophoresed on the Applied Biosystems 3130*xl* Genetic Analyzer.

Species Specificity

The Identifiler® Kit primers were designed to be primate-specific with minimal cross-reaction with other animal or microbial species (4,5). The Identifiler® Kit primer sequences are maintained in the Identifiler® Plus Kit. The DNA samples from primates (1 ng each from gorilla, chimpanzee, orangutan, and macaque), nonprimates (10 ng each from mouse, dog, pig, cat, horse, hamster, rat, chicken, and cow), and pooled microorganisms (*c.* 10^5 copies each from *Candida albicans*, *Staphylococcus aureus*, *Escherichia coli*, *Neisseria gonorrhoeae*, *Bacillus subtilis*, *and Lactobacillus rhamnosus*) were subjected to PCR amplification using the Identifiler® Plus Kit in triplicate at 28 PCR cycles. Electrophoresis was performed on the Applied Biosystems 3130*xl* Genetic Analyzer.

Sensitivity Study

Assessing amplification performance with a range of DNA input amounts is helpful to understand the potential interpretational limitations of a PCR-based typing system. Because of system variations, sensitivity studies aimed at producing interpretation guidelines are best performed by the individual testing laboratory. To demonstrate the performance of the Identifiler® Plus Kit, PCR amplification was performed in replicates of four on two genomic DNA samples (Control DNA 9947A and 007) that were serially diluted to provide amounts of 1 ng, 500, 250, 125, 62.5, and 31.25 pg per amplification. Both the 28-cycle and the 29-cycle PCR protocols were evaluated.

Stability Study

Stability studies using the 28-cycle PCR protocol were carried out to characterize amplification performance in the presence of either inhibited or degraded DNA, both of which have been known to change the efficiency of the PCRs (19–21). Artificially degraded DNA samples were prepared by sonicating the Raji DNA followed by 20-min incubation with varying amounts (0–6 U) of DNase I (Ambion Inc., Austin, TX). Two known PCR inhibitors, hematin and humic acid, were added to the PCR to simulate PCR inhibition. Stock hematin and humic acid solutions of high concentration were prepared by dissolving hematin (Sigma, St. Louis, MO) in 0.1 N NaOH and humic acid (Sigma) in TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). Further dilutions were made with water.

Mixture Studies

Mixtures of two genomic DNA samples were examined at various ratios (1:1, 3:1, 7:1, 10:1, and 15:1) while maintaining the total amount of DNA input constant at 1 ng. For example, a 1:1 mixture ratio contains 500 pg of each individual. Mixture ratios of 3:1, 7:1, 10:1, and 15:1 contain 250, 125, 91, and 62.5 pg of the minor component, respectively. The two individuals comprising the mixture were carefully selected to minimize both the number of overlapping alleles and the number of minor contributor alleles coinciding with the stutter position of major contributor alleles. Amplifications were performed in triplicate using both the 28-cycle and 29-cycle PCR protocols.

Population and Concordance Studies

Genomic DNA from 500 population samples (125 Caucasian, 125 African American, 125 Hispanic, and 125 Asian) was extracted, purified, and quantitated from whole blood samples, followed by amplification using the Identifiler® Kit and the Identifiler® Plus Kit with 1 ng of DNA input for 28 PCR cycles. All amplified PCR products were electrophoresed on the Applied Biosystems 3130*xl* Genetic Analyzer. A subset of the amplified population samples were electrophoresed on the Applied Biosystems 3100 Genetic Analyzer ($N = 200$) and on the ABI PRISM® 310 Genetic Analyzers ($N = 42$). Data analysis was performed using both GeneMapper® *ID* Software v3.2.1 and GeneMapper® *ID-X* Software v1.0.1. Genotype concordance was checked between the Identifiler® Kit and the Identifiler® Plus Kit, the three capillary electrophoresis (CE) platforms, and the two data analysis software packages.

Statistical Analysis

Intralocus peak height ratio (also known as heterozygote peak height ratio) was calculated by dividing the lower allele peak height of a heterozygous individual by the higher allele peak height, and the result was expressed as a percentage. Intracolor peak height balance was calculated by first averaging heterozygous peaks and dividing homozygous peaks in half. Once normalized for diploidy, the lowest score for a locus labeled with a given dye was divided by the highest, and the result was reported as a percentage. The errors reported for intralocus and intracolor peak height ratios are expressed as ± 1 standard deviation (SD).

Results

Primer Set Concentration and PCR Master Mix Components

A representative Identifiler® Plus Kit profile generated using 1 ng of Control DNA 9947A and 28 PCR cycles is shown in Fig. 1. To ensure utmost robustness and reproducibility, studies were performed by varying the concentrations of primers in the Identifiler® Plus Primer Set and the individual components within the Identifiler® Plus Master Mix. The studies covered a range of −30% to + 30% for each of the individual components. Full STR profiles were generated at the concentrations tested without any spurious amplification or detrimental effect on sample peak heights, intralocus peak height ratios, and intracolor peak height balances. The mean intralocus peak height ratios ranged from 88.2 ± 7.6% (at −30%) to 92.4 ± 7.2% (at ±0%), while the intracolor peak height balances ranged from 72.0 ± 13.5% (at +30%) to 80.1 ± 8.4% (at ±0%).

Thermal Cycling Parameters

Cycle Number—Optimal thermal cycling parameters are determined to be in the middle of a window that balances specificity and sensitivity. As expected, each increase in cycle number led to a corresponding increase in overall peak height of *c.* twofold. Full STR profiles were obtained at all PCR cycle numbers tested (26–

31 cycles), and no nonspecific amplification products were observed. However, off-scale homozygote peaks were occasionally detected at 29 PCR cycles and frequently observed at ≥30 PCR cycles (DNA input = 1 ng). Based on these observations, the optimal PCR cycle number was determined to be 28, where the overall allele peak heights were the highest without observing any off-scale homozygote peaks. By excluding the off-scale data from data analysis, changes in PCR cycle number did not show any significant effect on intralocus peak height ratios and intracolor peak height balances for all three Control DNA samples tested. Replicates of NTC samples amplified for 26–31 PCR cycles did not show any nonspecific or artifact peaks.

Denaturation Temperature—Two denaturation temperatures, 92.5 and 95.5°C, were tested against the standard 94°C. Full STR profiles were obtained at all denaturation temperatures tested. The STR profiles generated showed similar sample peak heights, and the intralocus peak height ratios and intracolor peak height balances were also comparable. The mean intralocus peak height ratios ranged from 93.4 ± 6.5% (at 92.5°C) to 95.8 ± 7.2% (at 95.5°C), while the intracolor peak height balances ranged from 79.2 ± 9.5% (at 92.5°C) to 79.9 ± 9.8% (at 94°C).

Annealing Temperature—Optimal annealing temperature is identified as the temperature that delivers a balance of specific

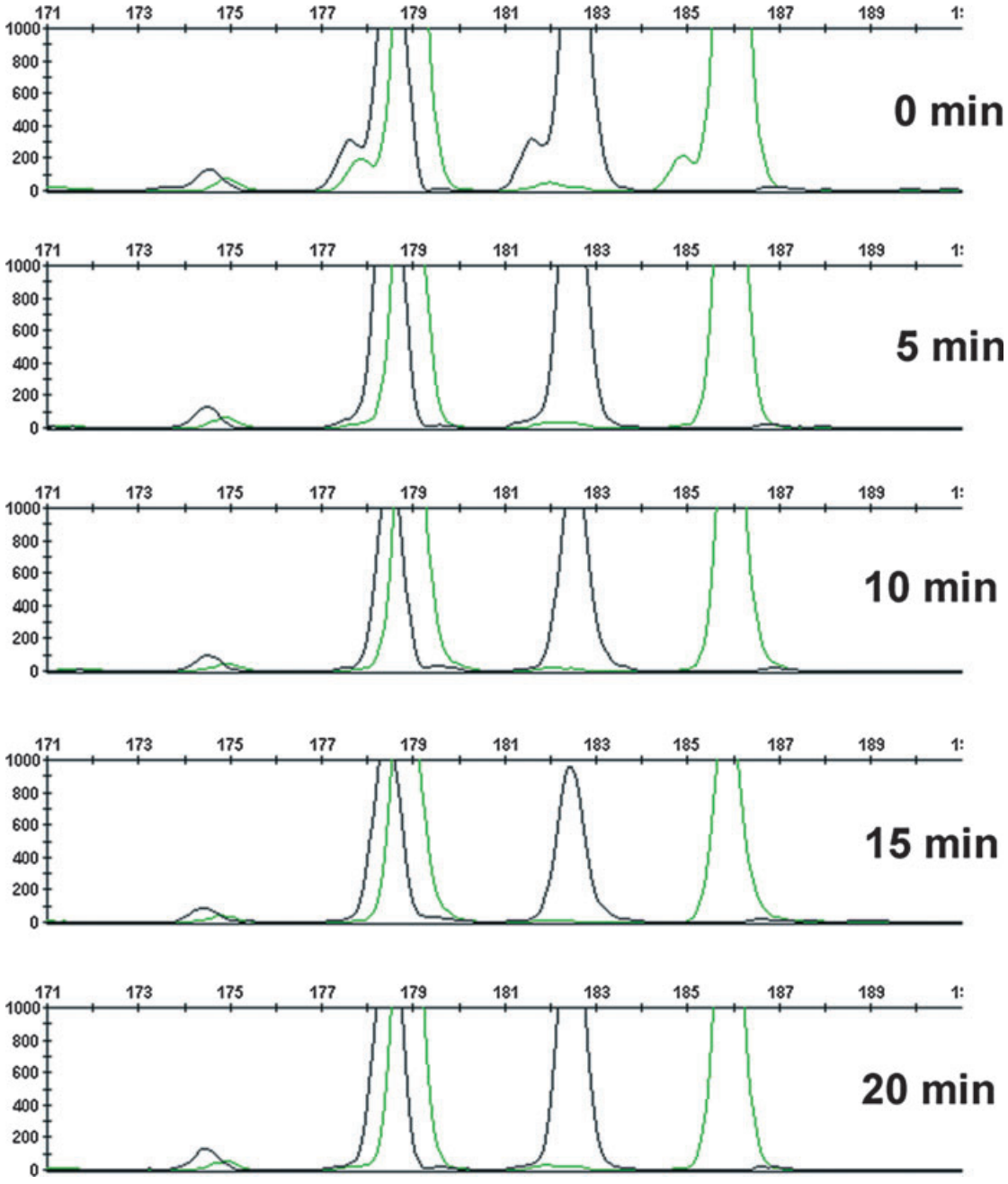

FIG. 3—*Effect of shortening final extension time. With 0 min of final extension, loci such as vWA (black) and TH01 (green) showed incomplete terminal nucleotide addition. The remaining loci did not exhibit incomplete terminal nucleotide addition. With final extension time ≥5 min, no incomplete terminal nucleotide addition was observed.*

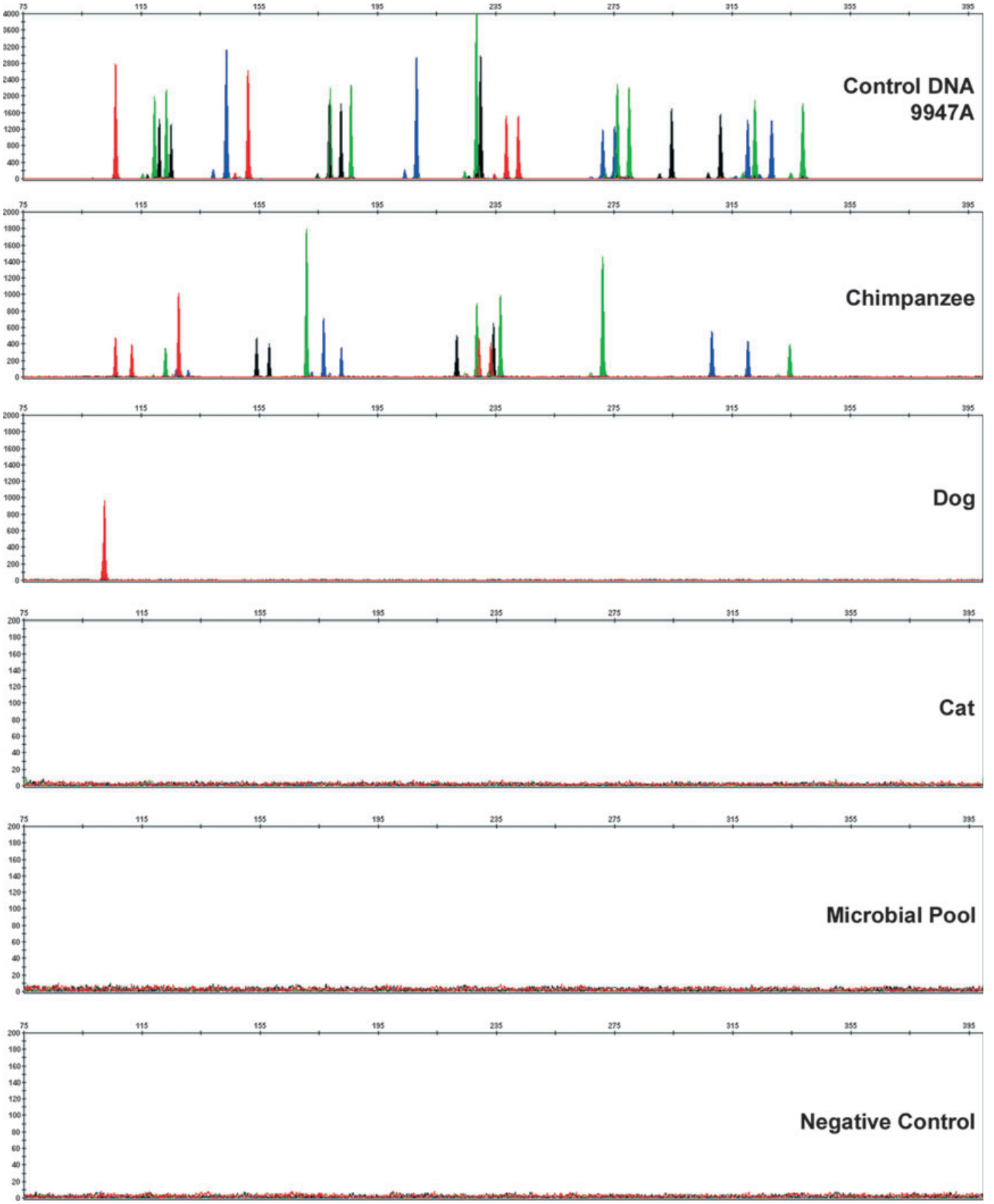

FIG. 4—*Representative electropherograms from a species specificity study including positive and negative controls. From top to bottom: 1 ng of control DNA 9947A (human), 1 ng of chimpanzee DNA, 10 ng of dog and cat DNA, a microbial pool (c. 10^5 copies each of* Candida albicans, Staphylococcus aureus, Escherichia coli, Neisseria gonorrhoeae, Bacillus subtilis*, and* Lactobacillus rhamnosus*) and a negative control. Different relative fluorescence units scales (Y-axis) are shown to demonstrate specificity. Except for the monomorphic, Amelogenin-like product seen in panel 3, amplification was only observed for primates among all tested species.*

amplification of human DNA, sensitivity, and reproducible intracolor peak height balance. No allele dropout events or artifact peaks were observed with an annealing temperature between 55 and 61°C. At 63°C, a significant decrease in peak heights was observed in several loci with occasional allele dropouts in D7S820 and D13S317 for all three Control DNA samples (Fig. 2). Between annealing temperatures of 55–61°C, intracolor peak height balance >50% was observed reproducibly. The mean intralocus peak height ratios were mostly unaffected and ranged from 93.2 ± 6.5% (at 57°C) to 94.8 ± 6.7% (at 63°C).

Final Extension Time—The Identifiler® Plus Kit maintains the same primer sequences found in the Identifiler® Kit, which was optimized for nontemplated terminal nucleotide addition by AmpliTaq Gold® DNA polymerase (22,23). By utilizing a robust PCR master mix formulation along with an optimized PCR cycle protocol, the final extension time designed to ensure complete terminal nucleotide addition was reduced from 60 min (Identifiler® Kit) down to 10 min (Identifiler® Plus Kit). With 0 min of final extension, incomplete terminal nucleotide addition was observed at the vWA and TH01 loci (Fig. 3). With just 5 min of final extension, the profiles generated did not show any incomplete terminal nucleotide addition for all three Control DNA samples studied (Fig. 3). Increasing the final extension time to 20 min did not provide worthwhile benefits in the Control DNA samples tested and was not detrimental to the chemistry. Changes in final extension time did not show any significant effect on sample peak heights, intralocus peak height ratios, and intracolor peak height balances. The mean intralocus peak height ratios ranged from 90.6 ± 6.7% (at 15 min) to 95.3 ± 7.4% (at 10 min), while the intracolor peak height balances ranged from 77.3 ± 9.2% (at 0 min) to 81.6 ± 10.6% (at 15 min).

TABLE 1—*Stutter averages, ranges, and standard deviations for the Identifiler® Plus Kit.*

Locus	Number of Observations (*n*)	Stutter Mean (%)	Stutter Range (%)	SD	Mean + 3 SD
CSF1PO	675	5.17	1.76–11.39	1.34	9.20
D13S317	693	4.88	1.51–9.60	1.68	9.93
D16S539	703	5.69	2.20–9.85	1.57	10.39
D18S51	810	7.87	3.13–17.96	1.94	13.68
D19S433	704	7.03	2.69–12.68	1.39	11.21
D21S11	790	7.18	3.92–10.70	1.16	10.67
D2S1338	841	7.75	4.36–13.44	1.56	12.44
D3S1358	631	8.02	0.97–11.62	1.42	12.27
D5S818	661	5.84	1.73–9.75	1.41	10.06
D7S820	703	4.79	1.54–10.07	1.63	9.69
D8S1179	717	6.50	2.22–10.96	1.27	10.32
FGA	772	7.63	2.75–19.94	1.80	13.03
TH01	509	1.91	0.67–4.00	0.72	4.08
TPOX	660	3.03	1.05–7.93	1.12	6.38
vWA	527	6.45	1.60–13.34	1.99	12.45

All loci have tetranucleotide repeats yielding −4 bp stutters. Stutters were determined for those samples with peak heights between 400 and 5000 RFU. The threshold minimum stutter peak height was 20 RFU. The recommended stutter filter to be used for the GeneMapper® software was calculated using the formula of the mean stutter value plus three times the standard deviation. RFU, relative fluorescence units.

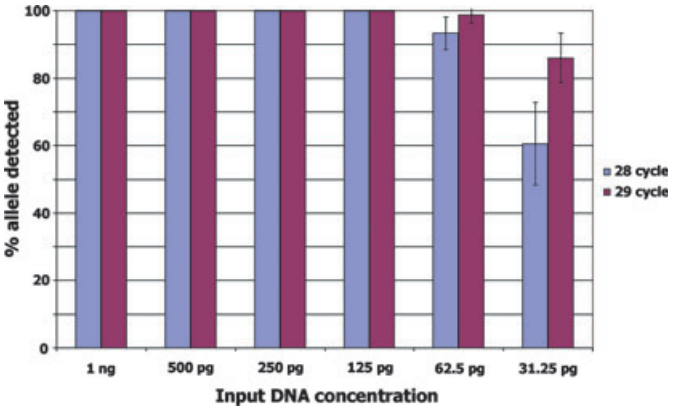

FIG. 5—*Amplification with serially diluted Control DNA (from 1 ng down to 31.25 pg) were performed with the Identifiler® Plus Kit. The percentage of alleles called was calculated by dividing the number of alleles observed over the expected number of alleles in a full short tandem repeat profile. The average percentage of alleles called when using the 28-cycle and 29-cycle protocols are shown (N = 8 for each protocol). Error bars show the standard deviation.*

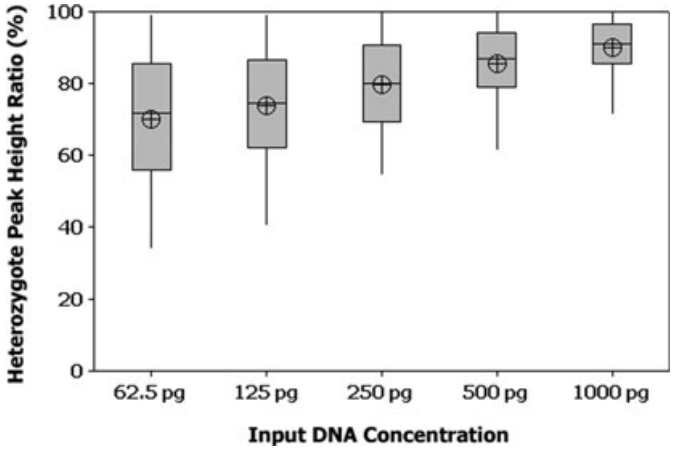

FIG. 6—*Heterozygote peak height ratios with varying inputs of template DNA. The results depicted are from four amplifications of two Control DNA samples at 62.5, 125, 250, 500, and 1000 pg using the 28-cycle PCR protocol. The grey boxes represent the 25th to the 75th percentile of the data, the lines within the boxes represent the median, and the whiskers show the range of the data. The median and mean values for each DNA input are indicated by the horizontal line within the box and the cross symbol, respectively.*

Accuracy, Precision, and Stutter Studies

Determining sizing accuracy and precision includes evaluation of measurement error and assessing performance for accurate and reliable genotyping. Two hundred genomic DNA samples were used to measure the deviation of each sample allele size from the corresponding allele size in the allelic ladder. All sample alleles tested were within ±0.5 bp of the corresponding alleles in the allelic ladder (15). Multiple injections of the Identifiler® Plus Allelic Ladder were analyzed to calculate the allelic ladder sizing precision. The SD of the mean was calculated and shown to be 0.15 bp or less (15), demonstrating adequate precision for sizing microvariants and off-ladder alleles.

Because of the repetitive nature of STRs, stutter products are often observed as the result of strand slippage during PCR amplification (24–26). The most common stutter product is 1 unit in length smaller than the true allele, resulting in a peak that could be, for example, four nucleotides smaller for any given tetranucleotide repeat marker in the Identifiler® Plus Kit. Percent stutter was calculated on a population composed of 500 genomic DNA samples. All loci showed the trend of increasing stutter percentages with increasing allele size (15,27). The stutter averages, ranges, and SD are shown in Table 1.

Species Specificity

A variety of animal and microbial species were tested to assess the human specificity of the Identifiler® Plus Kit. When tested with a range of nonhuman and microbial DNA templates, amplification was observed only among primates with one notable exception as indicated below. One nanogram of primate DNA (chimpanzee, gorilla, orangutan, and macaque) produced partial STR profiles. At most, 19 alleles were amplified for chimpanzee and gorilla DNA. This is not unexpected because the degree of STR homology between chimpanzee and human has been previously documented (28). The STR profiles generated were comparable with that of the Identifiler® and Identifiler® Direct Kits (4,5,14). The only nonprimate amplification observed was for an *c.* 103-bp-sized, PET® dye-labeled fragment in equine, porcine, and canine samples. With increased template concentration, amplification of this fragment was also observed in bovine and ovine samples. This PCR fragment has been attributed to a monomorphic product amplified from certain mammals by the Amelogenin primers (29). No detectable DNA profiles were observed in mouse, cat, hamster, rat, chicken, and pooled microorganisms comprising of *c.* 10^5 copies each from *C. albicans*, *S. aureus*, *E. coli*, *N. gonorrhoeae*, *B. subtilis*, and *L. rhamnosus*. Representative electropherograms are shown in Fig. 4.

Sensitivity Study

Sensitivity studies were performed, in replicates of four, using two Control DNA samples (9947A and 007) that were serially diluted to give 1 ng down to 31.25 pg of DNA per amplification. The optimal quantity of template DNA for the Identifiler® Plus Kit ranged from 0.5 to 1 ng using the 28-cycle PCR protocol and from 0.125 to 0.5 ng using the 29-cycle PCR protocol. With the 28-cycle PCR protocol, full profiles were obtained consistently at 0.125 ng, but occasional partial profiles missing anywhere from one to three alleles were observed at 0.062 ng. With the 29-cycle PCR protocol, full profiles were frequently obtained at 0.062 ng, but occasional partial profiles missing anywhere from one to five alleles were observed at 0.031 ng. The results of the sensitivity study are summarized in Fig. 5. Intralocus peak height ratio analysis was performed and the results shown in the box plot in Fig. 6 (28-cycle PCR data). Consistent with prior observations, the intralocus peak height ratio is positively correlated to the amount of DNA input (4,5). In contrast, the intracolor peak height balance is not greatly affected by the amount of DNA input.

To demonstrate the improved sensitivity of the Identifiler® Plus Kit over the Identifiler® Kit, a sensitivity study was also performed on the Identifiler® Kit using the same Control DNA samples, CE instrument, and injection protocol. The sensitivity results of the Identifiler® Kit, which utilized a standard PCR cycle number of 28, was compared with the sensitivity results of the Identifiler® Plus Kit with the 28-cycle PCR protocol. Samples amplified with the Identifiler® Plus Kit yielded peak heights 40–100% higher than the Identifiler® Kit. On average, the Identifiler® Plus Kit peak heights were *c.* 1.7 times higher than Identifiler® Kit peak heights (Fig. 7).

Inhibited DNA Study

Forensic DNA samples may contain inhibitors that cannot be effectively removed after the extraction and purification procedures. These inhibitors can interfere with PCR amplification and reduce PCR efficiency by varying degrees, at worst causing complete amplification failure. Heme compounds in bloodstains and humic acids in soil samples are known examples (18–21). The effect of hematin and humic acid on the amplification efficiency of the Identifiler® Plus Kit was examined. Complete STR profiles were reproducibly obtained in the presence of up to 300 μM of hematin, with little effect on the overall allele peak heights. Full STR profiles were also reliably obtained in the presence of up to 150 ng/μL of humic acid. However, at 150 ng/μL of humic acid, an indication of PCR inhibition was evident by a slight drop in peak heights for the allele peaks that are <220 bp and a larger drop in peak heights for the allele peaks that are >220 bp (Fig. 8). The results demonstrated a great improvement in inhibitor tolerance when compared to the Identifiler® Kit. The inhibited DNA study for the Identifiler® Kit showed partial STR profile was observed in the presence of 15 μM of hematin or 10 ng/μL of humic acid, and complete PCR inhibition was seen at 60 μM of hematin or 40 ng/μL of humic acid (Fig. 8).

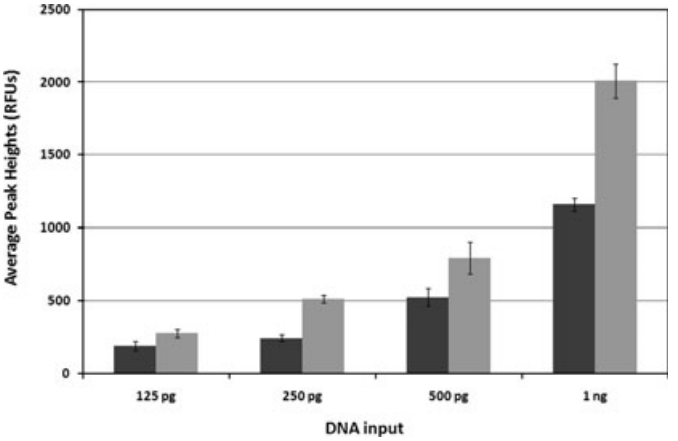

FIG. 7—*Illustration of the improved sensitivity of the Identifiler® Plus Kit. The figure shows the average peak heights obtained for four different dilutions of a Control DNA sample amplified for 28 cycles using the Identifiler® Kit (dark bars) and Identifiler® Plus Kit (light bars). On average, the Identifiler® Plus Kit peak heights were c. 1.7 times higher than Identifiler® Kit peak heights.*

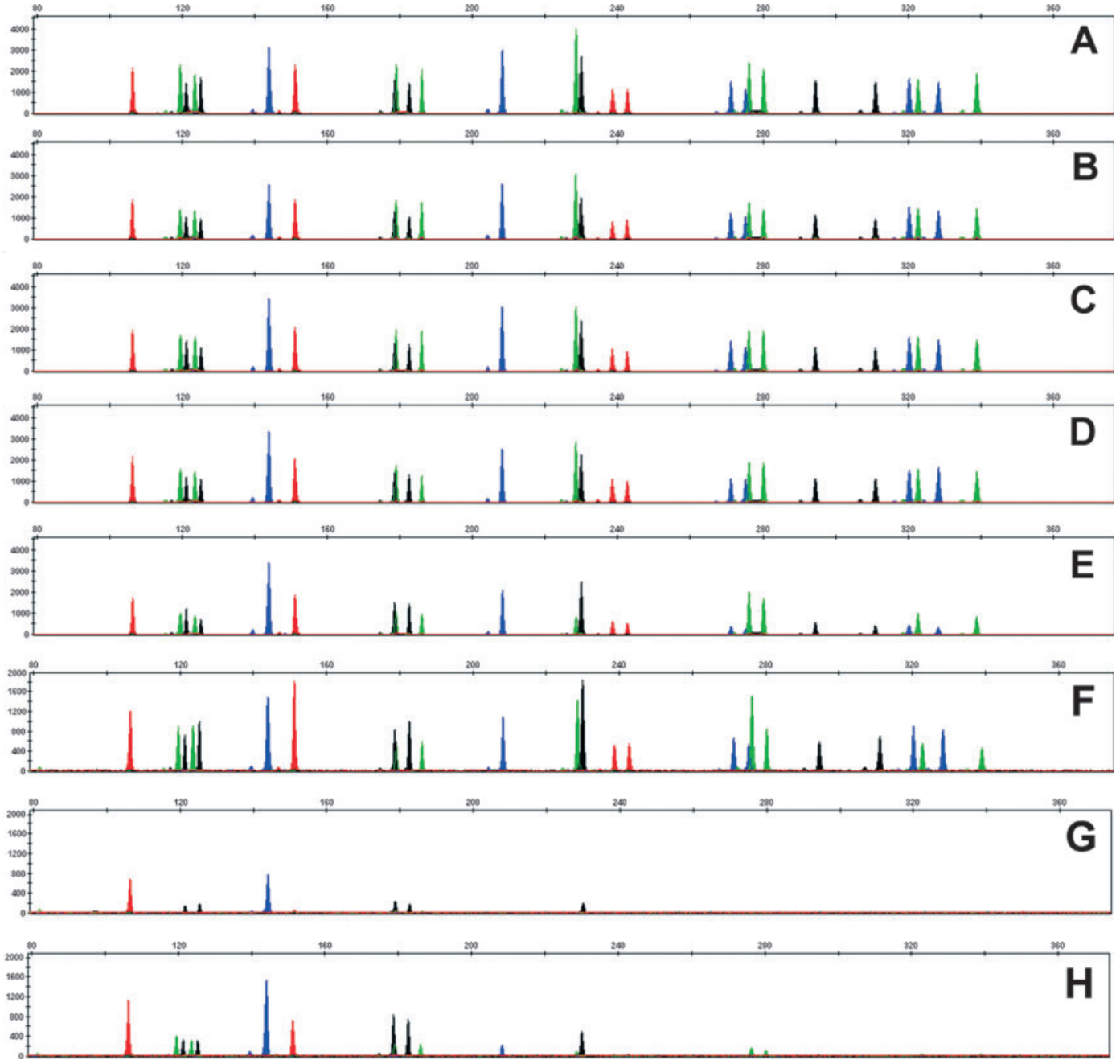

FIG. 8—*Representative electropherograms from the simulated inhibition study using the 28-cycle PCR protocol. Panels A, B, C, D, and E correspond to DNA amplified with the Identifiler® Plus Kit. Panels F, G, and H correspond to DNA amplified with the Identifiler® Kit. Each amplification contained 1 ng of Control DNA 9947A in the absence of inhibitor (A) and (F) or in the presence of (B) 200 μM hematin, (C) 300 μM hematin, (D) 100 ng/μL humic acid, (E) 150 ng/μL humic acid, (G) 30 μM hematin, and (H) 20 ng/μL humic acid. The Y-scales of the electropherograms for Identifiler® Plus and Identifiler® profiles were set at 5000 and 2000 RFU, respectively. RFU, relative fluorescence units.*

Degraded DNA Study

The common observations in forensic evidence samples are the fragmentation of full-length genomic DNA and the reduction of overall concentration of amplifiable DNA because of extended exposure to environmental insults. The effect of DNA degradation on the amplification efficiency of the Identifiler® Plus Kit was examined. To simulate DNA degradation, genomic Raji DNA was sonicated and incubated with varying amounts of DNase I for a period of 20 min. Complete STR profiles were obtained reproducibly when the amount of DNase I used was at 3 units or less (Fig. 9). When >3 units of DNase I were used, the number of allelic dropout events was directly proportional to the concentration of the DNase I. The trend was for the larger loci to drop out first (Fig. 9), consistent with the notion that larger genomic targets are more affected because of the nonspecific cleavage mechanism of the DNase I enzyme (30,31).

Mixture Study

Forensic casework samples that originate from more than one individual are often encountered, thus it is essential that typing

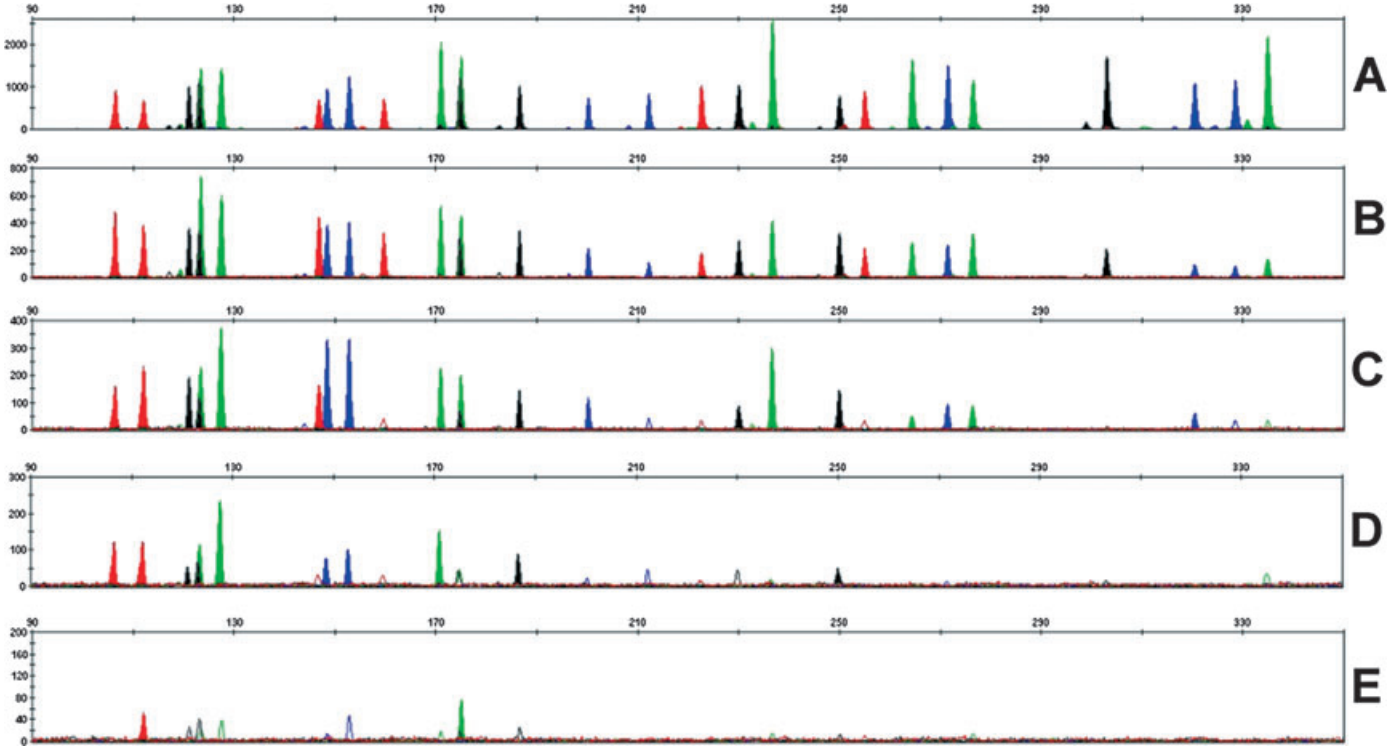

FIG. 9—*Representative electropherograms from the artificially degraded DNA study. The performance of the Identifiler® Plus Kit was assessed using artificially degraded DNA samples. The DNA was treated with increasing concentrations of DNase I: (A) untreated, (B) 3 units, (C) 4 units, (D) 5 units, and (E) 6 units. The Y-scale varies per panel and ranges from 200 RFU (panel E) to 2500 RFU (panel A). RFU, relative fluorescence units.*

systems can accurately detect mixtures. Figure 10 illustrates the genotyping results for several mixture ratios of two individuals using the 28-cycle PCR protocol. The two individuals were carefully selected so that most of the alleles at each locus do not overlap, and most of the alleles of the minor contributor do not reside at the stutter positions of the alleles from the major contributor. In Fig. 10, the alleles from the minor contributor (not overlapping with the major contributor) were highlighted as solid peaks to aid with the visualization. As the mixture ratios became higher, there was a decrease in the peak height of the minor contributor. However, even at the 15:1 ratio, where the minor component is *c.* 62.5 pg, all the alleles of the minor contributor were detected. These data demonstrate the lower limits of mixture detection observed in this study.

Population and Concordance Studies

Allele frequency distributions in major population groups and relevant statistics for the loci amplified by the Identifiler® Kit are fully documented in the AmpFℓSTR® Identifiler® Kit User's Manual (4). Because the primer sequences for the Identifiler® Plus Kit have not changed from those used in the Identifiler® Kit, it is expected that all the population data (such as allele frequency, heterozygosity, *p*-value and mutation rate for each of the 15 STR loci, the probability of identity, and probability of paternity exclusion) for the Identifiler® Kit remain the same for the Identifiler® Plus Kit. These population distribution data are documented in the AmpFℓSTR® Identifiler® Plus Kit User Guide (15).

Five hundred population samples were amplified using both the Identifiler® Kit (1 ng DNA input for 28 PCR cycles) and the Identifiler® Plus Kit (1 ng DNA input for 28 PCR cycles). CE of the population samples was performed on the Applied Biosystems 3130*xl* Genetic Analyzer. As expected, full genotype concordance was observed between the two chemistries. The population data amplified using the Identifiler® Plus Kit were also analyzed for intralocus peak height ratio at each of the 15 STR loci (Fig. 11 and Table 2) and for intracolor peak height balance in each dye channel (Fig. 12). The mean intralocus peak height ratios indicate that the two alleles of a heterozygous individual are generally very well-balanced (*c.* 90%). However, occasional low peak height ratios were observed as outlying data points as shown in the column labeled as "Minimum" in Table 2. The mean intracolor peak height balances are between 68.7% and 87.6%, indicating that the peak heights within a dye channel are also well-balanced.

The PCR products of a subset of the 500 population samples were also electrophoresed on the Applied Biosystems 3100 Genetic Analyzer ($N = 200$) and on the ABI PRISM® 310 Genetic Analyzers ($N = 42$). All three sets of CE data were analyzed using GeneMapper® *ID-X* Software v1.0.1, and full concordance was observed between the three CE platforms. Likewise, full concordance was observed with the CE data collected using the Applied Biosystems 3130*xl* Genetic Analyzer and analyzed using both the GeneMapper® *ID* Software v3.2.1 and GeneMapper® *ID-X* Software v1.0.1 (data not shown).

Discussion

To fully maximize the utility of DNA databases for identifying matches from the suspects to crime stains, the increased efficiency of database sample processing must also be accompanied by high performance chemistries for casework analysis. The more complete the profile generated from casework samples, the more informative the match to a database sample will be in terms of providing investigative leads. Forensic DNA casework laboratories have always encountered a percentage of challenging samples that are difficult

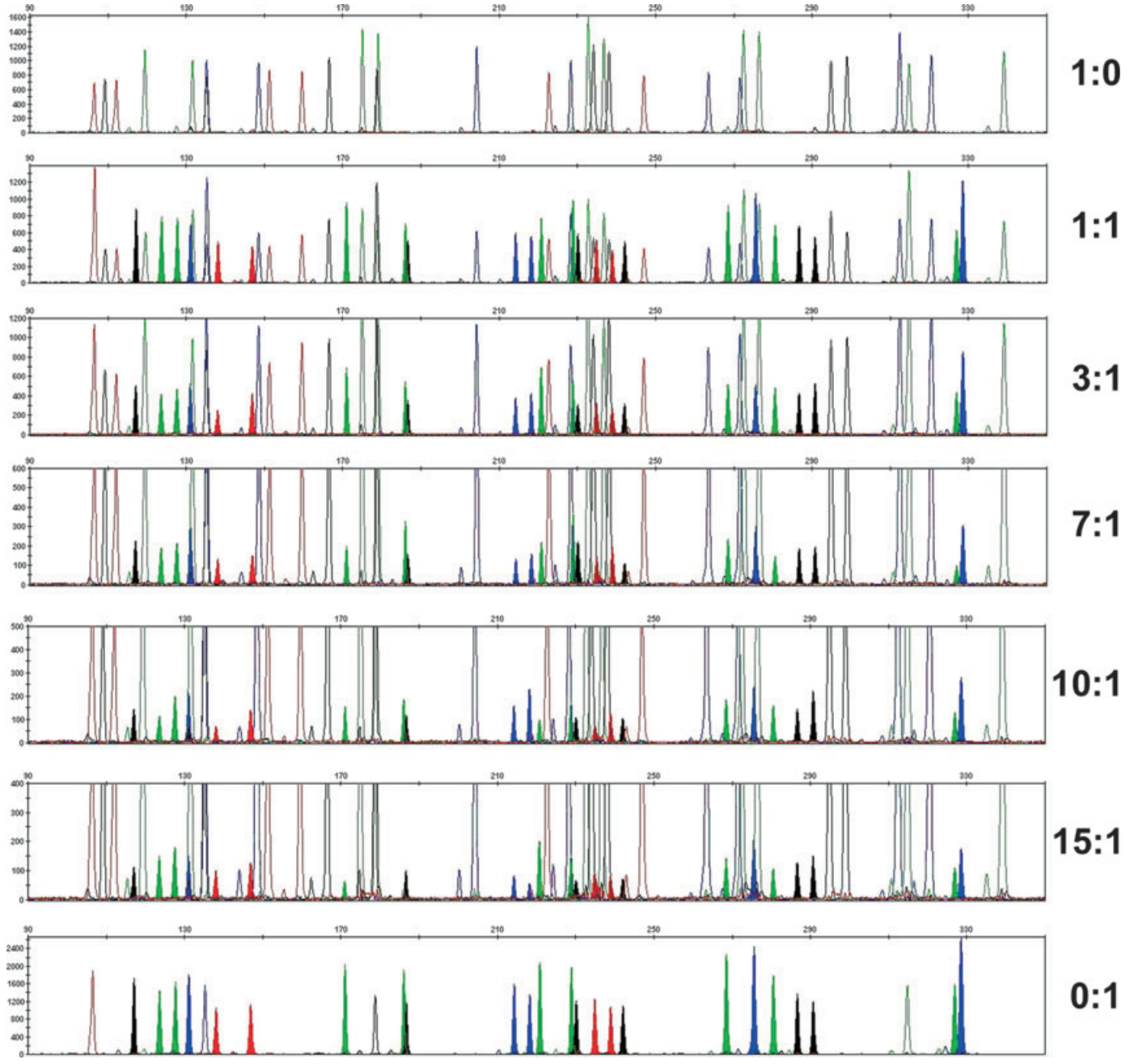

FIG. 10—*Representative electropherograms from the mixture study. The performance of the Identifiler® Plus Kit on mixture samples was evaluated using two individuals. The ratios examined were: 1:0 (major contributor profile only), 1:1, 3:1, 7:1, 10:1, 15:1, and 0:1 (minor contributor profile only). With the threshold of detection set at 50 RFU, the alleles from the minor contributor not overlapping with the major contributor are highlighted as solid peaks. Different RFU scales (Y-axis) are shown to aid the visualization of the alleles from the minor contributor. RFU, relative fluorescence units.*

to analyze successfully. However, as DNA analysis is sought to be used in an expanded set of crimes (such as property crimes and lesser felonies), it is likely that greater numbers of difficult samples afflicted by multiple challenges, such as low-level DNA, degradation, inhibition, and complex mixtures will be encountered. An improved version of the Identifiler® Kit was developed to specifically address these concerns.

The Identifiler® Plus Kit is an enhanced version of the Identifiler® Kit. Primer sequences have been maintained to ensure complete concordance with historical data sets, simplifying both the implementation and future use of the systems. Superior performance is achieved through a combination of improved master mix formulation, optimized PCR cycling condition, and flexibility of cycle number, providing options for both routine (0.5–1 ng of DNA input) and high sensitivity (<0.5 ng) applications. The standard thermal cycling conditions in the 9600 emulation mode consisted of enzyme activation at 95°C for 11 min, followed by 28 (0.5–1 ng of DNA input) or 29 cycles (<0.5 ng of DNA input) of denaturation at 94°C for 20 sec, and annealing/extension at 59°C for 3 min. A final extension step was performed at 60°C for 10 min, followed by a final hold at 4°C. The validation of the Identifiler® Plus Kit encompassed the verification of the best reaction conditions and reagent concentrations for the amplification of pristine as well as compromised DNA. The performance criteria assessed included overall peak heights, intralocus peak height ratio, intracolor peak height balance, and an

investigation of cross-reactive peaks in the presence of nonhuman DNA.

The enhanced sensitivity of the Identifiler® Plus Kit is evident from the peak height results of the sensitivity study. It confirmed the reliable generation of full STR profiles within the recommended input range for the Identifiler® Plus Kit (28 cycles for 0.5–1 ng and 29 cycles for 0.125–0.5 ng of DNA input). The sensitivity study results also demonstrated the ability to produce full profiles from samples with lower DNA input than the recommended amount (Fig. 5). A head-to-head comparison was made between the Identifiler® Kit using standard condition and the Identifiler® Plus Kit using the 28-cycle PCR protocol. The results showed an average increase in peak heights by 1.7-fold in the STR profiles generated using the Identifiler® Plus Kit under the various DNA input concentrations (Fig. 7).

The innovation in master mix formulation along with the optimized PCR cycling condition also extended the ability of the Identifiler® Plus Kit to overcome high levels of PCR inhibition. Using known forensic inhibitors to simulate PCR inhibition, the performance of the improved chemistry is unaffected even in the presence of 300 μM of hematin or 150 ng/μL of humic acid (Fig. 8). These results demonstrated an improvement of several orders of magnitude over the previously reported inhibitor tolerance of the Identifiler® Kit, where partial STR profiles were observed in the presence of 15 μM of hematin or 10 ng/μL of humic acid (Fig. 8).

Alongside the enhanced sensitivity and greater ability to overcome inhibition, the new chemistry also maintained the high-quality intralocus peak height ratios and intracolor peak height balances when compared to the Identifiler® Kit. Based on the results of the population study, the mean intralocus peak height ratios of the 15 STR loci ranged from 89.8 to 91.6% (Fig. 11 and Table 2), and the intracolor peak height balances of the four dye channels ranged from 68.7 to 87.6% (Fig. 12). These results showed improvement over the previously reported values for the Identifiler® Kit, where the intralocus peak height ratios ranged from 86.0 to 90.0% and the intracolor peak height balances were only >50% (4,5).

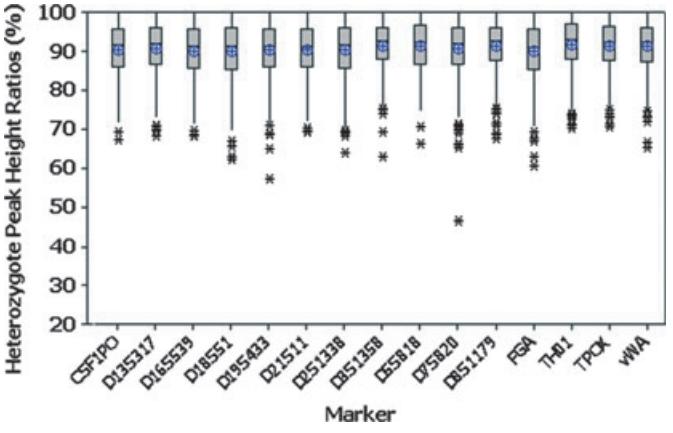

FIG. 11—*Heterozygote peak height ratios for 15 short tandem repeat loci from 500 correctly genotyped population samples (*n = *333–436, depending on locus). The X-axis indicates the locus and the Y-axis indicates the heterozygote peak height ratio observed at each individual marker. The grey boxes represent the 25th to the 75th percentile of the data, the whiskers show the range of the data, while the points outside the whisker are suspected outliers (data points that are different than the mean by more than twice the pooled standard deviation). Locus median and mean values are indicated by the horizontal line within the box and the blue cross symbol, respectively.*

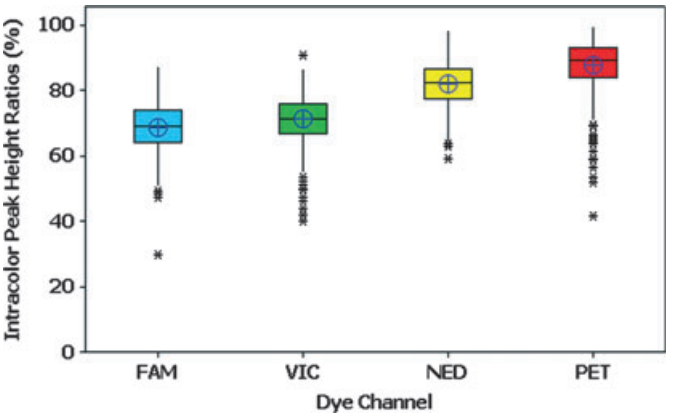

FIG. 12—*Intracolor peak height ratios for each dye channel from 500 correctly genotyped population samples (*n = *500). The X-axis indicates the dye channel and the Y-axis indicates the intracolor peak height balance observed at each dye channel. The colored boxes represent the 25th to the 75th percentile of the data, the whiskers show the range of the data, while the points outside the whisker are suspected outliers (data points that are different than the mean by more than twice the pooled standard deviation). The median and the mean values for each dye channel are indicated by the horizontal line within the box and blue cross symbol, respectively.*

TABLE 2—*Heterozygote peak height ratio calculations for 15 loci obtained from genotyped population samples (DNA input = 1 ng).*

Locus	Number of Observations (*n*)	Mean (%)	Median (%)	Minimum (%)	Maximum (%)
CSF1PO	378	90.4	91.6	67.2	100
D13S317	375	90.6	92.1	68.3	99.9
D16S539	389	90.0	91.1	68.1	99.9
D18S51	432	90.1	91.2	62.3	100
D19S433	399	90.2	91.2	57.3	100
D21S11	428	90.3	91.1	69.1	100
D2S1338	436	90.4	91.6	63.9	100
D3S1358	356	91.4	92.7	62.8	99.9
D5S818	357	91.1	92.2	66.1	100
D7S820	395	90.5	92.1	46.6	99.9
D8S1179	396	91.2	92.7	67.4	100
FGA	429	89.8	91.1	60.4	99.9
TH01	362	91.6	93.0	70.1	100
TPOX	333	91.2	92.2	70.6	100
vWA	414	91.2	92.3	65.2	100

Heterozygote peak height ratios were determined only on heterozygous samples with peak heights ≥50 relative fluorescence units.

Casework mixture samples often contain PCR inhibitors and the minor component may be present at very low level. The enhanced sensitivity, greater capability to overcome inhibition, and improved heterozygote and intracolor balance afforded by the Identifiler® Plus Kit will allow the analyst to analyze and interpret mixtures more easily and reliably than was previously possible. The mixture study (Fig. 10) demonstrated that the Identifiler® Plus Kit is capable of producing profiles for the minor component even at ratios of 15:1, where the minor contributor is present at a concentration of only *c.* 62.5 pg.

The studies described here are appropriate for a reagent manufacturer, but do not act as a substitute for a laboratory's internal validation. It is recommended that the laboratory conduct internal validation according to FBI/National Standards and SWGDAM guidelines (16,17).

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Additional information and reprint requests:
Dennis Y. Wang, Ph.D.
Staff Scientist
Life Technologies
850 Lincoln Centre Drive, M/S 404-1
Foster City, CA 94404
E-mail: dennis.wang@lifetech.com