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PAPER

CRIMINALISTICS

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Development and Validation of the AmpFℓSTR® Identifiler® Direct PCR Amplification Kit: A Multiplex Assay for the Direct Amplification of Single-Source Samples*,†

ABSTRACT: The AmpFℓSTR® Identifiler® Direct PCR Amplification Kit is a new short tandem repeat multiplex assay optimized to allow the direct amplification of single-source blood and buccal samples on FTA® card without the need for sample purification and quantification. This multiplex assay has been validated according to the FBI/National Standards and SWGDAM guidelines. Validation results revealed that slight variations in primer concentration, master mix component concentration, and thermal cycling parameters did not affect the performance of the chemistry. The assay's sensitivity was demonstrated by amplifying known amounts of white blood cells spotted onto FTA® cards, and the assay's specificity was verified by establishing minimal cross-reactivity with nonhuman DNA. No effect on the age of the sample stored on the FTA® substrate was observed and full concordance was established in the population study. These findings of the validation study support the use of the Identifiler® Direct Kit for forensic standards and database samples genotyping.

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

Short tandem repeat (STR) markers are the primary means used today for human identification and forensic DNA testing (1,2). STRs are highly polymorphic and capable of generating typing results from very little material through multiplex amplification using the polymerase chain reaction (PCR). Blood or buccal cells are the most common biological samples collected by database laboratories to extract DNA for STR analysis. However, crude biological samples such as blood and buccal cells contain many substances that can inhibit PCR (3,4). Furthermore, many database laboratories use FTA® cards (Whatman®, Inc., Piscataway, NJ) for archiving blood or buccal samples at room temperature to allow re-interrogation of the DNA profiles at any time. FTA® cards are treated with proprietary chemicals and nucleases that lyse cells, inactivate pathogens, and inhibit the growth of bacteria (5,6). As a result, an extensive wash and dry procedure is required to remove PCR inhibitors for biological samples deposited on FTA® cards prior to PCR amplification. The wash and dry procedure takes approximately 1–3 h or more depending on the protocol used (manual vs. automated) and the number of samples being processed. The wash and dry procedure is laborious, time-consuming, and each additional purification step introduces the potential risk of contamination between the samples.

The AmpFℓSTR® Identifiler® Direct PCR Amplification Kit (Applied Biosystems, Foster City, CA) is a new STR multiplex assay optimized to allow direct amplification of single-source blood and buccal samples on FTA® card without the need for sample purification. Unlike a previously reported attempt in direct amplification (7), where an FTA® elutent was used for PCR amplification, the Identifiler® Direct Kit directly amplifies STR loci directly from the unpurified FTA® disc. The Identifiler® Direct Kit utilizes an optimized PCR buffer formulation and an improved PCR cycling protocol designed specifically to overcome the PCR inhibitors present within the crude sample and contained within the FTA® card. The Identifiler® Direct Kit amplifies in a single reaction 15 autosomal loci (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, and FGA) and the sex-determining marker, amelogenin, and employs the same primer sequences used in the AmpFℓSTR® Identifiler® PCR Amplification Kit (8).

This article describes the developmental validation of the AmpFℓSTR® Identifiler® Direct PCR Amplification Kit. The experiments were performed according to the guidelines issued by the Director of the FBI (9) and the revised guidelines published by the Scientific Working Group on DNA Analysis Methods (SWGDAM) (10). The results confirm the reliability of the Identifiler® Direct Kit as required for forensic standards and database sample genotyping.

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Materials and Methods

DNA Samples

Anonymous whole-blood samples were purchased from Seracare Life Sciences (Oceanside, CA) or Interstate Blood Bank, Inc. (Memphis, TN), and the control DNA 9947A was purchased from Marligen Biosciences (Ijamsville, MD). The quantity of the control DNA 9947A was determined prior to amplification using the Quantifiler® Duo DNA Quantification kit (Applied Biosystems) on the ABI PRISM® 7500 Sequence Detection System (Applied Biosystems) according to manufacturer's recommended procedures. FTA® cards, Indicating FTA® cards, and EasiCollect™ devices were purchased from Whatman®, Inc. Blood on FTA® cards was prepared by spotting 75–80 μL of whole blood onto the center of the sampling spot. Buccal cells were collected using either EasiCollect™ devices or foam swabs, followed by contact transfer to the Indicating FTA® cards. Microbial pool DNA was created by combining several common microorganisms found in the oral cavity (11,12). Individual microbial DNA was purchased from ATCC (Manassas, VA).

PCR Amplification

Unless stated otherwise, the protocols in the Identifiler® Direct Kit User Guide were followed (13). The PCR was prepared in a volume of 25 μL containing 12.5 μL of AmpFℓSTR® Identifiler® Direct Master Mix and 12.5 μL of AmpFℓSTR® Identifiler® Direct Primer Set. Samples were amplified in a MicroAmp® Optical 96-well reaction plate (Applied Biosystems) in the GeneAmp® PCR system 9700 with a gold-plated silver or silver block (Applied Biosystems). The standard thermal cycling condition in the 9600 emulation mode consisted of enzyme activation at 95°C for 11 min, followed by 27 cycles of denaturation at 94°C for 20 sec, annealing at 59°C for 2 min, and extension at 72°C for 1 min. A final extension step was performed at 60°C for 25 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 ABI PRISM® 3100, Applied Biosystems 3130*xl*, or Applied Biosystems 3730 Genetic Analyzers using the specified G5 variable binning modules as described in the AmpFℓSTR® Identifiler® Direct Kit User Guide (13). 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 and then chilled on ice for 3 min. For Applied Biosystems 3130*xl* Genetic Analyzers, 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. For Applied Biosystems 3730 Genetic Analyzer, samples were injected at 2 kV for 10 sec and electrophoresed at 15 kV for 1200 sec in Performance Optimized Polymer-7 (POP-7™ polymer, Applied Biosystems) with a run temperature of 66°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). Allele peaks were interpreted when the peak heights were greater than or equal to 50 relative fluorescence units (RFU).

Primer Set Concentration

The Identifiler® Direct Kit employs the same primer sequences as used in the Identifiler® PCR Amplification Kit; however, the primer concentrations in the AmpFℓSTR® Identifiler® Direct Primer Set required a number of adjustments to address the formulation changes in the AmpFℓSTR® Identifiler® Direct Master Mix. For the evaluation of primer mix performance, five DNA samples (two blood samples on FTA® cards, two buccal samples on Indicating FTA® cards, and control DNA 9947A) were amplified in triplicate at the standard primer mix concentration and at 10% intervals up to ±30% levels (by volume).

PCR Master Mix Components

The PCR master mix components of the AmpFℓSTR® Identifiler® Direct Master Mix included AmpliTaq Gold® DNA polymerase, buffer, salts, dNTPs, carrier protein, detergents, and 0.05% sodium azide. The individual master mix components were tested at increments of ±10%, ±20%, and ±30% (by volume) from the standard formulation to test for reliability and robustness of the master mix configuration. Five DNA samples (two blood samples on FTA® cards, two buccal samples on Indicating FTA® cards, and control DNA 9947A) were tested 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 establish the optimal performance window of amplification for the Identifiler® Direct Kit. Cycling parameters around the standard set of conditions were tested. For each study, five samples (two blood samples on FTA® cards, two buccal samples on Indicating FTA® cards, and control DNA 9947A) were tested in triplicate for each thermal cycling parameter.

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

- Cycle number: 25, 26, **27**, 28, and 29 cycles.
- Denaturing temperature: 92.5, **94**, and 95.5°C.
- Annealing temperature: 55, 57, **59**, 61, and 63°C.
- Final extension time: 5, 15, **25**, 35, and 45 min.

Accuracy, Precision, and Stutter Studies

Two hundred DNA samples (whole blood spotted on FTA® card) from Seracare Life Sciences and Interstate Blood Bank, Inc. were used to measure the accuracy—the deviation of each sample allele size from the corresponding allelic ladder allele size. The blood on FTA® samples (1.2-mm FTA® discs) was amplified with the Identifiler® Direct Kit using the standard PCR condition. Allelic ladder sizing precision was measured by calculating the standard deviation in the size values obtained for an allele that is run in several injections on a capillary instrument. All data were electrophoresed on the Applied Biosystems 3130*xl* Genetic Analyzer.

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 amplified samples (370 blood samples on FTA® card and 299 buccal samples on Indicating FTA® card) at the loci found in the Identifiler® Direct Kit. All Identifiler Direct® Kit 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. All data were electrophoresed on the Applied Biosystems 3130*xl* Genetic Analyzer.

Species Specificity

The Identifiler® Kit primers were designed to be primate specific with no cross-reaction with other animal or microbial species (8,14). The primer sequences used in the Identifiler® Direct Kit are identical to those found in the Identifiler® 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 oral microorganisms (*c.* 10^5 copies each from *Candida albicans, Enterococcus faecalis, Escherichia coli, Fusobacterium nucleatum, Lactobacillus casei, Staphylococcus aureus, Streptococcus mitis, Streptococcus mutans, Streptococcus salivarius,* and *Streptococcus viridans*) were subjected to PCR amplification using the Identifiler® Direct Kit in replicates of three at 28 PCR cycles. Electrophoresis was performed on an 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. Owing to sample-to-sample and instrument-to-instrument variations, sensitivity studies aimed at producing interpretation guidelines are best performed by the individual testing laboratory. To demonstrate the performance of the Identifiler® Direct Kit on buccal samples deposited on Indicating FTA® cards, white blood cells (WBCs) from three individuals were purchased from AllCells, LLC (Emeryville, CA), resuspended in TE buffer, and quantitated using a hemacytometer. Following quantitation, a dilution series starting with 4×10^5 WBCs were spotted on the swab of the EasiCollect™ device, followed by contact transfer to saturate the sampling spot of the Indicating FTA® card. Upon drying, amplifications were performed in replicates of four using 1.2-mm discs punched from the prepared Indicating FTA® cards.

Stability Study

Stability studies were conducted to examine the sample-on-substrate stability. Aged blood on FTA® cards was prepared by spotting finger-prick blood onto FTA® cards for three individuals over the course of 30 weeks. Aged buccal cells on Indicating FTA® cards were prepared by cheek swab using the EasiCollect™ device and subsequently transferred onto the Indicating FTA® cards for three individuals over the course of 30 weeks. The collected samples were allowed to dry and then stored in a drawer at room temperature. At the end of the sample collection period, amplifications were performed in replicates of four using 1.2-mm discs punched from the aged FTA® and Indicating FTA® cards.

Population and Concordance Studies

A small population sample of 200 was employed in the concordance study. Pure genomic DNAs were purified and quantitated from 200 whole-blood samples, followed by amplification using the Identifiler® Kit with 1 ng of DNA input for 28 PCR cycles. The same 200 whole-blood samples were also spotted onto FTA® cards, and upon drying, amplification using the Identifiler® Direct Kit was performed using 1.2-mm discs punched from the FTA® cards. Electrophoresis was performed on both the Applied Biosystems 3130*xl* Genetic Analyzer and the Applied Biosystems 3730 Genetic Analyzer. 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 two STR assays, the two capillary electrophoresis (CE) platforms, and the two data analysis software packages. Heterozygote peak height ratios were determined only on the heterozygous samples with peak heights ≥ 50 RFU.

Statistical Analysis

Intralocus balance (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 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 reported as a percentage.

Results

Primer Set Concentration and PCR Master Mix Components

Full STR profiles were generated at all primer set concentrations and PCR master mix components tested without any spurious amplification or detrimental effect on intralocus balance. Between the standard PCR condition and the ±10% concentrations tested, no significant deviation was observed in peak heights and intracolor peak height balance (data not shown).

Thermal Cycling Parameters

Cycle Number—A representative Identifiler® Direct Kit profile generated using 4 ng of control DNA 9947A and 27 PCR cycles is shown in Fig. 1. The optimal thermal cycling parameters were determined to be in the middle of a window that balances differences in sample type (control DNA 9947A, blood on FTA® cards, and buccal sample on Indicating FTA® cards), specificity, and sensitivity. As expected, each increase in cycle number led to a corresponding increase in overall peak height of approximately twofold. Full STR profiles were obtained at all PCR cycle numbers tested (25–29 cycles). However, a few off-scale homozygote peaks were observed at 28 PCR cycles and several more off-scale homozygote peaks were observed at 29 PCR cycles. Excluding any off-scale data from the calculation, changes in PCR cycle number did not show any significant effect on the intralocus and intracolor peak height balances (Table 1).

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. Minimal effects were observed on intralocus balance for all three sample types. Minimal effects were also observed on intracolor peak height balance on control DNA 9947A and buccal samples on Indicating FTA® cards. With blood samples on FTA® cards, the intracolor peak height balances at 92.5 and 94°C were indistinguishable. However at 95.5°C, the peak heights of the large loci were lower than expected, leading to an intracolor peak height balance of <40%. For overall peak heights, denaturation at 94°C yielded the highest peak heights, followed by 92.5 and 95.5°C.

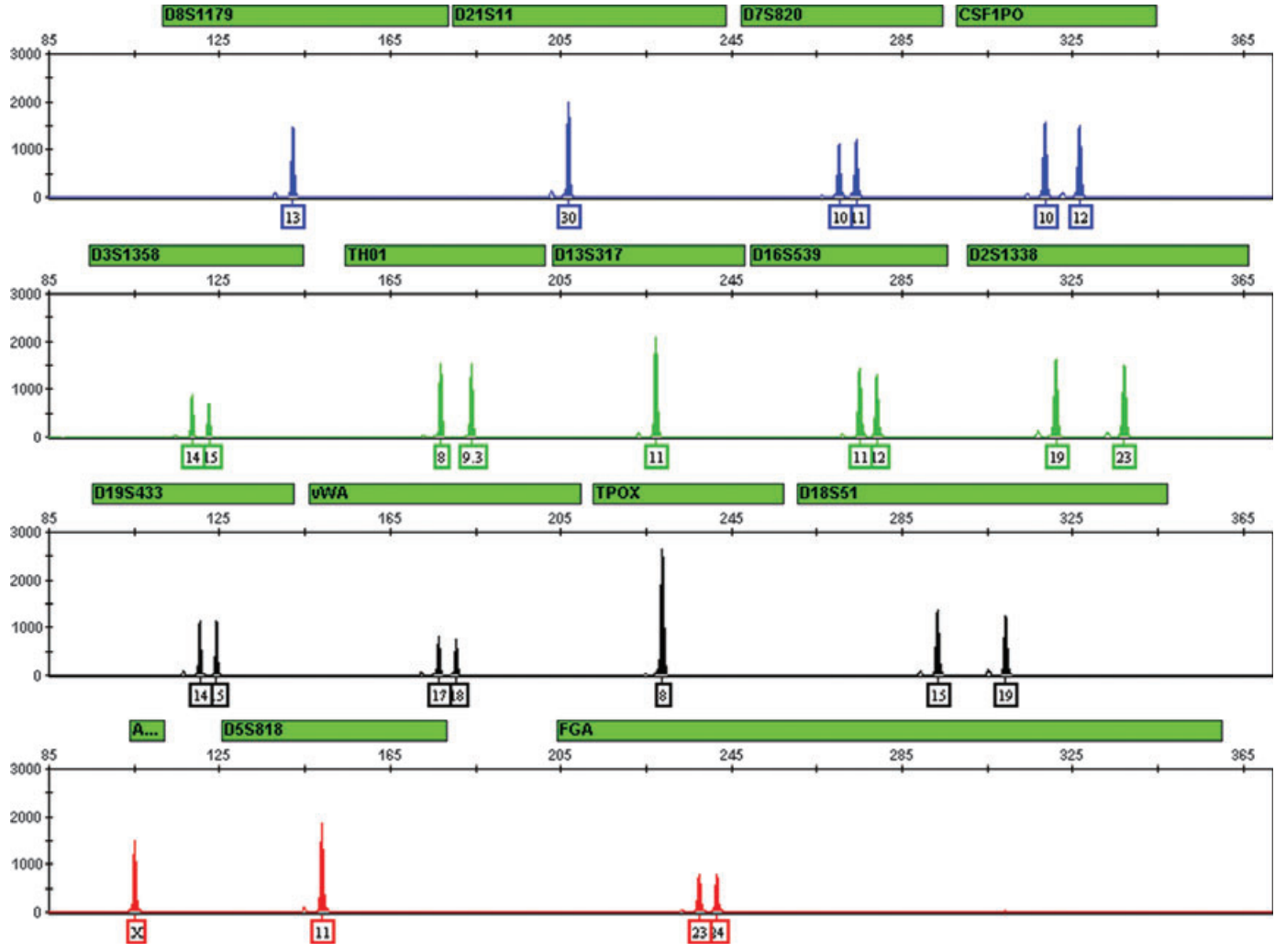

FIG. 1—*Representative electropherogram showing the profile of 4 ng of control DNA 9947A amplified with the Identifiler® Direct Kit for 27 PCR cycles. The four panels correspond to (from top to bottom) 6-FAM™, VIC®, NED™, and PET® dye-labeled peaks. The genotype is shown with the allele number displayed underneath each peak.*

TABLE 1—*PCR cycle number study: observed intralocus and intracolor peak height balances. The values are expressed as mean (%) ± SD.*

	Cycle No.				
	25	26	27	28	29
Intralocus balance	91.73 ± 5.82	91.80 ± 5.36	91.01 ± 5.75	90.37 ± 6.25	89.40 ± 6.72
Intracolor balance					
FAM™	60.69 ± 6.22	62.34 ± 8.98	58.91 ± 8.71	57.91 ± 8.44	56.81 ± 9.13
VIC®	60.71 ± 9.79	55.89 ± 7.37	55.20 ± 9.29	53.86 ± 7.14	52.84 ± 9.94
NED™	68.51 ± 9.08	71.11 ± 7.50	65.63 ± 7.90	68.21 ± 7.73	69.08 ± 9.82
PET®	66.46 ± 11.94	64.94 ± 9.99	60.46 ± 11.26	64.24 ± 13.37	68.49 ± 12.82

Annealing Temperature—The optimal annealing temperature was identified as the temperature that delivered a balance of specific amplification of 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 the smaller loci with occasional allele dropouts in D7S820, D3S1358, and D13S317 for all three sample types (Fig. 2). At the standard annealing temperature of 59°C, intracolor peak height balance >40% was observed reproducibly for all three sample types.

Final Extension Time—The Identifiler® Direct Kit maintains the same primer sequences found in the Identifiler® Kit, which was optimized for nonspecific terminal nucleotide addition by AmpliTaq Gold® DNA polymerase (15,16). In the presence of a more robust PCR master mix formulation together with an optimized

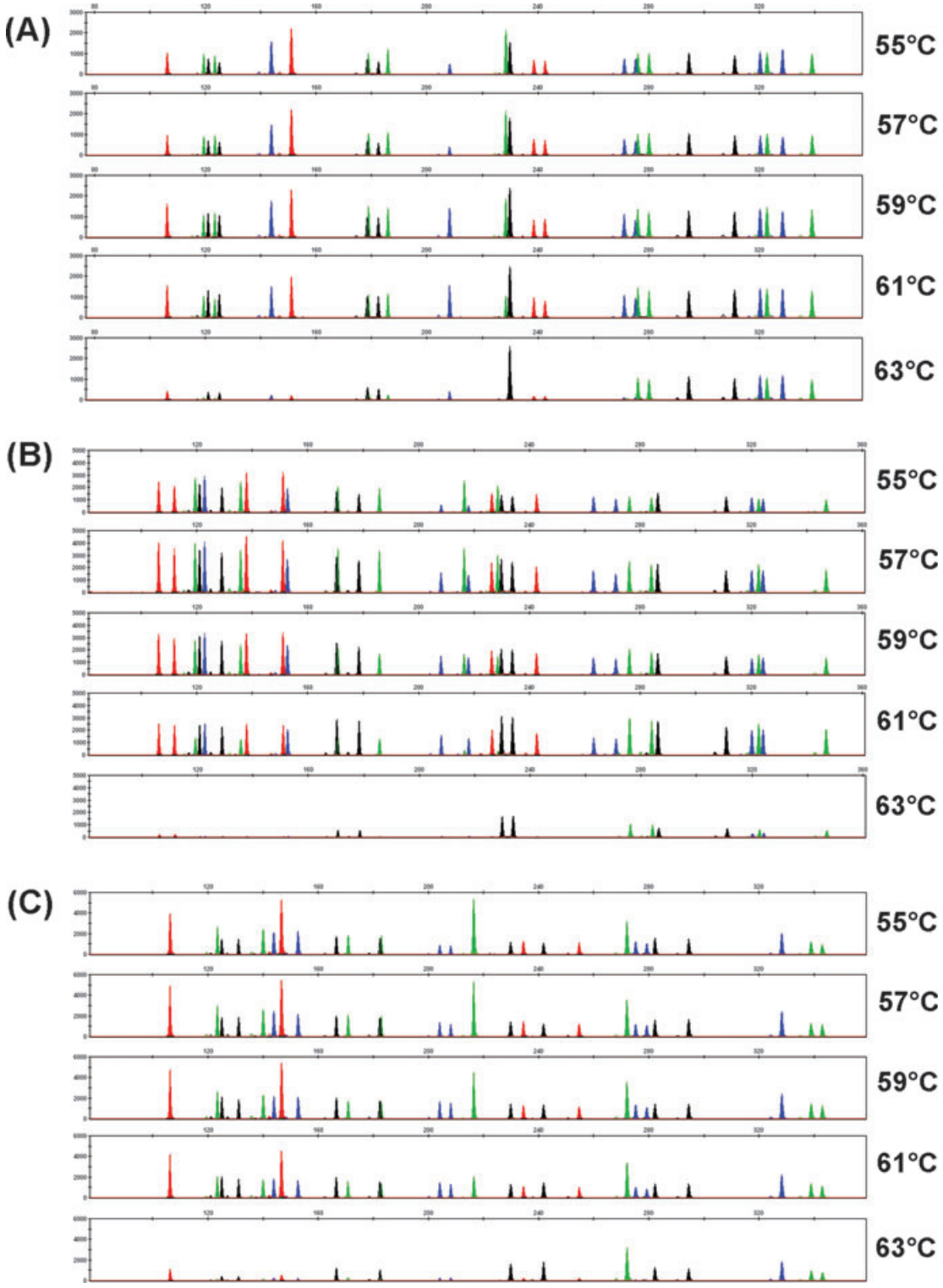

FIG. 2—*Representative electropherograms from the annealing temperature study of the (A) control DNA 9947A, (B) blood samples on FTA® card, and (C) buccal samples on Indicating FTA® cards. The samples were amplified with the Identifiler® Direct Kit at the indicated annealing temperatures. Peak heights were measures in relative fluorescent units (RFU).*

PCR cycle conditions, the final 60°C hold was reduced from 60 min (Identifiler® Kit) down to 25 min (Identifiler® Direct Kit) to ensure complete terminal nucleotide addition. With only 5 min of final extension, terminal nucleotide addition was incomplete at the D3S1358, D8S1179, D5S818, vWA, and TH01 loci (Fig. 3). At 15-min final extension, profiles generated did not exhibit incomplete terminal nucleotide addition for all three sample types. Final extension time longer than 25 min did not provide worthwhile benefits and was not detrimental to the assay. Changes in final extension time did not show any significant effect on intralocus and

intracolor peak height balances for all three sample types tested (Table 2).

Accuracy, Precision, and Stutter Studies

Determining sizing accuracy and precision includes the evaluation of measurement error and assessing performance for accurate and reliable genotyping. Two hundred blood samples on FTA® card were used to measure the deviation of each sample allele size from the corresponding allelic ladder allele size. All sample alleles tested were within ±0.5 bp of the corresponding allele in the allelic ladder (13). Allelic ladder sizing precision was calculated from multiple injections of allelic ladder. The standard deviation (SD) of the mean was calculated and shown to be 0.15 bp or less (data not shown).

Stutter products are the result of strand slippage during PCR amplification (17–19). The most common stutter product is one unit in length smaller than the true allele resulting in a product that could be, for example, four nucleotides smaller for any given tetranucleotide repeat marker in the Identifiler® Direct Kit. Percent stutter was calculated on a population sample composed of 370 blood samples on FTA® card and 299 buccal cells on Indicating FTA® card. Consistent with prior observations on stutters, all loci showed the trend of increasing stutter percentages with increasing allele size (8,20). The stutter average, range, and standard deviation (SD) are shown in Table 3. On average, the stutter product formation for the Identifiler® Direct Kit is slightly higher than that of the Identifiler® Kit (8). This could be attributed to the higher $MgCl_2$ concentration in the Identifiler® Direct Master mix, contributing to more slippage events by the polymerase (19,20).

Species Specificity

When tested with a range of animal and microbial DNA templates, amplification was observed only among primates with one notable exception. 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 as the degree of STR homology between chimpanzee and human has been previously documented (21). The only nonprimate amplification observed was for an approximately 103-bp-sized, PET® dye-labeled fragment in equine, porcine, and canine samples. With increased template concentration, amplification of this fragment was also observed with bovine and ovine samples. This PCR fragment has been attributed to a monomorphic product amplified from certain mammals by the amelogenin primers (22). The remainder of the tested species exhibited no amplification (mouse, cat, hamster, rat, chicken, and pooled oral microorganisms comprising of $\sim 10^5$ copies each from *Candida albicans, Enterococcus faecalis, Escherichia coli, Fusobacterium nucleatum, Lactobacillus casei, Staphylococcus aureus, Streptococcus mitis, Streptococcus mutans, Streptococcus salivarius,* and *Streptococcus viridans*). Representative electropherograms are shown in Fig. 4.

Sensitivity Study

Sensitivity studies were performed using three WBC samples that were quantitated, serially diluted, spotted onto EasiCollect™ swab, and contact-transferred onto Indicating FTA® cards. Assuming 100% transfer efficiency, each Indicating FTA® card contained from 4×10^5 WBCs (neat) down to 1.25×10^4 WBCs (1:32

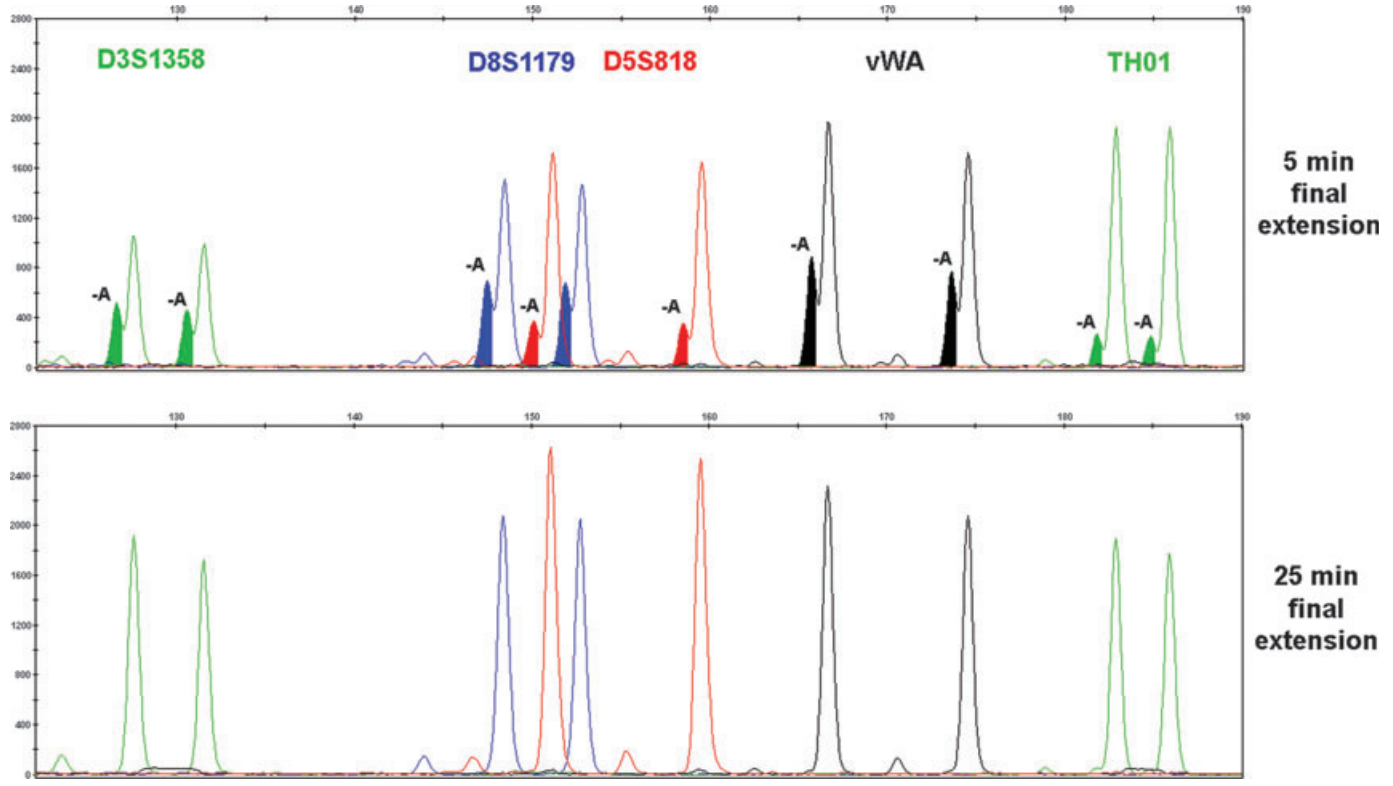

FIG. 3—*Effect of shortening final extension time. With only 5 min final extension, five loci showed incomplete terminal nucleotide addition. The remaining loci did not exhibit incomplete terminal nucleotide addition.*

TABLE 2—*PCR extension time study: observed intralocus and intracolor peak height balances. The values are expressed as mean (%) ± SD.*

	Extension Time				
	5	15	25	35	45
Intralocus balance	91.34 ± 6.09	90.78 ± 6.19	90.99 ± 6.14	91.20 ± 5.41	91.50 ± 5.19
Intracolor balance					
FAM™	57.63 ± 6.95	57.65 ± 7.88	53.74 ± 8.71	55.02 ± 7.60	54.76 ± 6.23
VIC®	52.54 ± 9.11	51.10 ± 8.81	52.81 ± 9.03	50.48 ± 9.97	56.05 ± 5.86
NED™	63.70 ± 8.56	66.92 ± 7.24	67.39 ± 5.23	69.59 ± 7.63	67.91 ± 7.31
PET®	59.62 ± 12.06	62.99 ± 11.27	61.21 ± 9.66	60.79 ± 11.50	61.20 ± 11.47

TABLE 3—*Stutter average, range, and standard deviation for the Identifiler® Direct Kit.*

Locus	Number of Observations (*n*)	Stutter Mean (%)	Stutter Range (%)	SD	Mean + 3 SD*
CSF1PO	756	4.87	1.80–12.07	1.20	8.48
D13S317	823	4.87	1.18–9.47	1.51	9.39
D16S539	914	5.05	2.01–11.38	1.46	9.42
D18S51	1034	7.37	3.06–15.27	1.84	12.89
D19S433	964	6.69	2.19–13.29	1.49	11.15
D21S11	988	6.54	3.22–11.33	1.29	10.42
D2S1338	1065	7.18	3.53–12.06	1.53	11.77
D3S1358	932	7.46	2.37–12.20	1.33	11.45
D5S818	858	5.77	1.55–9.88	1.37	9.89
D7S820	783	4.36	1.33–8.41	1.41	8.60
D8S1179	918	5.93	2.37–9.66	1.20	9.54
FGA	1019	6.71	2.87–14.15	1.64	11.62
TH01	424	2.16	0.67–11.11	0.87	4.76
TPOX	640	2.50	0.65–6.55	0.92	5.27
vWA	943	6.61	1.84–13.54	1.79	11.99

*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.

dilution). By calculating the surface area of the Indicating FTA® card sampling spot (radius = 9 mm) and the surface area of a single 1.2-mm FTA® disc (radius = 0.6 mm), it was determined that one FTA® sampling spot can yield approximately 225 1.2-mm discs. WBCs per 1.2-mm FTA® disc can be estimated for each WBC dilution series by dividing the total number of WBCs by 225. For each WBC dilution series, four replicates of amplification were performed for three different WBC samples using 1.2-mm Indicating FTA® discs and 27 PCR cycles. Full STR profiles were obtained consistently with 1:8 dilution and more than half of the samples in 1:16 dilution yielded full STR profile (Table 4). Based on this study, the sensitivity of the Identifiler® Direct Kit for WBCs spotted on Indicating FTA® card is between 100 and 200 cells (Fig. 5). However, the true sensitivity is likely to be even lower because it is unlikely that the transfer efficiency between the EasiCollect™ swab and the Indicating FTA® card was 100%.

Stability Study

The Identifiler® Direct Kit was developed to directly amplify samples (blood on FTA® cards or buccal cells on Indicating FTA® cards) that are too inhibitory for direct amplification using the existing STR chemistries. A stability study was performed to examine the sample-on-substrate stability as per SWGDAM guidelines (10). Blood on FTA® cards and buccal cells on Indicating FTA® cards stored at room temperature for 0, 42, 84, 126, 168, and 210 days were amplified using the Identifiler® Direct Kit. As illustrated in Fig. 6, the performance of the Identifiler® Direct Kit is not affected by the age of the FTA® samples. The calculated average peak height, intralocus balance, and intracolor peak height balance values were maintained, irrespective of the age of the sample.

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 (8). Because the primer sequences for the Identifiler® Direct 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, and the probability of identity and probability of paternity exclusion) for the Identifiler® Kit remain the same for the Identifiler® Direct Kit. These population distribution data are documented in the AmpFℓSTR® Identifiler® Direct Kit User Guide (13).

A population sample of 200 was amplified using both the Identifiler® Kit (1 ng DNA input for 28 PCR cycles) and the Identifiler® Direct Kit (1.2-mm blood on FTA® disc for 27 PCR cycles). As expected, full genotype concordance was observed between the two chemistries. The population data amplified using the Identifiler® Direct Kit were also analyzed for heterozygote peak height ratios at each of the 15 STR loci (Table 5) and for intracolor peak height balance in each dye channel (Fig. 7). The mean heterozygote peak height ratios indicate that the two alleles of a heterozygous individual are generally very well balanced (ranging from 87.98% to 95.22%). However, occasional low peak height ratios were observed as outlying data points as shown in the column labeled as "Minimum" in Table 5. The mean intracolor peak height balances indicate that the peak heights within a dye channel are also well balanced (between 55 and 80%).

Capillary electrophoresis of the 200 population samples amplified by the Identifiler® Direct Kit was performed on both the Applied Biosystems 3130*xl* and 3730 Genetic Analyzers. Both sets of CE data were analyzed using GeneMapper® *ID-X* Software v.1.0.1, and full concordance was observed between the two 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

Forensic DNA databases are being created or expanded in many countries worldwide. Today, the National DNA Index System (NDIS) in the United States contains more than 8 million offender DNA profiles (23). As law enforcement agencies and the general public recognize the power of DNA technology, the types of

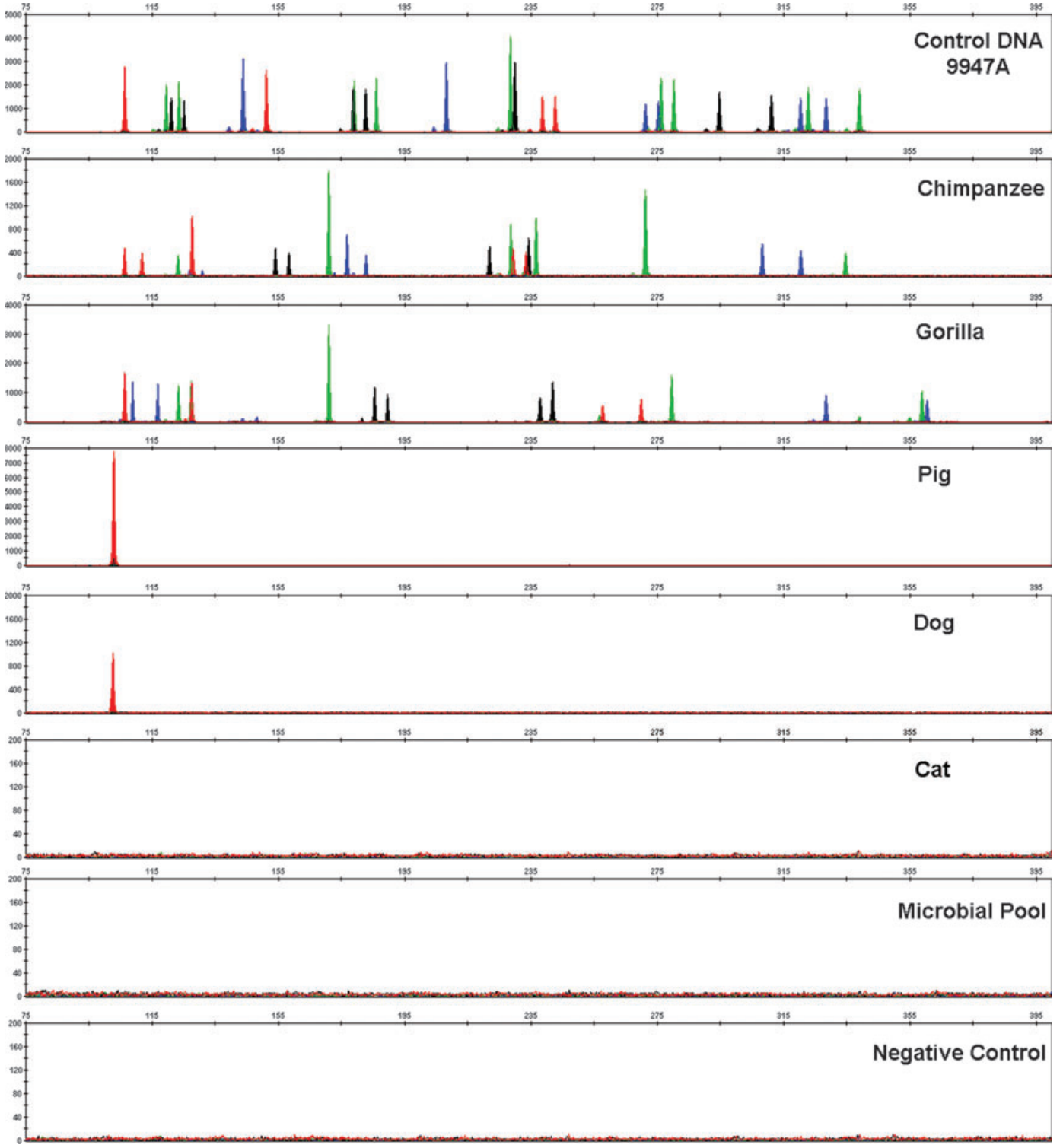

FIG. 4—*Representative electropherograms from a species specificity study including positive and negative controls. From top to bottom: 1 ng of control DNA 9947A (human), 1 ng of chimpanzee and gorilla DNA, 10 ng of pig, dog, and cat DNA, a microbial pool (*c. 10^5 *copies each of* Candida albicans, Enterococcus faecalis, Escherichia coli, Fusobacterium nucleatum, Lactobacillus casei, Staphylococcus aureus, Streptococcus mitis, Streptococcus mutans, Streptococcus salivarius, *and* Streptococcus viridans*) and a negative control. Different RFU scales (*Y*-axis) are shown to demonstrate specificity. Except for the monomorphic, amelogenin-like product seen in panels 4 and 5, amplification was only observed for primates among all tested species.*

offenders in the database are no longer limited to violent criminals. Many states now have legislation(s) that mandates the collection of DNA from individuals who have been convicted of numerous non-violent felonies, such as drug possession, burglary, and robbery. Further expansion is expected in the next few years, as more states and countries continue to pass legislation that allows for the collection of DNA from felony arrestees and for an expanded list of offenses. To keep up with the increase in the number of DNA

database samples requiring analysis, many laboratories have implemented automation into their workflow. Significant strides have been made, but critical process bottlenecks remain, such as DNA extraction and data analysis. The AmpFℓSTR® Identifiler® Direct PCR Amplification Kit has been developed specifically to help address the DNA extraction and purification bottleneck.

TABLE 4—*Amplification efficiency of serially diluted WBC samples on Indicating FTA® cards.*

Dilution Series	WBC Sample No 1 (n = 4), %	WBC Sample No 2 (n = 4), %	WBC Sample No 3 (n = 4), %
Neat	100	100	100
1:1 dilution	100	100	100
1:4 dilution	100	100	100
1:8 dilution	100	100	100
1:16 dilution	100	73 ± 4.5	100
1:32 dilution	47 ± 15	6 ± 3	92 ± 9

The Identifiler® Direct Kit amplifies the 16 loci included in the Identifiler® Kit from single-source samples spotted on FTA® cards, without the need to perform any DNA extraction or purification. Blood or buccal samples on FTA® cards can be punched into PCR plates or tubes, and taken directly to PCR amplification, enabling users to obtain results quickly, without any loss of data quality. By eliminating the tedious steps involved in DNA extraction and purification, automation of the process becomes much easier and requires a less sophisticated and less expensive robot. As the time to result is greatly reduced, so is the potential for sample contamination or other procedural errors that can occur during the process.

During the development of the Identifiler® Direct Kit, several critical factors were identified that can affect the performance of

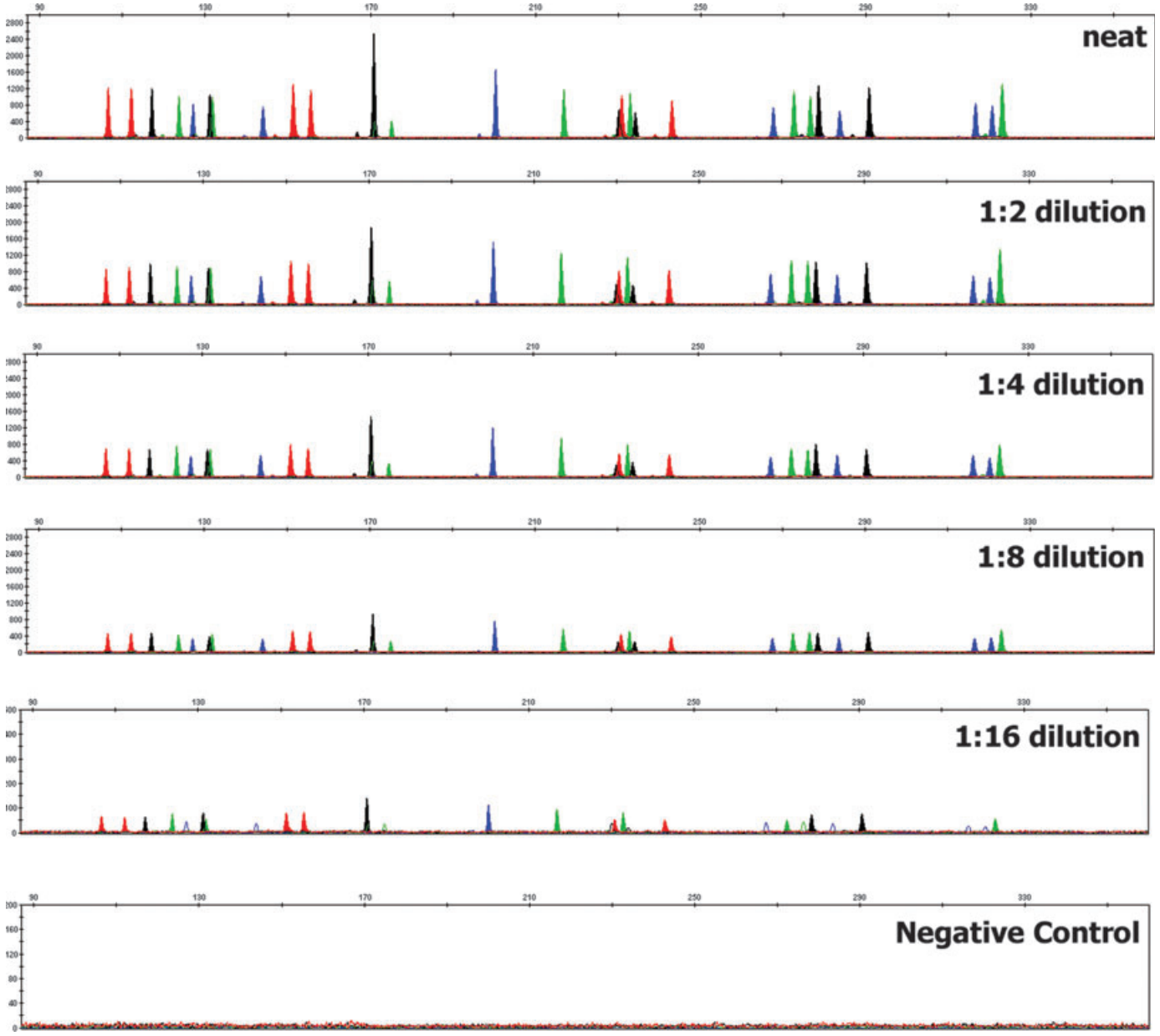

FIG. 5—*Effect of varying inputs of WBCs on Indicating FTA® cards on peak heights. The results depicted are representative of the 27-cycle amplification on one of the three WBC samples at the indicated input amounts. The data were analyzed with a peak amplitude threshold of 50 RFU.*

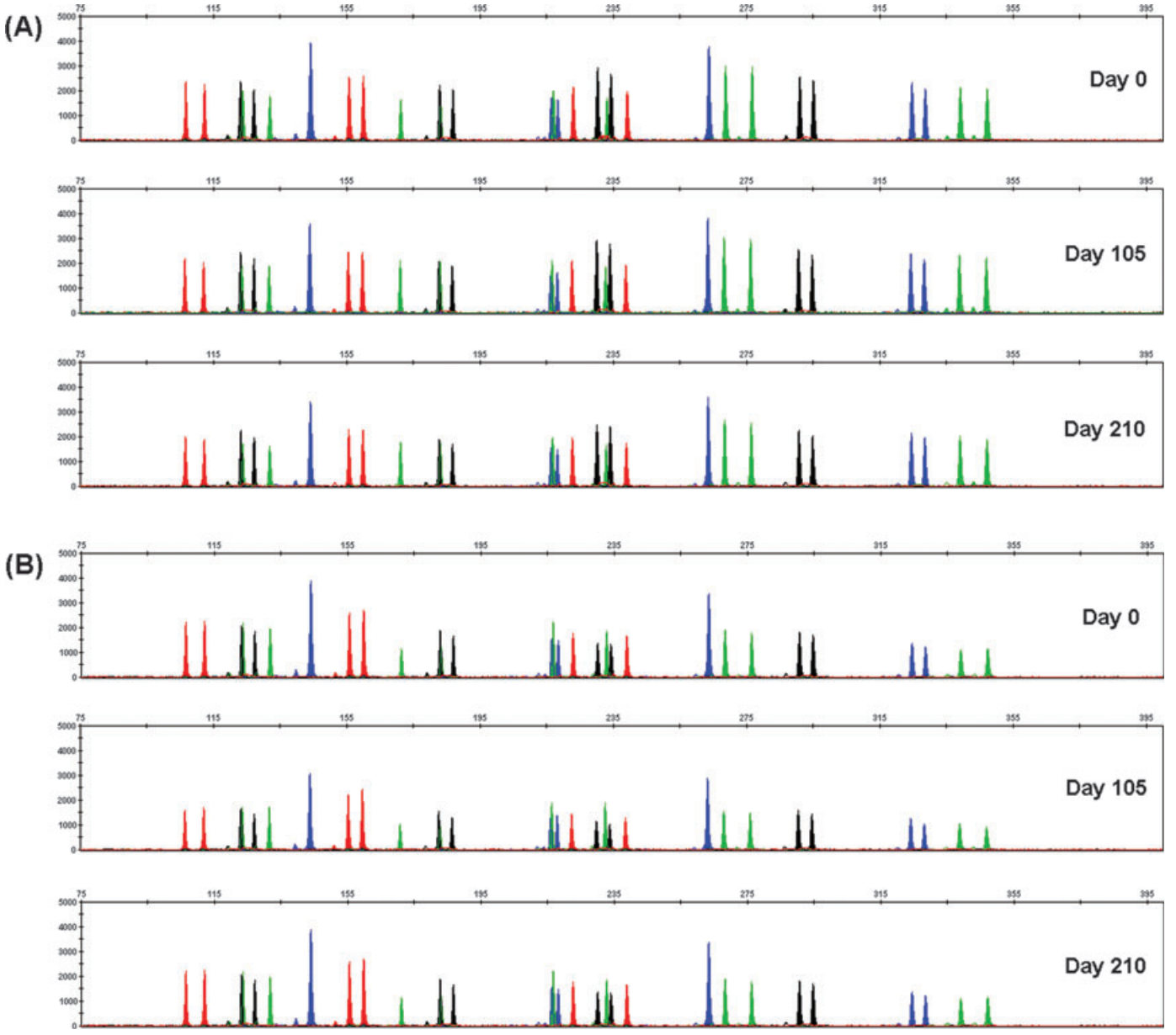

FIG. 6—*Representative electropherograms from the stability study. The performance of the Identifiler® Direct Kit did not show any variation when amplifying fresh and aged blood samples on FTA® cards (A) or fresh and aged buccal samples on Indicating FTA® cards (B).*

the chemistry. These factors include the size of the FTA® disc and the sampling location of the FTA® card. Internal and external testing revealed that the most consistent results were generated using 1.2-mm FTA discs with the optimal punch location at the center of the blood or buccal stain (24). Furthermore, external testing also highlighted the importance of internal validation studies performed by individual laboratories to identify optimum conditions for amplification (e.g., PCR cycle number), based on their own sample type(s) and capillary electrophoresis instruments.

The validation of the Identifiler® Direct Kit reported here encompassed the verification of the best reaction conditions and reagent concentrations for the direct amplification of blood or buccal cells on FTA® cards as well as pristine DNA for use as a positive control. The performance criteria assessed included overall peak heights, intralocus balance, intracolor peak height balance, and lack of cross-reactive peaks in the presence of nonhuman DNA. The experiments demonstrated that the Identifiler® Direct Kit is a robust, reliable assay, capable of handling samples of varied quantity and quality. Furthermore, the assay can tolerate moderate changes to the recommended protocol without compromising the quality of the results.

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TABLE 5—*Heterozygote peak height ratio for 15 loci obtained from genotyped blood on FTA® population samples.*

Locus	Number of Observations (*n*)	Mean (%)	Median (%)	Minimum (%)	Maximum (%)
CSF1PO	148	91.97	92.04	83.50	99.86
D13S317	159	91.30	91.42	77.16	99.74
D16S539	149	91.06	90.97	78.97	99.78
D18S51	167	89.84	90.30	64.90	99.96
D19S433	151	91.95	91.59	81.22	99.88
D21S11	171	92.42	92.39	83.56	99.88
D2S1338	176	88.49	89.11	66.59	99.92
D3S1358	138	91.85	91.79	76.68	99.33
D5S818	150	90.56	91.86	73.61	99.90
D7S820	161	92.80	92.75	82.28	99.86
D8S1179	155	87.98	89.31	72.33	99.32
FGA	167	90.98	91.41	68.71	99.44
TH01	143	95.22	95.21	88.62	99.94
TPOX	136	93.64	94.21	80.56	99.81
vWA	169	91.15	90.91	66.00	99.32

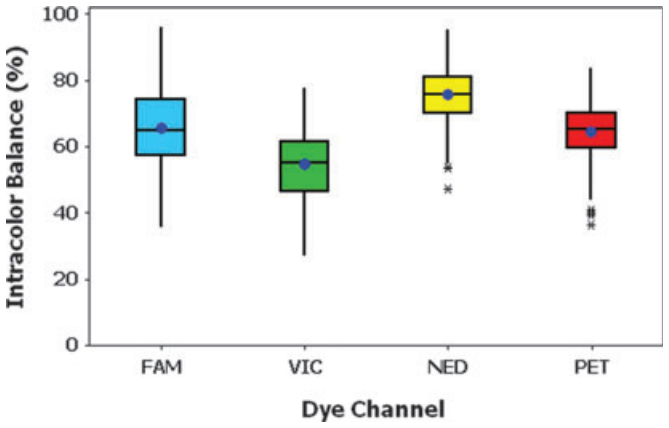

FIG. 7—*Intracolor peak height ratios for each dye channel from 200 correctly genotyped population samples (*n = *200). The* X*-axis indicates the dye channel and the* Y*-axis indicates the intracolor peak height balance observed at dye channel. The colored boxes represent the 25th to the 75th percentile of the data, the whiskers show the range of the data, while the points outside the whisker are suspected outliers. The mean values for each dye channel are indicated by the blue circle.*

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