TECHNICAL NOTE

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Validation of the AmpFℓSTR™ Profiler Plus PCR Amplification Kit for Use in Forensic Casework

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ABSTRACT: According to TWGDAM guideline 4.5 (1), prior to implementing a new DNA analysis procedure or an existing DNA analysis procedure developed by another laboratory, the forensic laboratory must first demonstrate reliability of the procedure in-house. Seven phases were designed to validate the use of the AmpFℓSTR Profiler Plus PCR Amplification Kit, as well as the PE Applied Biosystems 310 Genetic Analyzer. This report summarizes the results obtained for each of the seven phases of the validation study which included the following evaluations: polymer, reproducibility, sensitivity, stutter, precision, mixtures and nonprobative casework.

KEYWORDS: forensic science, deoxyribonucleic acid (DNA), DNA typing, validation, short tandem repeat (STR), capillary electrophoresis, polymerase chain reaction (PCR), AmpFℓSTR Profiler Plus

The AmpFℓSTR Profiler Plus PCR Amplification Kit (PE Applied Biosystems, Foster City, CA) is a multiplex PCR reaction kit designed to amplify nine tetrameric repeat loci on nine separate chromosomes plus a homologous region of the Amelogenin gene on the X and Y chromosomes (2). The STR fragments amplified by the AmpFℓSTR Profiler Plus PCR Amplification Kit range in length from approximately 107 to 341 base pairs. The primer sets used in this kit are labeled with one of three fluorescent dyes. Loci labeled with the same fluorescent dye do not overlap in base pair size.

The fluorescently labeled PCR products can be genotyped using the PE Applied Biosystems 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA). The PE Applied Biosystems 310 Genetic Analyzer (ABI 310 CE) is a capillary electrophoresis based instrument. Fragments labeled with one of three fluorescent dyes are excited by an argon laser on the instrument and the resulting emission spectra are detected by a CCD camera. Fragments are sized using an internal lambda DNA standard labeled with a fourth fluorescent dye. An allelic ladder is used to assign genotypes to the samples.

Analysis of STR loci for identification studies has been reported. These studies include the loci contained in the AmpFℓSTR Profiler Plus Kit as well as other STR loci (3). The use of fluorescent technology and STR analysis in forensic science has been applied to multiplexes such as those designated multiplex 1A (Amelogenin, HUMD21S11 and HUMFGA) and multiplex 1B (HUMD3S1358, HUMD21S11, and HUMFGA) (4). Because STR multiplexes have demonstrated stability, reproducibility, sensitivity, and a high power of discrimination they are of interest for use in forensic science (3,4,6). However, according to TWGDAM guideline 4.5, before a new DNA typing system can be utilized in casework it must be demonstrated in-house that the procedure is reliable.

The following validation phases were completed at the Illinois State Police Research and Development Laboratory (R&D) (Springfield, IL) and the Forensic Science Center at Chicago (FSC-C) using the AmpFℓSTR Profiler Plus PCR Amplification Kit to satisfy TWGDAM guideline 4.5 : 1) polymer evaluation, 2) reproducibility study, 3) sensitivity study, 4) precision study, 5) stutter evaluation, 6) mixture study, and 7) an evaluation of nonprobative casework. Upon completion of each analytical phase, all data was collected and summarized into a combined report. A draft protocol was then agreed upon for use in casework.

Materials and Methods

The AmpFℓSTR Profiler Plus PCR Amplification Kit was utilized to amplify the Amelogenin locus and the following tetrameric STR loci: D3S1358, vWA, FGA, D8S1179, D21S11, D18S51, D5S818, D13S317, and D7S820. Amplifications were conducted according to manufacturer recommendations (5) using the PE Applied Biosystems model 480 thermal cycler (PE Applied Biosystems, Foster City, CA). Population database samples were selected from an Illinois population study. The database samples utilized in this study had previously been amplified and profiled using the AmpFℓSTR Blue PCR Amplification Kit (PE Applied Biosystems, Foster City, CA) (7) and the AmpFℓSTR Green II PCR Amplification Kit. The samples had also been amplified and profiled using the PowerPlex PCR Amplification Kit (Promega, Madison, WI) (8). Samples amplified using the AmpFℓSTR PCR amplification kits were analyzed using PE Applied Biosystems GeneScan 2.1 and

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Genotyper 2.1 software programs (PE Applied BioSystems, Foster City, CA) (9). Genotyper analysis was defined by the KAZAM macro in Genotyper 2.1 software, with minimum allele threshold detection set at 150 relative fluorescent units (rfu). While no upper bound for rfu was established for allele calls to be made in Genotyper, values greater than 4500 rfu were interpreted with caution (5). Samples amplified using the PowerPlex PCR Amplification Kit were analyzed using the Hitachi FMBIO Gel Electrophoresis System and STaR CALL software program (10) (Hitachi, South San Francisco, CA). The following seven studies were conducted.

Polymer

A sample with a known 9.3, 10 genotype at the HUMTH01 locus was amplified twice using the AmpFℓSTR Profiler PCR Amplification Kit (PE Applied Biosystems, Foster City, CA) (11,12). The PCR product was analyzed on the ABI 310 CE using the proprietary polymers *POP4* (13) and *POP6* (14) (PE Applied Biosystems, Foster City, CA).

Reproducibility (TWGDAM 4.1.5.2 and 4.1.5.4)

A. Interlaboratory reproducibility: 50 samples were amplified in the Research and Development (R&D) Laboratory and the same 50 samples were amplified at the Forensic Science Center at Chicago (FSC-C) using the AmpFℓSTR Profiler Plus PCR Amplification Kit.
B. Gel electrophoresis versus capillary electrophoresis: Ten samples previously characterized by vertical gel electrophoresis using the Hitachi FMBIO 100 gel electrophoresis system and STaR CALL software were analyzed at the R&D Laboratory. A second set of the same ten samples were analyzed at the FSC-C. Each laboratory extracted and purified the samples (15). All samples were analyzed and genotypes determined using the ABI 310 CE.
C. Evaluation of different extraction methods: The Puregene (Gentra Systems, Minneapolis, MN) (16) nonorganic extraction protocol was compared to a phenol chloroform based organic method (15). Twenty samples were extracted using both the nonorganic method and the organic method.

Sensitivity (TWGDAM Guideline 4.1.5.10)

A. Sensitivity between different capillary electrophoresis instruments: To compare the difference in sensitivity obtained between CEs, a dilution series was prepared from 10 ng to 36 pg for four different samples quantitated by slot-blot analysis using the QuantiBlot™ Human DNA Quantitation kit (PE Applied Biosystems, Foster City, CA) (17) in the R&D laboratory. Aliquots from the dilution series were amplified using the AmpFℓSTR Profiler Plus PCR Amplification Kit and run on two different CEs in the R&D laboratory and one CE at FSC-C.
B. Injection time: Five, ten, and twenty second injections were made at FSC-C and in the R&D Laboratory on samples from a dilution series of samples that contained from 0.6 ng to 36 pg of input DNA.

Stutter or Strand Slippage

Five samples were selected for each of the nine tetrameric repeat loci of the AmpFℓSTR Profiler Plus PCR Amplification Kit. Samples selected exhibited heterozygous loci in which the sister alleles were separated by at least two repeat units. A sample's percent stutter was calculated by determining the proportion of the stutter fragment to the true allele (5). This data was then compared to the stutter data published by PE Applied Biosystems (5).

Precision (TWGDAM Guideline 4.1.5.3)

A. Precision of allele determination: Five known samples were injected twenty times to establish the precision in the base pair sizing of the AmpFℓSTR Profiler Plus PCR products using the ABI 310 CE. Base pair size and genotype data were collected and precision data for one allele at each locus was calculated (Table 1). Each laboratory also collected sizing data for the first allele of the allelic ladder for D3S1358, Amelogenin and D5S818 from 100 allelic ladder runs (Table 2).

TABLE 1—*Precision study—database samples (base pairs).*

Locus	FSC-allele C	3SD	SD	R&D	3SD	SD
D3S1358	14	0.24	0.08	18 allele	0.38	0.13
vWA	16	0.21	0.07	16 allele	0.46	0.15
FGA	24	0.20	0.07	21 allele	0.55	0.18
Amelogenin	X	0.22	0.07	X allele	0.36	0.12
D8S1179	12	0.25	0.08	15 allele	0.44	0.15
D21S11	30	0.18	0.06	28 allele	0.30	0.10
D18S51	19	0.31	0.10	12 allele	0.98	0.33
D5S818	13	0.23	0.08	13 allele	0.39	0.13
D13S317	11	0.25	0.08	9 allele	0.44	0.15
D7S820	12	0.27	0.09	11 allele	0.53	0.18

TABLE 2—*Precision study—allelic ladder (base pairs).*

Locus	Allele	FSC-C 3SD	FSC-C SD	Allele	R&D 3SD	R&D SD
D3S1358	12	0.35	0.12	12	0.56	0.19
Amelogenin	X	0.36	0.12	12	0.44	0.15
D5S818	7	0.27	0.09	7	0.47	0.16

B. Precision of relative peak heights of alleles comprising a heterozygote pair: Samples from the reproducibility phase, the stutter evaluation and part A of the precision validation phase were selected for this study. Approximately 425 comparisons were made from heterozygous pairs to determine an average heterozygote peak height ratio. This data was then compared to the PE Applied Biosystems report (5) and to peak height precision results from the nonprobative casework phase.

Mixtures (TWGDAM Guideline 4.1.5.5)

Two samples were mixed together at known proportions to determine the ratio at which the major and minor components of a mixture might be resolved. Mixtures were produced by holding the concentration of one sample constant at 10 ng and increasing the second component from 1:200, 1:100, 1:20, 1:10, 1:2, and 1:1. Two nanograms of DNA from each mixture was amplified. The minor components were evaluated in relation to peak height and position of the major components.

Nonprobative Casework (TWGDAM Guideline 4.1.5.8)

Nineteen nonprobative cases were analyzed. Nonprobative case samples included blood standards for comparison to semen stains or bloodstains from adjudicated cases. Nine of the cases examined had previously been analyzed using the Amplitype PM + DQA1 PCR Amplification Kit (PE Applied Biosystems, Foster City, CA) (18) and a D1S80 (20) PCR analysis system. The results from the nine cases analyzed using the AmpFℓSTR Profiler Plus PCR Amplification Kit were compared to the results obtained using the Amplitype PM + DQA1 PCR Amplification Kit and the D1S80 analysis system.

Discussion

The first phase of the validation study was designed to examine the resolution of the two commercially available polymers *POP4* and *POP6* (PE Applied Biosystems, Foster City, CA). The AmpFℓSTR Profiler PCR Amplification Kit was selected to evaluate the polymers. The AmpFℓSTR Profiler PCR Amplification Kit amplifies ten loci—one of which is HUMTH01. The 9.3 and 10 alleles at the HUMTH01 locus are separated by one base pair. A 1 ng and a 2 ng aliquot of a sample with a HUMTH01 9.3,10 genotype was amplified using the AmpFℓSTR Profiler PCR Amplification Kit and run on the ABI 310 CE. Each amplification was analyzed after being electrophoresed through *POP4* or *POP6* polymer solutions. Genotyper identified the correct genotype for all samples. Neither polymer resolved the two peaks of the alleles to baseline. PE Applied Biosystems recommends *POP4* for genotyping applications and *POP6* for sequencing applications. Given that both polymers revealed similar resolution, PE Applied Biosystem's recommendation to select *POP4* for genotyping was accepted. The 4% polymer was utilized throughout the remainder of the validation study.

Interlaboratory reproducibility was demonstrated by both the R&D and FSC-C laboratories obtaining the same genotyping results for the 50 samples. In addition, consistent with previous report (21) no differences were observed in the genotypes for the ten samples defined by vertical gel electrophoresis or the capillary electrophoresis instrument. Lastly, the extraction procedure utilized had no effect on the genotype obtained with the AmpFℓSTR Profiler Plus PCR Amplification Kit.

In the sensitivity study, complete profiles were identified for all four samples down to 1.25 ng of input DNA on both ABI 310 CEs in the R&D laboratory. Complete profiles were detected at the 0.6 ng level on one ABI 310 CE. The Genotyper analysis of the four samples on the second ABI 310 at the 0.6 ng level showed incomplete results at the D7S820 locus for two of the samples and incomplete results at the D8S1179 locus for one of these two samples. Not all loci were detected at the 0.3 ng level for any sample on either of the ABI 310 CEs. Overall, one ABI 310 CE detected approximately two times the number of complete loci at the 0.3 ng level of input DNA therefore demonstrating sensitivity differences between instruments. Amplification of 1 to 2 ng of DNA often resulted in rfu values approximately equal to those produced by the AmpFℓSTR Profiler Plus PCR Amplification Kit positive control. Therefore, this range was selected as an optimum for input DNA.

An additional evaluation of sensitivity was conducted at FSC-C and in the R&D Laboratory which involved increasing injection time. A dilution series of samples ranging from 0.6 to 36 pg was analyzed. Ten second injections increased peak heights approximately 200% over five second injections for most samples with little to no artifacts identified. Twenty second injections provided an additional 100% increase in peak height over the ten second injection for most samples, however, stutter and pull-up artifacts were often pronounced.

An observation was noted during the sensitivity study at the FSC-C and in the R&D Laboratory on the differences in the sensitivity of dyes. To quantify this observation, rfu data was collected from samples of a dilution series where fluorescence values averaged approximately 1000 rfu. Ratios were established for the rfu values between the three fluorescent dyes. The loci labeled with the fluorescent dyes 5-FAM and JOE produced the greatest rfus followed by the fluorescent dye NED. Samples at FSC-C generally produced ratios of approximately 1.4:1.3:1 while the R&D Laboratory saw values in the range of 1.3:1.4:1 for loci labeled with 5-FAM, JOE, and NED.

When the input concentration of DNA to be amplified exceeded 1 to 2 ng and or injection time for the amplified DNA exceeded 10 seconds, the rfu values assigned to a particular allele may not be accurately measured as the detection system becomes saturated. Artifacts such as incomplete 3′ adenosine addition, stutter, or poor spectral separation also known as "pull-up" (5) were observed when conditions which result in alleles with rfu values greater than 4500 were in place. The linear range for rfu values for the ABI 310 CE is reported to be between 150 to 4500 (25). Our observation of PCR artifacts under the conditions of high input DNA concentration or increased PCR product injection time verify that caution must be exercised when interpreting genotypes outside the 4500 rfu range. Amplification of less than 1 ng of DNA may result in low rfu and maximum sensitivity not being obtained. Decreasing the concentration of input DNA can result in PCR product, which registers rfu values below the established threshold. Such alleles would not be called or "labeled" in Genotyper. These observations indicate that 1 to 2 ng of DNA should be targeted for amplification when possible. Additionally, injection times greater than 10 seconds would not be recommended.

Stutter (strand slippage) is an artifact of the PCR process (5,22). Stutter products are generally one repeat unit smaller than the actual size of the true allele. Amplification conditions, input DNA

concentration, and rfu threshold can influence the amount of stutter identified in a sample. When the amplification product for an allele exceeds the linear detection limits for the ABI 310 CE, minor components such as stutter products artificially rise in proportion to the allele due to system saturation. Increasing the amount of DNA amplified, and or the injection time, can therefore increase the amount of stutter identified. In addition, software parameters used to analyze the data can determine the amount of stutter that will be interpreted.

Samples selected to evaluate stutter were heterozygous at the majority of loci. Additionally, sister alleles were separated by at least two repeat units. It has been reported that as fragment size increases so does the percent stutter for the alleles of a given locus (5). Therefore, samples with higher molecular weight alleles were selected over those with lower molecular weight alleles for each locus. A sample's percent stutter was calculated as reported by PE Applied Biosystems. The proportion of stutter fragment peak height to that of the true allele was calculated (5). The AmpFℓSTR Profiler Plus User's Manual reports the maximum percent expected stutter observed for each of the STR loci in the AmpFℓSTR Profiler Plus PCR Amplification Kit. Our results indicated that stutter was not often seen under standard laboratory amplification and ABI 310 instrument specifications. In order to produce stutter, up to 5 to 10 ng of DNA was amplified. PCR product was injected on the ABI 310 up to 10 to 20. These conditions increased the number of loci for which stutter alleles were labeled in Genotyper. The only percentage stutter identified in either laboratory which was outside the range reported by PE Applied Biosystems was for an allele pair in which the ISP laboratory calculated the proportion of stutter for a higher molecular weight allele. In data reported by PE Applied Biosystems for the FGA, locus stutter was reported to be <10% for the 28 allele. The FSC-C studied the 29 allele, calculated the observed percentage of stutter as done by PE Applied Biosystems, and reported <11.4% stutter. The R&D Laboratory identified <12.2 percent stutter for a high molecular weight off ladder FGA allele larger than the allele used by PE Applied BioSystems for the study. The guidelines for stutter definitions established in the AmpFℓSTR Profiler Plus User's Manual (5) were accepted for the draft protocol.

Precision with respect to the determination of allele size and precision concerning the relative peak heights of alleles comprising a heterozygote pair was evaluated. Samples from Phases 3, 4, and 5 were selected for relative peak height evaluation. From these studies 425 comparisons were conducted. Ninety-seven percent of the peak height comparisons revealed peaks of a heterozygous pair are within 70% of one another. Of the 3% that fell outside the 70% window, five samples showed fluorescent signals which were outside the linear range of the instrument. Additionally, two had one off ladder allele in the heterozygote pair and seven were from the amplification of less than 1 ng of DNA. These observations are in agreement with the conditions described by PE Applied Biosystems which may cause peak height ratios to fall below 70% (5).

The result of peak height precision studies from the amplification of 2.5, 1.25, and 0.6 ng of ten samples is listed in Table 3. Five samples were amplified and analyzed at the R&D Laboratory and five samples were amplified and analyzed at FSC-C. Such amplifications are expected to produce rfu values in the linear range of the instrument and peak height ratios at or above 70% for heterozygous loci (5). The mean value for all observations was above 70%. An evaluation of peak height ratios from nonprobative casework samples is listed in Table 4. The mean value for peak height ratios for nonprobative casework samples was also above 70%. However,

TABLE 3—*Peak height ratios—database samples.*

Locus	Mean Value	Mean—3SD	SD	Number Analyzed
D3S1358	0.93	0.75	0.06	26
vWA	0.91	0.76	0.05	23
FGA	0.86	0.62	0.08	23
D8S1179	0.93	0.75	0.06	27
D21S11	0.90	0.72	0.06	26
D18S51	0.89	0.65	0.08	26
D5S818	0.93	0.75	0.06	24
D13S317	0.89	0.65	0.08	24
D7S820	0.88	0.64	0.08	23

TABLE 4—*Peak height ratios—nonprobative casework samples.*

Locus	Mean Value	Mean—3SD	SD	Number Analyzed
D3S1358	0.93	0.80	0.04	153
vWA	0.90	0.70	0.07	174
FGA	0.89	0.64	0.08	374
D8S1179	0.91	0.71	0.07	256
D21S11	0.90	0.73	0.06	217
D18S51	0.89	0.68	0.07	333
D5S818	0.92	0.75	0.06	322
D13S317	0.90	0.75	0.05	439
D7S820	0.90	0.75	0.06	577

when three standard deviations (SD) were subtracted from the mean observations for data in Table 3 the results at FGA, D18S51, D13S317, and D7S820 fell below 70%. When three SD were subtracted from the mean observations in Table 4 the results at FGA and D18S51 fell below 70%. These results were incorporated into the guidelines for mixture interpretation.

In the evaluation of sizing precision, data showed the maximum range of base pair deviation for some alleles to exceed 0.5 bp, however; three standard deviations of all size determinations were found to be within one base pair and would therefore result in the correct assignment of alleles and genotypes. The precision data documented by PE Applied Biosystems in the Profiler Plus User's Manual (5) reports similar findings.

The quantitative results from the precision and stutter data was next applied to evaluate mixed samples. Both laboratories initially detected some loci of the minor component of mixed samples at the 1:20 dilution. However, if the peak height of a minor allele is relatively low in relation to a major allele and the minor allele is one repeat unit shorter than the major allele, the minor component could be interpreted as stutter (5). The rfu value of minor component alleles in a mixture should also be evaluated with respect to the results from the peak height ratio portion of the precision study. Both alleles of a heterozygote might not be labeled in Genotyper if the proportion of the minor component results in rfu values near the threshold. If a 70% peak height ratio can be expected, a minimum peak height of 214 rfu would be necessary to result in the second allele of a heterozygote being labeled with a 150 rfu threshold. At loci where mixtures were expected to produce four alleles and the minor component was less than 10% of the input DNA for amplification, allele and locus dropout were noted. The number of alleles detected at each locus and the proportions of peak heights of the alleles at each locus is presented in the PE Applied BioSystems Profiler Plus User's Manual (5) in a guide to mixture interpretation.

Prior to starting the nonprobative casework phase, the data collected from each validation phase and the AmpFℓSTR Profiler Plus User's Manual (5) was used to construct the laboratory's initial procedures manual to be utilized for nonprobative casework. Samples from nineteen nonprobative cases were analyzed. The results from nine cases analyzed using the AmpFℓSTR Profiler Plus PCR Amplification Kit were compared to the results previously obtained using the Amplitype PM PCR Amplification Kit and the D1S80 analysis system. No DQA1 results were produced for the comparison. The same match assessments were made concerning the unknown samples and the standard samples when profiles were defined using either the AmpFℓSTR Profiler Plus or the Amplitype PM/D1S80 PCR amplification sets. For each of the nine cases analyzed, the expected frequency of occurrence associated with the DNA profile in Caucasian population (5,19) defined using AmpFℓSTR Profiler Plus PCR Amplification Kit was found to be on average 1.8×10^9 times lower than the Amplitype PM profile. The AmpFℓSTR Profiler Plus PCR Amplification Kit was used to amplify DNA from ten nonprobative cases with semen stains. A differential extraction procedure (23) was used to isolate epithelial cell DNA and sperm cell DNA for these amplifications. Consistent with studies of different tissue types (24), no differences were noted between blood standard DNA profiles and the corresponding epithelial cell DNA profile defined by the AmpFℓSTR Profiler Plus PCR Amplification Kit.

In conclusion, the completion of these validation studies permitted the Illinois State Police to comply with TWGDAM guideline 4.5. Once these studies were finished, analysts at the FSC-C and the R&D Laboratory completed an internal proficiency test and an external proficiency test. Casework sample analysis using the AmpFℓSTR Profiler Plus PCR Amplification Kit began on May 1, 1998.

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Note

TWGDAM Guidelines include 4.1.5.6 (environmental effects), 4.1.5.7 (matrix effects) and 4.1.5.9 (evaluation of nonhuman DNA sources). These parameters had been evaluated on other multiplex systems in the R&D Laboratory and at FSC-C and did not effect genotyping results. They were not included in this study.

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