TECHNICAL NOTE

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TWGDAM Validation of the AmpFℓSTR Profiler Plus and AmpFℓSTR COfiler STR Multiplex Systems Using Capillary Electrophoresis*

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ABSTRACT: Prior to forensic implementation, a profiling system requires validation following the recommendations presented by the Technical Working Group on DNA Analysis Methods (TWGDAM). In this work two such systems, AmpFℓSTR Profiler Plus and AmpFℓSTR COfiler have been validated according to the guidelines provided by TWGDAM. Profiler Plus and COfiler simultaneously amplify nine and six STR loci respectively; both also amplify a portion of the amelogenin gene. Performance of the two STR multiplex systems under conditions set forth by TWGDAM was robust and reproducible, indicating that these systems are suitable for use in forensic analysis. Additionally, specific sections of the TWGDAM validation guidelines are especially valuable in terms of familiarizing users with particular limitations of the systems prior to taking on casework.

KEYWORDS: forensic science, DNA, polymerase chain reaction, AmpFℓSTR Profiler Plus, AmpFℓSTR COfiler, validation, capillary electrophoresis

DNA profiling using short tandem repeat (STR) loci has become a useful tool for human identification. STR loci provide a rich source of polymorphic markers resulting from variation in the number of copies of the repeated motif. Reliable amplification requires little template DNA and the products can be analyzed using several instrumental platforms and well-characterized allelic ladders (1–3). Statistical analysis for these loci is simplified by the use of precise allelic designations (4–6).

The AmpFℓSTR Profiler Plus PCR Amplification kit (PE Biosystems, Foster City, CA) supports simultaneous amplification of nine tetrameric repeat loci plus a homologous region of the amelogenin gene on the X and Y chromosomes (7). The STR fragments amplified by the AmpFℓSTR Profiler Plus PCR amplification kit range in length from approximately 111 to 341 bases (8). The AmpFℓSTR COfiler multiplex (PE Biosystems, Foster City, CA) amplifies six STRs along with the portion of the X-Y homologous amelogenin gene. The alleles vary in length from approximately 111 to 317 bases (9).

The relatively small size ranges of the target loci have allowed development of multiplex sets of STR loci; these systems offer an accurate and rapid method of analysis for STR loci (10–13). Multiplex sets coupled with a multicolor fluorescence detection system provide a high throughput method of DNA identification with significant power of discrimination suitable for forensic analysis.

Prior to forensic implementation, a profiling system requires validation following the recommendations presented by the Technical Working Group on DNA Analysis Methods (TWGDAM) (14–16). In this work we present our validation experiences with the two amplification kits and observations that we hope will be useful to other users.

Materials and Methods

Biological material collected mostly on paper, fabric, or swabs was allowed to dry before storage. The samples were maintained prior to extraction at −20°C or −80°C. The DNA was extracted by organic methods; proteinase *K* (Life Technologies, Inc., Gaithersburg, MD) digestions of the samples were extracted with phenol/chloroform/isoamyl alcohol (Life Technologies, Inc., Gaithersburg, MD) followed by a Microcon-100 (Amicon, Inc., Beverly, MA) cleanup of the aqueous portion (17). Mini-gel electrophoresis with the DNA dye DAPI (Sigma-Aldrich, St. Louis, MO) was used to quantify the resulting DNA by comparison with K562 DNA standards (Life Technologies, Inc., Gaithersburg, MD) (18). Unless indicated to the contrary, the DNA extracts were diluted to approximately 0.1 to 0.2 ng/μL with Tris-EDTA buffer.

AmpFℓSTR Profiler Plus PCR kits were used to amplify a portion of the X-Y homologous amelogenin gene and the following tetrameric STR loci: D3S1358, vWA, FGA, D8S1179, D21S11, D18S51, D5S818, D13S317, and D7S820. The nine STR loci amplified by this kit are on separate chromosomes as are those amplified by the AmpFℓSTR COfiler kit. The amelogenin gene portion and the following tetrameric STR loci: D3S1358, D16S539, THO1, TPOX, CSF1PO, and D7S820 were amplified using COfiler PCR kits. Overlapping loci are distinguished from

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one another by utilizing primers tagged with one of three fluorescent dyes.

Amplifications were conducted according to manufacturer recommendation (8,9) with one minor exception; total amplification volume was halved to 25 μL. Unless noted otherwise, the target amount of DNA amplified was 1 to 2 ng. The thermal cycler used was a PE Biosystems Applied Biosystems model 9600 (PE Biosystems, Foster City, CA) set to the parameters indicated by the kit instructions.

Samples were prepared for capillary electrophoresis by combining 1 μL of amplified product with 12.0 μL of deionized formamide (Amresco, Solon, OH) and 0.5 μL of GS ROX 500 internal lane standard (PE Biosystems, Foster City, CA). Prepared samples were denatured at 95°C for 3 min and snap-cooled in an ice-bath for 3 min. Samples were run on an ABI PRISM Genetic Analyzer 310 capillary electrophoresis (CE) instrument equipped with a 47-cm, 50-μm i.d. capillary, 1X concentration Genetic Analyzer Buffer with EDTA, and the proprietary separation medium POP-4™ polymer (all from PE Biosystems, Foster City, CA). Samples were analyzed with Collection 1.0.4, Genescan 3.1, and Genotyper 2.0 software programs (PE Biosystems, Foster City, CA) (19).

Sample peak heights were assessed for the minimum sample (TWGDAM 4.1.5.10) and the population sample (TWGDAM 4.1.5.3) studies detailed below. Population study samples were used to calculate stutter (20) and allele balance for developing interpretation guidelines and so had to comply with Vermont Forensic Laboratory (VFL) guidelines; peak heights had to be within 150 to 5500 relative fluorescent units (rfu) for STRs and 75 to 5500 rfus for amplified portions of the amelogenin gene.

Results and Discussion

The following studies were performed according to guidelines defined by TWGDAM (14) to validate the Profiler Plus and COfiler multiplex systems. Unless otherwise stated, results described were obtained with both multiplex systems.

Standard Specimens

Organically extracted DNA from the blood, saliva, hair, and skin of a single donor was amplified using both the Profiler Plus and COfiler kits. Identical genotypes were obtained from the amplified products derived from these same-source biological materials (data not shown).

Consistency

To test the consistency of results derived with the Profiler Plus and COfiler multiplex kits, six sample sets were used. The processing of each sample set is detailed separately in the following paragraphs.

Ten duplicate bloodstains patched out on both S/S (Scheicher & Schuell, Keene, NH) and FTA™ (Flinders/Fitzco, Maple Plain, MN) papers were received as part of a TWGDAM study. Two extraction methods were used. Organic extraction methods were used to free the DNA from the S/S paper prior to Profiler Plus/COfiler amplification. A 1-mm Harris punch was used to remove circular cuttings from the bloodstained FTA™ papers. Following preparation of the cuttings according to the manufacturer's instructions, DNA was amplified directly from the processed FTA™ paper cuttings using the two kits. Results between the FTA and S/S paper sample groups were in agreement with one another and with the genotype information provided as part of the study (data not shown).

A total of seven DNA extracts from two sets of samples received from National Institute of Standards and Technology (NIST) (Gaithersburg, MD) were amplified using Profiler Plus and COfiler PCR kits. Blood stains received were extracted both organically and with Chelex (Bio-Rad Laboratories, Hercules, CA). The samples were meant to resemble casework and included mixed stains from two or more individuals requiring differential extraction. The resulting profiles matched the NIST results for both sample sets (data not shown).

Portions of 20 bloodstain cards used in deriving population data were sent to the Broward County Sheriff's Office in Fort Lauderdale, FL. There, DNA from the bloodstains was extracted, amplified, and analyzed. Genotypes obtained by that laboratory matched the results from the Vermont Forensic Laboratory (data not shown).

Eight DNA proficiency test blood samples received from Collaborative Testing Services, Inc. (Herndon, VA) were typed using the COfiler and Profiler Plus kits. Identical results to those received from the sample manufacturer were obtained (data not shown).

Amplifications of samples from five different starting concentrations of DNA were run through the capillary electrophoresis analyzer multiple times. Typing results were reproducible except in samples with low input DNA concentrations. These samples showed peak imbalance and allele dropout, especially in the higher molecular weight alleles. Details of this study and results are found under the heading "Minimum Sample."

A Profiler Plus and a COfiler allelic ladder were injected ten times each to evaluate consistency. The capillary on which the samples were run had over 250 injections. Average sizing (in bases) for each allele was calculated, as were the standard deviations for each. The largest standard deviation for the Profiler Plus alleles was 0.15 demonstrated by the D18S51 21 repeat. The COfiler allele exhibiting the largest standard deviation was CSF1PO 15 with 0.11 (Tables 1*A,* 1*B*).

Population Studies

Genotypes were determined for 107 Caucasians (presumed to be unrelated). The alleles common to the Profiler Plus and the COfiler multiplex systems were found to match. Stutter peak averages and heterozygous allele balances were calculated for both systems. Stutter was calculated by dividing the height of the peak in the stutter position (four bases shorter than the true allele) by the height of the true allele peak. The highest stutter peaks observed were higher than the maximum reported by the manufacturer for all Profiler Plus loci except D87S820 and for all COfiler loci except D16S539 (Table 2).

Heterozygote allele balances were calculated for each locus by dividing the peak height of the second peak of a heterozygous pair by the height of its first peak and expressing this value as a percent. The average heterozygous balance was at least 90% for all loci, and ranged from a low of 58% to a high of 144%. The highest standard deviation for heterozygote balance demonstrated by any locus in the two multiplex systems was that of 0.12 for Profiler Plus' D5S815 (Table 3).

Preliminary review of allele frequency data compares favorably with published values.

TABLE 1*A*—*Ladder study.*

	Profiler Plus				
	Alleles	Average	Std. Dev.	Minimum	Maximum
D3S1358	12	111.40	0.07	111.32	111.50
	15	123.41	0.05	123.31	123.48
	19	139.99	0.05	139.95	140.07
vWA	11	154.65	0.06	154.61	154.80
	16	175.34	0.08	175.24	175.49
	21	195.02	0.07	194.92	195.15
FGA	18	216.41	0.06	216.34	216.51
	25	244.66	0.07	244.57	244.80
	30	265.05	0.09	264.94	265.26
D13S317	8	205.30	0.08	205.19	205.40
	11	217.31	0.05	217.24	217.39
	15	233.20	0.07	233.09	233.33
Amelogenin	X	103.60	0.07	103.50	103.70
	Y	109.27	0.08	109.17	109.41
D8S1179	8	123.81	0.06	123.73	123.89
	14	149.41	0.04	149.39	149.52
	19	170.67	0.08	170.59	170.83
D21S11	24.2	187.33	0.05	187.26	187.41
	31	212.70	0.04	212.65	212.79
	38	240.42	0.05	240.37	240.51
D5S818	7	131.41	0.05	131.34	131.47
	11	148.57	0.04	148.54	148.69
	16	169.81	0.07	169.72	169.96
D7S820	6	256.18	0.07	256.09	256.29
	10	272.35	0.06	272.27	272.46
	15	292.60	0.10	292.52	292.83
D18S51	9	270.58	0.07	270.50	270.71
	21	321.62	0.15	321.45	321.87
	26	342.14	0.11	342.01	342.40

The average, standard and deviation, minimum, and maximum size in bases of duplicate injections ($N = 10$) of the ladders for Profiler Plus were calculated. Three alleles for each locus (first, middle, and last) are presented.

TABLE 1*B*—*Ladder study.*

	COfiler				
	Alleles	Average	Std. Dev.	Minimum	Maximum
D3S1358	12	111.45	0.05	111.39	111.53
	15	123.46	0.03	123.38	123.51
	19	140.02	0.05	139.94	140.07
D16S539	5	229.37	0.05	229.27	229.43
	11	253.40	0.06	253.30	253.48
	15	269.63	0.07	269.50	269.73
Amelogenin	X	103.66	0.04	103.59	103.72
	Y	109.32	0.04	109.25	109.37
THO1	5	167.13	0.07	167.04	167.24
	8	179.23	0.05	179.17	179.31
	10	187.14	0.05	187.06	187.19
TPOX	6	215.16	0.06	215.07	215.25
	10	230.97	0.05	230.88	231.08
	13	242.92	0.06	242.82	243.00
D7S820	6	256.27	0.05	256.18	256.36
	11	276.53	0.07	276.41	276.63
	15	292.73	0.06	292.66	292.82
CSF1PO	6	279.73	0.06	279.60	279.82
	10	296.03	0.05	295.96	296.16
	15	317.79	0.11	317.58	317.92

The average, standard deviation, minimum, and maximum size in bases of duplicate injections ($N = 10$) of the ladders for COfiler were calculated. Three alleles for each locus (first, middle, and last) are presented.

TABLE 2—*Stutter values.*

	VFL avg.	VFL max.	VFL std. dev.	Perkin-Elmer max (8,9)
Profiler Plus				
D3S1358	0.068	0.122	0.015	<0.10
vWA	0.067	0.127	0.016	<0.10
FGA	0.067	0.108	0.015	<0.10
D8S1179	0.057	0.136	0.019	<0.09
D21S11	0.065	0.197	0.018	<0.10
D18S51	0.081	0.152	0.022	<0.13
D5S818	0.047	0.084	0.011	<0.08
D13S317	0.047	0.087	0.013	<0.08
D7S820	0.044	0.078	0.012	<0.08
COfiler				
D3S1358	0.070	0.119	0.014	<0.10
D16S539	0.053	0.097	0.015	<0.10
THO1	0.022	0.049	0.009	<0.04
TPOX	0.025	0.040	0.008	<0.04
CSF	0.046	0.076	0.010	<0.07
D7S820	0.043	0.080	0.011	<0.08

The average, maximum, and standard deviation of stutter values resulting from amplification of population samples with Profiler Plus and COfiler are shown with the manufacturer's reported highest stutter values.

TABLE 3—*Summary of population study heterozygote balance.*

	Avg.	Min.	Max.	Std. Dev.	Avg. + 3 Std. Dev.	Avg. − 3 Std. Dev.
Profiler Plus locus						
D3S1358	0.96	0.73	1.14	0.08	1.21	0.71
vWA	0.92	0.58	1.23	0.09	1.19	0.65
FGA	0.91	0.68	1.44	0.11	1.24	0.58
D8S1179	0.95	0.73	1.20	0.09	1.21	0.69
D21S11	0.94	0.68	1.43	0.11	1.26	0.61
D18S51	0.90	0.64	1.34	0.11	1.22	0.58
D5S818	0.95	0.60	1.52	0.12	1.31	0.59
D13S317	0.92	0.59	1.23	0.09	1.19	0.66
D7S820	0.95	0.76	1.50	0.11	1.28	0.62
COfiler locus						
D3S1358	0.95	0.79	1.31	0.10	1.26	0.65
D16S539	0.94	0.75	1.36	0.10	1.24	0.63
THO1	0.95	0.70	1.24	0.09	1.23	0.68
TPOX	0.93	0.67	1.14	0.09	1.19	0.66
CSFPO1	0.95	0.73	1.30	0.11	1.28	0.61
D7S820	0.93	0.68	1.18	0.10	1.23	0.63

The population samples with heterozygous alleles were evaluated for peak balance. Ratios between the two peaks of the heterozygous pair were calculated and averaged per locus.

Mixed Specimen Studies

DNA was extracted from bloodstains of two different individuals, one male and one female. At six of the 13 core loci, the two individuals shared no alleles; at another six, the two shared one allele and at one out of the 13, both had the same type. The extracted DNA was diluted to approximately 1.0 ng/μL and combined as follows: 1:2, 1:5, 1:10, and 1:20. The mixes were then amplified using both PCR kits with 1 ng of template DNA entering each amplification reaction. Following analysis on the capillary electrophoresis unit, the resulting profiles were evaluated for the presence of the following mixture indicators: more than two peaks at a locus, peaks at stutter positions higher than typical stutter peaks, and heterozygote peak imbalance.

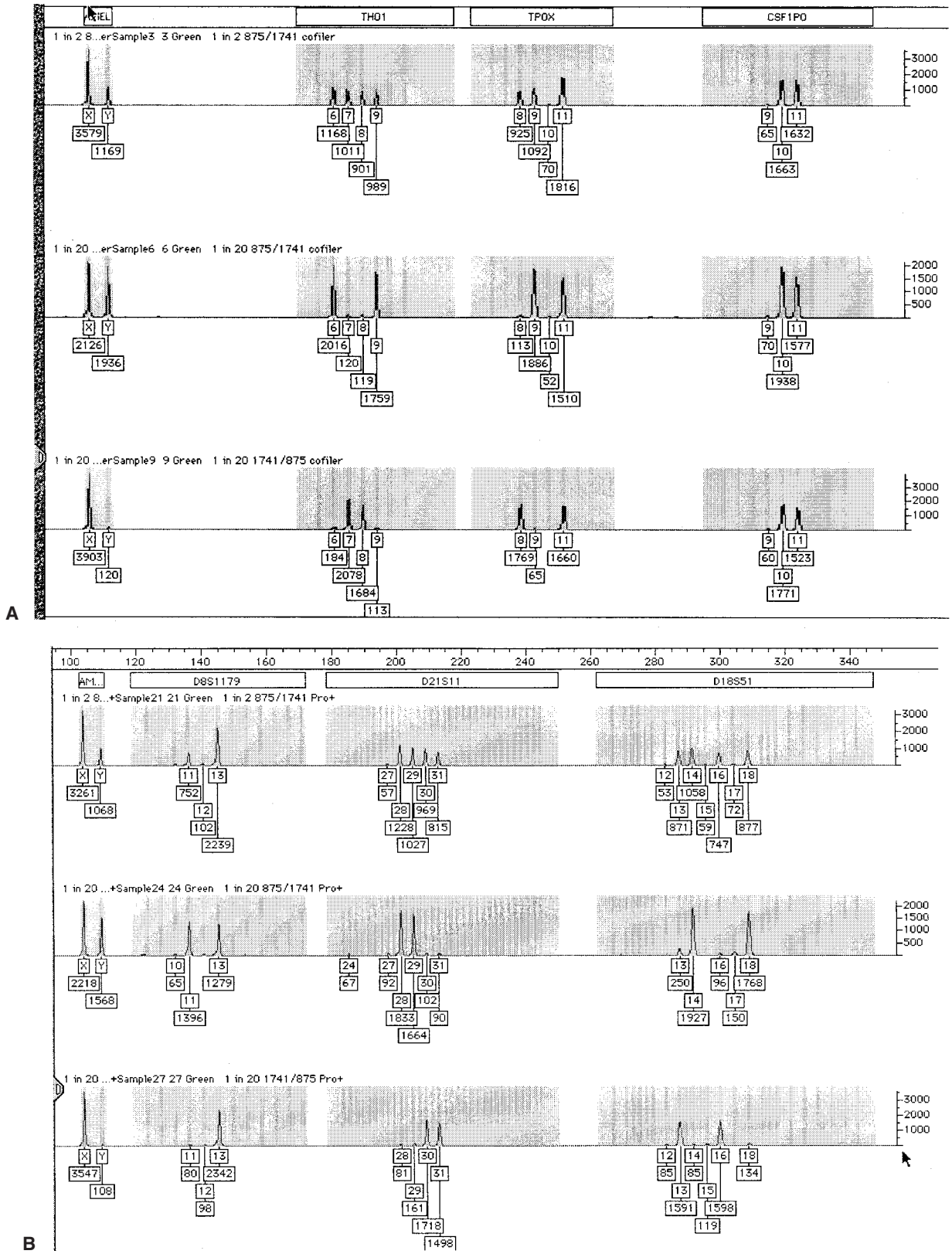

FIG. 1*A, B—Mixed specimen studies—Two samples (numbered 875 and 1741) were combined at the following ratios: 1:2, 1:20, and the reciprocal 1:20 mixture. The top panel in each set is the 1:2 mixture. Electropherograms from samples amplified with COfiler (Fig. 1*A*) and Profiler Plus (Fig. 1*B*).*

Specified amplified fragments derived from each sample were generally observed in proportion to the presence of that DNA sample in the mixture. While it was possible to discern a mixture was present in the samples combined 1:20, the minor components were, for the most part, either below the 150 rfu minimum needed to call a peak or were at stutter positions at levels characteristic of, or slightly higher than, true stutter.

The peak heights of the minor profile of a 1:10 mixture were above, or nearing the 150 rfu limit established for interpretation. Minor profile peaks at stutter positions were higher than would be expected for true stutter. Similar results were seen for the 1:5 combinations as well, though all of the minor profile peaks in this sample group were higher than 150 rfus.

Predicting the genotype of the minor component was easiest for the 1:10 and 1:5 combinations, largely due to the disparity in peak heights, though loci with two or three alleles could not always be definitively assessed. The 1:2 combination was undoubtedly a mixture, with little ambiguity as to the true alleles represented, but the overall equality of peak heights made separation of the two genotypes uncertain (Fig. 1).

Environmental Studies

DNA samples derived from bloodstains exposed to variations in environmental conditions that can affect the integrity of forensic samples were evaluated using both the Profiler Plus and the COfiler multiplex systems. Blood from four different donors was placed on white cloth and allowed to dry. The bloodstains then began exposure to varying conditions of heat, light, and temperature for varying periods of time. The treatments were as follows:

1. room temperature, sunlight, four weeks
2. room temperature, sunlight, three months
3. 37°C, dark, four weeks
4. room temperature, dark, three months
5. 37°C, dark, three months

Once the prescribed time intervals had passed, the samples were then preserved at −80°C until extraction, amplification with the two kits, and subsequent CE analysis. For several random samples, complete profiles were obtained only after extraction and amplification steps were repeated. Despite the treatments, typing was not compromised; those profiles generated from samples of a common source matched. Not surprisingly, some of the larger alleles did not amplify as strongly as their smaller counterparts, presumably due to condition-induced sample degradation. This resulted in a "typical degradation" pattern; the smaller alleles exhibit taller peaks than the larger sized alleles in the electropherogram so that the peaks graduate downward from left to right (Fig. 2). Typically, the highest molecular weight alleles displayed peak heights approximately 50% less than the smallest molecular weight alleles tagged with a common fluorescent dye for all Profiler Plus loci and for the COfiler loci tagged with the green fluorescent dye. Though results did not seem to vary significantly among the treatment groups, a trend toward lower peak heights in the samples exposed to sunlight for three months was observed (data not shown).

Matrix Studies

Possible degradation of sample material or inhibition of amplification due to contact with various matrix supports was tested. Blood from a single individual was spotted on common substrates, including denim, glass, cardboard, and metal. Blood from this same individual was also spotted on white cotton cloth, allowed to dry and treated with the following chemical contaminants: 5% detergent, 5% bleach, 10% EDTA, 3% H_2O_2, 0.02 M $CuCl_2$, 0.02 M

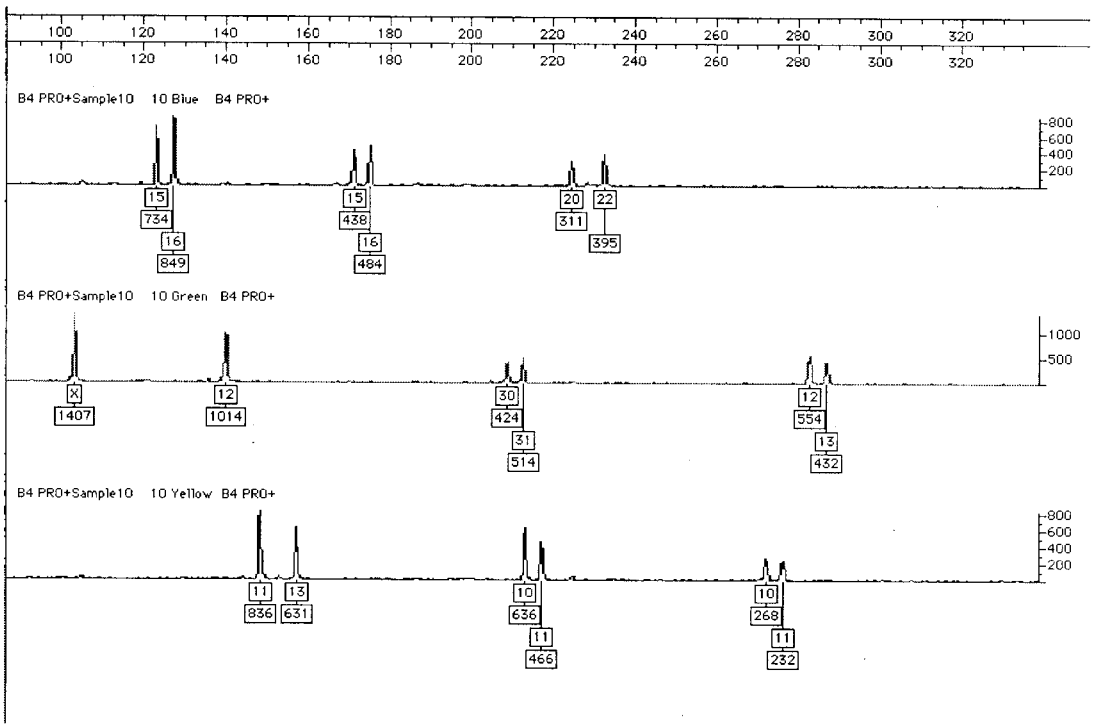

FIG. 2—*Environmental studies—Profiler Plus electropherograms demonstrate the environmental effects on the results of a blood sample exposed to room temperature and sunlight for three months. These electropherograms show a degraded sample pattern.*

$MgCl_2$, 0.02 M $MnCl_2$, and 0.02 M $NiCl_2$. DNA was extracted from the bloodstained substrates and from the chemically treated stains. The DNA was amplified using the Profiler Plus and COfiler kits and the PCR products were analyzed by capillary electrophoresis. STR profiles matching the untreated control were obtained for all of the samples in this set, though some samples from both the chemical contaminant and the substrate set required re-extraction/re-amplification to yield complete profiles (data not shown).

Nonprobative Evidence

Nonprobative cases were examined using the Profiler Plus and the COfiler multiplex systems. Four cases for a total of 21 samples were evaluated with Profiler Plus; these included blood standards, a mixed bloodstain, and differential extractions from three sexual assaults. Three sexual assault cases totaling nineteen samples were done using COfiler. The conclusions reached from the results obtained were consistent with those drawn using PM/DQA1 (data not shown).

Nonhuman Studies

Nonhuman sources of DNA were examined to determine the species specificity of the Profiler Plus and COfiler multiplex systems as well as the gender identification locus amelogenin. DNA was extracted from muscle tissue or from blood of various nonhuman animals that had been absorbed onto paper or cloth and stored at −80°C. Animals tested were bear, goat, moose, rabbit, sheep, swine, turkey, chicken, cat, dog, cow, horse, and deer.

No detectable amplification products were observed for any of these nonprimate species with the Profiler Plus or the COfiler systems with the exception of a reproducible peak sizing at approximately 99 bases (21). This peak, tagged with the green fluorescent dye JOE, is apparently associated with the amelogenin gene and was present in more than half of the species tested (Fig. 3).

Minimum Sample

Previously extracted DNA from blood was serially diluted from 0.25 ng/μL to 0.0005 ng/μL. Ten microliters each of the diluted

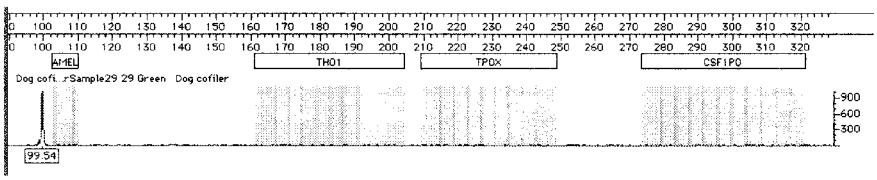

FIG. 3—*Nonhuman studies—The electropherogram demonstrates an amelogenin-related peak (99 bases) for dog DNA.*

TABLE 4*A*—*Heterozygote balance as a function of template DNA for COfiler.*

Dye	Locus	ng input DNA	Average	Min.	Max.	Std. Dev.
Blue	D3S1358	1.00	1.03	0.90	1.18	0.11
		0.50	0.70	0.46	0.87	0.15
		0.25	1.12	0.77	1.73	0.35
		0.125*	1.15	0.44	1.96	0.71
		0.06*	0.95	0.59	1.29	0.28
	D16S539	1.00	0.86	0.77	0.92	0.05
		0.50	0.97	0.59	1.30	0.24
		0.25	0.83	0.50	1.12	0.21
		0.125*	1.18	0.25	4.00	1.13
		0.06*	0.88	0.57	1.33	0.28
Green	THO1	1.00	1.17	0.85	1.61	0.28
		0.50	0.65	0.51	0.82	0.11
		0.25	1.29	0.67	2.12	0.57
		0.125*	0.83	0.32	1.24	0.33
		0.6	no result	no result	no result	no result
	CSFPO1	1.00	1.02	0.94	1.07	0.04
		0.50	0.93	0.62	1.54	0.31
		0.25	0.94	0.60	1.26	0.21
		0.125	1.33	0.23	3.13	1.01
		0.06*	1.14	0.85	1.32	0.18
Yellow	D7S820	1.00	0.79	0.67	0.89	0.08
		0.50	1.03	0.69	1.59	0.30
		0.25	0.72	0.48	1.14	0.24
		0.125*	1.09	0.66	1.50	0.30
		0.06	no result	no result	no result	no result

The evaluation of input template amount on heterozygote balance employing the COfiler system. Ratios between the two peaks of the heterozygous pair were calculated and averaged per locus.

* Indicates values based on fewer than ten samples.

TABLE 4*B*—*Heterozygote balance as a function of template DNA for Profiler Plus.*

Dye	Locus	ng input DNA	Average	Min.	Max.	Std. Dev.
Blue	D3S1358	1.00	0.79	0.73	0.89	0.05
		0.50	0.98	0.65	1.17	0.18
		0.25	0.83	0.54	1.20	0.27
		0.125	1.07	0.79	1.46	0.24
		0.06	no result	no result	no result	no result
	vWA	1.00	0.95	0.76	1.08	0.12
		0.50	1.25	0.89	1.61	0.29
		0.25	1.11	0.69	1.78	0.37
		0.125*	0.95	0.87	1.07	0.09
		0.06	no result	no result	no result	no result
	FGA	1.00	0.85	0.74	0.96	0.08
		0.50	0.96	0.71	1.26	0.24
		0.25	0.69	0.37	1.25	0.33
		0.125*	0.77	0.65	0.91	0.11
		0.06	no result	no result	no result	no result
Green	D18S51	1.00	0.85	0.72	0.94	0.08
		0.50	1.19	0.91	1.60	0.25
		0.25	0.88	0.58	1.29	0.24
		0.125*	1.61	1.09	2.06	0.49
		0.06	no result	no result	no result	no result
Yellow	D7S820	1.00	0.97	0.82	1.34	0.20
		0.50	0.80	0.65	0.93	0.11
		0.25	1.11	0.54	2.71	0.65
		0.125*	1.24	0.55	1.66	0.60
		0.06	no result	no result	no result	no result

The evaluation of input template amount on heterozygote balance employing the Profiler plus system. Ratios between the two peaks of the heterozygous pair were calculated and averaged per locus.

* Indicates values based on fewer than ten samples.

extracts were then amplified using the Profiler Plus and COfiler amplification kits.

Consistent allele calls and heterozygote allele balance were observed in all samples amplified from 2.5 ng of DNA down to 0.3 ng. Both heterozygote imbalance and peaks under 150 rfus were seen in Profiler Plus and COfiler samples derived from 0.16 ng of DNA and less (data not shown).

Homozygous peak heights obtained from the highest template concentration (2.5 ng) were generally above 5500 rfus. This sample group suggests that best results may be obtained from samples amplified from template input of at least 0.3 ng to 1.25 ng of DNA. Though not observed in this particular sample set, large non-A peaks (22), excessive stutter and peaks higher than 5500 rfus have all been encountered in samples amplified from 2.5 ng or more of DNA (data not shown).

Serial Dilution—Multiple Amplification Study

Control DNA taken from an STR amplification kit was serially diluted from its initial concentration of 0.1 ng/μL. Each dilution was amplified five times with DNA input at the following amounts: 0.50, 0.25, 0.13, and 0.06 ng/μL respectively. The PCR products were analyzed in duplicate by capillary electrophoresis. Analysis parameters were set to recognize peaks above a threshold value of 30 rfus, which is approximately twice the height of the observed background levels. The peak heights, stutter peak percentages, peak balance for alleles of common fluorescent dye tag, and heterozygote peak ratios for the ten results of each different DNA input were averaged.

For all heterozygote loci, input DNA of 0.25 ng or less in both Profiler Plus and COfiler samples resulted in average peak heights less than 150 rfus. At DNA input of 0.125 ng and less, total allele dropout was seen; that is, peak heights for some alleles fell to levels below twice the background, and consequently, these alleles went undetected.

Peak heights of homozygote loci dropped under 150 rfus when template DNA was 0.125 ng or less. Stutter bands (23,24) were displayed at multiple loci in samples amplified from 1.0 ng of DNA with both STR kits and were seen to a lesser extent in Profiler Plus samples derived from 0.5 ng of DNA. Stutter peaks were seen in COfiler samples amplified from less than 1.0 ng of DNA only at the D3S1358 locus. Stutter peaks were virtually absent in all samples amplified from less than 0.5 ng of template.

Heterozygote ratios calculated from both Profiler Plus and COfiler samples amplified from a 0.25 ng template tended to show higher standard deviations than those derived from samples amplified from templates of 0.5 and 1.0 ng. This implies that reduced template leads to greater variability and consequently less reliability for these ratios, an important consideration in the interpretation of mixtures (Table 4). These findings also helped to validate the 150 rfu peak height minimum adopted by the VFL as a reasonable lower threshold to avoid misinterpreting results.

Conclusion

The results of the TWGDAM validation studies performed here demonstrate the reliability, reproducibility, robust nature, and high discriminating power of the Profiler Plus and COfiler multiplex systems, as well as limitations that must be considered for successful use. Laboratories employing the multiplex kits for forensic use would be well advised to put emphasis on TWGDAM validation guidelines *Population Studies, Mixed Specimen Studies, Nonprobative Evidence,* and *Minimum Sample* before implementing the kits for casework. These areas are likely to provide the validating laboratory with the most relevant experience with regard to kits, instrumentation and "problem sample" exposure with which to evaluate and interpret results. These studies will help a laboratory establish interpretation criteria, such as minimum and maximum peak value thresholds, heterozygote balance ratio and stutter peak expectations.

Both the mixed sample studies, in which the input DNA is held constant, and the minimum sample studies give the laboratory valuable experience with samples that show heterozygote imbalance, total allele drop-out and peak heights below the laboratory-established minimum limits. Evaluating these samples using laboratory-established interpretation guidelines is necessary experience prior to taking on casework.

With few exceptions the samples tested in these studies were derived from single source DNA assumed to be "clean" and intact. True forensic samples may behave somewhat differently. Knowing when to expect allele dropout and skewed heterozygote ratios can help to avoid interpretational problems.

It should be noted here that variations in peak height can be due to one or more of the many steps inherent to the studies as well as performance differences between individual CE analyzers.

The multiplex systems coupled with CE instrumentation, provide sensitive, accurate results even when forensic samples are exposed to extreme conditions. These attributes make the Profiler Plus and COfiler amplification kits powerful, investigative tools for the analysis of forensic samples.

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