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Validation of a male-specific, 12-locus fluorescent short tandem repeat (STR) multiplex☆

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Abstract

Y chromosome-specific short tandem repeat (Y-STR) analysis has become another widely accepted tool for human identification. The PowerPlex® Y System is a fluorescent multiplex that includes the 12 loci: DYS19, DYS385a/b, DYS389I/II, DYS390, DYS391, DYS392, DYS393, DYS437, DYS438 and DYS439. This panel of markers incorporates the 9-locus European minimal haplotype (EMH) loci recommended by the International Y-STR User Group and the 11-locus set recommended by the Scientific Working Group on DNA Analysis Methods (SWGDAM). Described here are inter-laboratory results from 17 developmental validation studies of the PowerPlex® Y System and include the following results: (a) samples distributed between laboratories and commercial standards produced expected and reproducible haplotypes; (b) use of common amplification and detection instruments were successfully demonstrated; (c) full profiles were obtained with standard 30 and 32 cycle amplification protocols and cycle number (24–28 cycles) could be modified to match different substrates (such as direct amplification of FTA® paper); (d) complete profiles were observed with reaction volumes from 6.25 to 50 μL; (e) minimal impact was observed with variation of enzyme concentration; (f) full haplotypes were observed with 0.5–2× primer concentrations; however, relative yield between loci varied with concentration; (g) reduction of magnesium to 1 mM (1.5 mM standard) resulted in minimal amplification, while only partial loss of yield was observed with 1.25 mM magnesium; (h) decreasing the annealing temperature by 2–4 °C did not generate artifacts or locus dropout and most laboratories observed full amplification with the annealing temperature increased by 2 °C and significant locus dropout with a 4 °C increase in annealing temperature; (i) amplification of individual loci with primers used in the multiplex produced the same alleles as observed with the multiplex amplification; (j) all laboratories observed full amplification with ≥125 pg of male template with

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partial and/or complete profiles observed using 30–62.5 pg of DNA; (k) analysis of ≤500 ng of female DNA did not yield amplification products; (l) the minor male component of a male/female mixture was observed with ≤1200-fold excess female DNA with the majority of alleles still observed with 10,000-fold excess female; (m) male/male mixtures produced full profiles from the minor contributor with 10–20-fold excess of the major contributor; (n) average stutter for each locus; (o) precision of sizing were determined; (p) human-specificity studies displayed amplification products only with some primate samples; and (q) reanalysis of 102 non-probative casework samples from 65 cases produced results consistent with original findings and in some instances additional identification of a minor male contributor to a male/female mixture was obtained. In general, the PowerPlex® Y System was shown to have the sensitivity, specificity and reliability required for forensic DNA analysis.

Keywords: Forensic science; DNA typing; Short tandem repeat (STR); Y chromosome; Polymerase chain reaction (PCR); Validation; PowerPlex; European minimal haplotype

1. Introduction

Autosomal short tandem repeat (STR) analysis is the primary technology for genetic human identification (forensic science, paternity and some anthropological studies). However, the benefits of Y-specific analysis are widely recognized. Y chromosome-specific short tandem repeat (Y-STRs) markers have proven to be valuable tools for criminal investigations and paternity testing among other applications [1–5]. Because the Y-STRs reside on male-specific DNA, female DNA is not reactive. Consequently, Y-STRs can be exploited for use in the analysis and interpretation of male/female mixtures commonly encountered in casework. Moreover, the Y-STRs reside in the non-recombining portion of the Y-chromosome, which makes them useful for lineage and inheritance studies. To provide guidance in the use of Y-STRs, the International Y-STR User Group defined the European Minimal Haplotype (EMH) for Y-STRs. The EMH contains a core set of nine Y-STR loci: DYS19, DYS385a/b, DYS389I/II, DYS390, DYS391, DYS392 and DYS393 [6,7]. In 2003, the Scientific Working Group on DNA Analysis Methods (SWGDAM) also recommended the same nine loci plus the loci DYS438 and DYS439 [8] for use in forensic DNA analysis (personal communication, Jack Ballantyne). The PowerPlex® Y System (Promega, Madison, WI, USA) was developed to type these 11 loci plus the DYS437 locus [8].

The 12-locus PowerPlex® Y System [9,10] is a three-color fluorescent multiplex developed to analyze DNA samples typically encountered in forensic science, paternity and anthropological studies. In this multiplex, one of the two primers for DYS389I/II, DYS391 and DYS439 are labeled with fluorescein (FL); one primer specific for DYS19, DYS392, DYS437 and DYS438 is labeled with 6-carboxy-4′,5′-dichloro-2′,7′-dimethoxy-fluorescein (JOE); and one primer specific for DYS385a/b, DYS390 and DYS393 is labeled with carboxy-tetramethylrhodamine (TMR). Fragment sizing is calculated from an internal size standard (Internal Lane Standard 600) labeled with carboxy-X-rhodamine (CXR). Amplified samples are compared to the provided allelic ladder (Fig. 1) to designate the specific alleles present at each locus [11].

This study examines the consistency and robustness of the PowerPlex® Y System in order to support its use in human identification studies. In addition to population study data presented elsewhere [12], this work encompasses the ''developmental validation'' of this assay [13,14]. The primary goals are to demonstrate that the supplied protocols perform as described and to report the limitations of the system. This study examines the effect of DNA quality and quantity in addition to amplification and analysis variation on the assay. Also, average stutter, specificity and overall precision were evaluated. In total, 17 studies were performed to demonstrate the capability of this assay as a genotyping tool. Each laboratory employing the PowerPlex® Y System should consider the appropriate performance tests, establish in-house conditions (as recommended in the product instructions) and perform an ''internal validation'' specific to the laboratory. Guidelines that can outline what efforts might be valuable to the end-user laboratory have been issued by the Director of the FBI and further defined by SWGDAM [13,14].

2. Materials and methods

2.1. Protocol optimization

In general, each laboratory performed amplification and analysis according to the manufacturer's recommendations [10]. The supplied protocols allow some variation in amplification and analysis to optimize product performance for each laboratory. Data were produced after each laboratory performed this optimization. These optimizations included template quantity (0.5–1 ng), cycle number, injection time or loading volume and minimum peak threshold for interpretation.

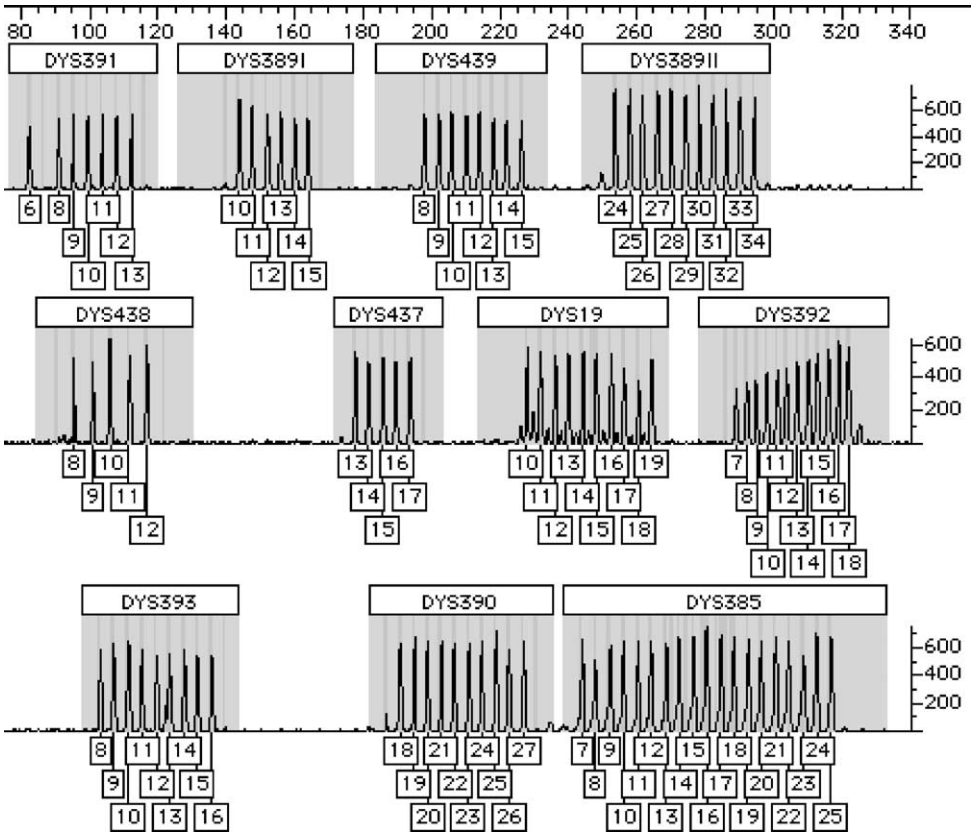

Fig. 1. The PowerPlex® Y Allelic Ladder Mix. An analysis of 1 μL of the PowerPlex® Y Allelic Ladder Mix is shown. Top: the blue fluorescein-labeled allelic ladder components (DYS389I/II, DYS391 and DYS439) and their allele designations. Middle: the green JOE-labeled allelic ladder components (DYS19, DYS392, DYS437 and DYS438) and their allele designations. Bottom: the yellow TMR-labeled allelic ladder components (DYS385a/b, DYS390 and DYS393) and their allele designations. Detection was performed using the ABI PRISM® 310 Genetic Analyzer.

2.2. Human DNA sources

Non-cell-line DNA provided by Promega Corporation for the proficiency, sensitivity and mixture testing was isolated using phenol/chloroform extraction [15]. DNA isolated from the RAJI, 9947A and 9948 cell-lines were also provided by Promega. Promega-supplied DNA samples were quantified by spectrophotometric analysis using A_{260} detection [15]. Sensitivity and mixture samples were also independently verified by quantitative, real-time polymerase chain reaction (PCR) [16]. DNA samples supplied by individual laboratories were extracted from liquid blood, buccal swabs or dried blood on FTA® paper (Whatman, Clifton, NJ, USA) or stains/swabs from non-probative casework. DNA purification was performed with phenol/chloroform, Chelex (Bio-Rad, Hercules, CA, USA) or the DNA IQ™ System (Promega Corporation). The extracted human DNA from most samples supplied by individual laboratories was quantified using the Quantiblot® Human DNA Quantitation Kit (Applied Biosystems, Foster City, CA, USA), AluQuant® Human DNA Quantitation System (Promega Corporation) or quantitative, real-time PCR [16]. Most sexual assault samples underwent differential lysis [17] during DNA purification. Microcon® devices (Millipore, Bedford, MA, USA) were used for sample concentration on some non-probative case samples.

2.3. Non-human DNA sources

Some DNA used for the non-human studies was isolated from liquid blood samples kindly provided by Dr. Robert F. Giddings (Kensington Bird and Animal Hospital, CT, USA). Other animal samples were obtained from BIOS Laboratories (New Haven, CT, USA). An isolate of *Candida albicans* was kindly provided by Dr. Kenneth W. Nickerson (University of Nebraska-Lincoln, USA). Isolates were generally purified using an organic extraction [15]. The DNA samples were quantified by spectrophotometric analysis using A_{260} detection [15] or comparison to 12.5–400 ng of DNA standards on an ethidium bromide-stained agarose gel [15] visualized with a Bio-Rad Gel Doc 2000. Some purifications used Millipore Microcon® devices for sample concentration.

2.4. Amplification

Y-STR amplification of the PowerPlex® Y System was performed according to the manufacturer's recommendations with minor modifications specific to individual participant laboratory optimization (described in the product instructions [10]). The amplification enzyme used was AmpliTaq Gold® DNA polymerase (Applied Biosystems). Thermal cycling was typically performed using the GeneAmp® PCR System 9600 and 9700 (Perkin-Elmer, Norwalk, CT, USA) although the GeneAmp® PCR System 2400 and Thermal Cycler Model 480 (Perkin-Elmer) were also evaluated (Section 3.8). Amplifications typically contained 0.5–1 ng of extracted DNA, except where otherwise noted. Final reaction volume used was 25 μL and cycling was performed as described in the manufacturers' instructions (generally 30 or 32 cycles), except where otherwise noted. Some adjudicated samples were previously tested with the D1S80 AmpF*l*P® (VNTR), Ampf*l*STR® Profiler® Plus and COfiler® Amplification Kits (Applied Biosystems) according to supplied protocols.

2.5. Amplification analysis

The systems tested all employ fluorescent labeling using up to four different fluorophores. Electrophoretic separation and sample detection were performed using the ABI PRISM® 310 or 3100 Genetic Analyzer or ABI PRISM® 377 DNA Sequencer (Applied Biosystems) according to manufacturers' instructions. Spectral resolution was performed using color matrix kits Matrix FL-JOE-TMR-CXR or PowerPlex® Matrix Standards, 3100 (Promega Corporation). Sizing was performed using the Internal Lane Standard 600 (ILS 600, Promega Corporation) size standard and the GeneScan® software package (Applied Biosystems). The minimum interpretation peak threshold used was 50–150 relative fluorescent units (RFU). Genotyping was generally performed using the provided PowerPlex® Y Allelic Ladder Mix (Promega Corporation) and Genotyper® software (Applied Biosystems) with the PowerTyper™ Y Macro (Promega Corporation). The effective assay range for the PowerPlex® Y System is 70–340 bases and observations outside of this size range were disregarded. For non-probative casework (Section 3.17) and the reaction volume study (Section 3.3), some laboratories utilized an agarose test gel [15] or an ABI PRISM® 377 DNA Sequencer-based 12 cm acrylamide test/yield gel to estimate total amplification product and accordingly adjusted the volume of post-amplification material to analyze.

2.6. Stutter calculation

A total of 412 male individuals were used to calculate average stutter [18,19] for each marker. The average percent stutter was calculated by dividing the peak height (RFU) of the stutter peak by the peak height of the true allele peak and subsequently multiplying by 100. Calculations were only performed using observable stutter peaks (0% stutter data points were not included) and signal greater then 6000 RFU (using the ABI PRISM® 310 or 3100 Genetic Analyzers) was not used to avoid data anomalies created by signal saturation.

3. Results and discussion

3.1. Proficiency testing and inter-laboratory consistency

Prior to participating in the validation study of the PowerPlex® Y System, each participant laboratory demonstrated proficiency by first correctly typing five blind samples provided by Promega Corporation (data not shown). These samples included four non-cell-line DNA samples and RAJI cell-line DNA with typically 0.5–1 ng template being used for amplification. Inter-laboratory comparison of the proficiency results demonstrated identical typing results for corresponding samples illustrated the typing consistency of the PowerPlex® Y System. The amplification of 1 ng of supplied 9947A DNA (female negative control) and 9948 male DNA (positive control) templates consistently rendered correct negative and positive results, respectively.

3.2. Variation of cycle number

The number of amplification cycles can directly impact the yield of a multiplex assay. Although the design of a multiplex may focus on optimization using a set cycle number or narrow range, alteration of cycle number can be used to change the assay sensitivity. For amplification of 0.25–1 ng of male template, 30–32 cycles are recommended (i.e., 10/20 or 10/22 cycling as described in the protocol supplied with the kit). The majority of testing in each laboratory was successfully performed within these ranges. Each laboratory determined the optimal cycle number for each type of instrument based on the desired amplification sensitivity relative to overall detection sensitivity. Near dropout of some loci was observed with $\leq$1 ng of template using 28 cycles (data not shown). Decreased cycle number (24–28 cycles) was evaluated with replicate 1.2, 1.25, 1.5 and 2.0 mm FTA® punches (blood and buccal swab transfers) added directly to the amplification mix (Table 1). At 27 or 28 cycles, all size punches yielded correct and complete profiles and as few as 24 cycles provided reliable typing results with 1.5 and 2.0 mm blood FTA® punches.

3.3. Variation of reaction volume

The change in volume of a reaction can have only subtle effects if all reagents maintain a constant concentration [20]. However, reduction in reaction volume can significantly increase the observed yield for a constant quantity of template. This can be useful when only minimal template is available. To evaluate the impact of reaction volume on the

Table 1
Variation of cycle number with different size punches of whole blood and buccal swab FTA® transfers/stains

Punch size (type)	Cycles				
	28	27	26	25	24
1.2 mm (buccal swab)	+	+	−	−	−
1.25 mm (buccal swab)	+	+	−	−	−
1.5 mm (buccal swab)	+	+	+/−	−	−
2.0 mm (buccal swab)	+	+	+	+/−	−
1.2 mm (blood)	+	+	+/−	−	−
1.25 mm (blood)	+	+	+/−	+/−	−
1.5 mm (blood)	+	+	+	+	+
2.0 mm (blood)	+	+	+	+	+

Analyses performed in duplicate: +, consistent full amplification; +/−, inconsistent (one negative reaction) or partial profile amplification; −, no amplification. Peak height threshold was 150 RFU.

PowerPlex® Y System, 50, 25, 15, 12.5 and 6.25 μL of reaction volumes were tested with constant template concentrations of 0.2–2.5 ng/25 μL (Fig. 2) and constant template quantities of 0.2–2.5 ng per reaction (data not shown). Prior to analysis, signal was normalized using a post-amplification test gel and 0.025–1 μL of amplified sample was analyzed on an ABI PRISM® 377 DNA Sequencer. Significant locus-to-locus imbalance was observed with the lowest quantities of DNA (50 pg) amplified in the smallest reaction volume (6.25 μL). However, all amplification reactions produced a full profile (Fig. 2). The peak height balance between the two products of DYS385a/b were generally >60%. As expected, the post-amplification gel analysis demonstrated that reduction of reaction volume with a constant template quantity increased the observed yield (concentration) in the 1 μL product analysis (data not shown).

3.4. AmpliTaq Gold® DNA polymerase titration

The concentration of enzyme used in a reaction has an impact on numerous factors of multiplex performance including yield and locus-to-locus signal balance. A titration of the AmpliTaq® Gold DNA polymerase was tested including 0.5×–1.5× enzyme (2.75U/25 μL is standard) at multiple template concentrations using 0.13–1 ng of template.

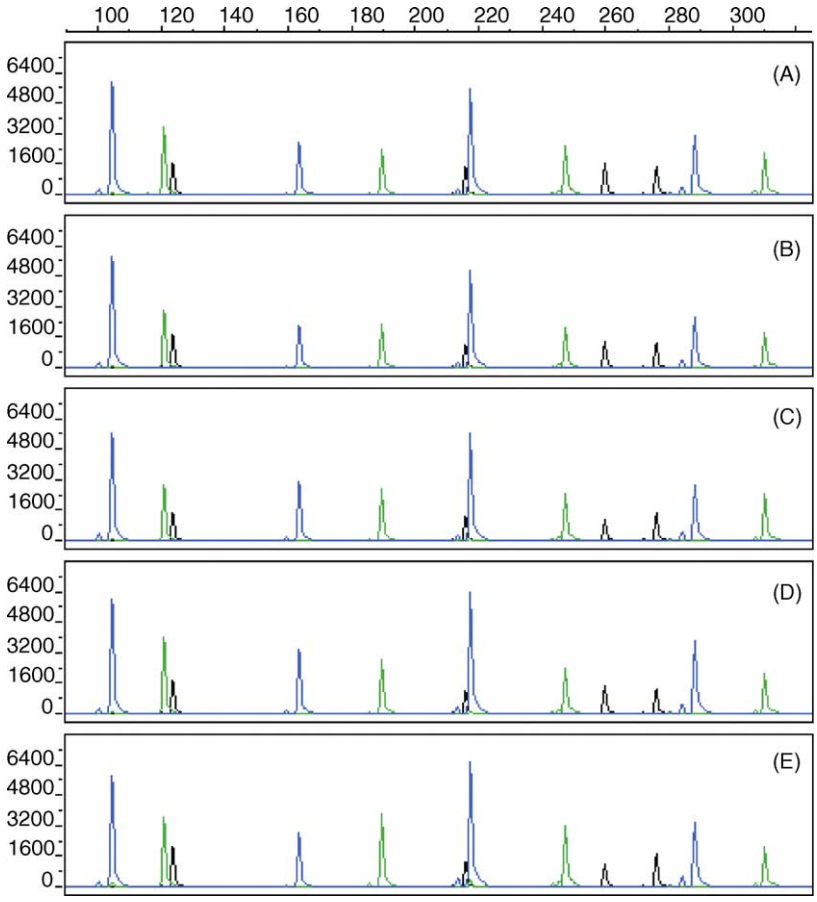

Fig. 2. Variation of reaction volume. Amplifications of 1 ng/25 μL DNA are shown using 50 μL of reaction volume (panel A), the standard 25 μL of reaction volume (panel B), 15 μL of reaction volume (panel C), 12.5 μL of reaction volume (panel D) and 6.25 μL of reaction volume (panel E) analyzed on the ABI PRISM® 377 DNA Sequencer.

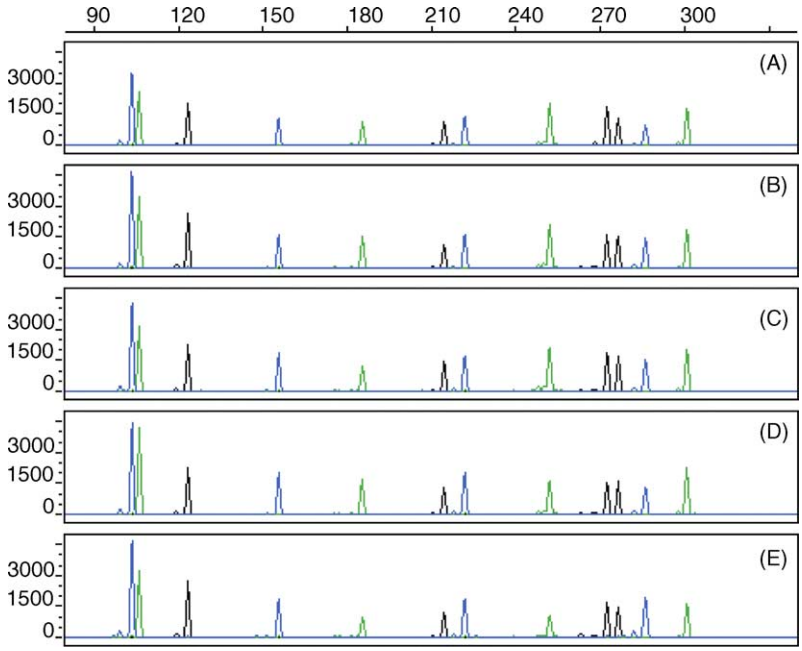

Fig. 3. Variation of AmpliTaq® Gold DNA polymerase. Amplifications of 0.5 ng DNA are shown using 1.38 U enzyme (panel A), 2.06 U enzyme (panel B), the standard 2.75 U enzyme (panel C), 3.44 U enzyme (panel D) and 4.13 U enzyme (panel E) analyzed on the ABI PRISM® 310 Genetic Analyzer.

Minimal change in yield and balance was observed from the standard at any of the concentrations tested (Fig. 3). Locus drop-out was not observed under any of the tested conditions. No notable amplification artifacts were observed with the different enzyme concentrations tested. While changes in concentration of enzyme are not recommended, the assay demonstrated little impact by small changes in enzyme concentration.

3.5. Primer pair titration

In order to achieve relative signal balance in a large multiplex, adjustment of the primer concentration for each marker is often required. To evaluate the impact of total primer concentration variation, a titration including 0.5×, 0.75×, 1×, 1.5× and 2× of the supplied 10× Primer Mix was tested using 0.13–1 ng of template. Relative yield of most loci varied unequally with change in primer concentration (Fig. 4) consistent with other studies [20]. Locus drop-out was not observed under any of the tested conditions. Consequently, such variations should not change the resultant haplotype obtained, however, for more balanced results it is not recommended to alter primer concentration.

3.6. Titration of magnesium

The concentration of magnesium in a PCR can impact the activity and fidelity of the polymerase [21]. This component is included within the supplied 10× Gold ST*R Reaction Buffer at a concentration of 15 mM (10×). A titration of magnesium from 1 to 2 mM per reaction was tested by using a magnesium-free Gold ST*R buffer (Promega Corporation) and 0.13–1 ng of template. Near or complete drop-out of multiple loci was observed in some instances with 1 mM magnesium with the DYS439 locus being the first to drop-out and the locus DYS393 the least affected (Fig. 5). A reduction in yield was observed at some loci with 1.25 mM magnesium. Correct and complete results were obtained with 1.5–2.0 mM magnesium. Some testers noted that yield increased in some instances with increased magnesium (i.e., >1.5 mM). However, locus-to-locus balance was reduced with increased magnesium. No additional amplification artifacts were observed at any magnesium concentration tested and all 9947A female control reactions were negative. Although magnesium cannot be intentionally decreased within the reaction buffer, the effective concentration could be impacted by a significant amount of magnesium-chelating inhibitors. For this reason we recommend that template DNA be stored in low-EDTA buffer such as TE^{-4} (Tris, pH 8.0; 0.1 mM EDTA).

3.7. Variation of annealing temperature

Five laboratories evaluated the effect of raising and lowering the recommended annealing temperature on the

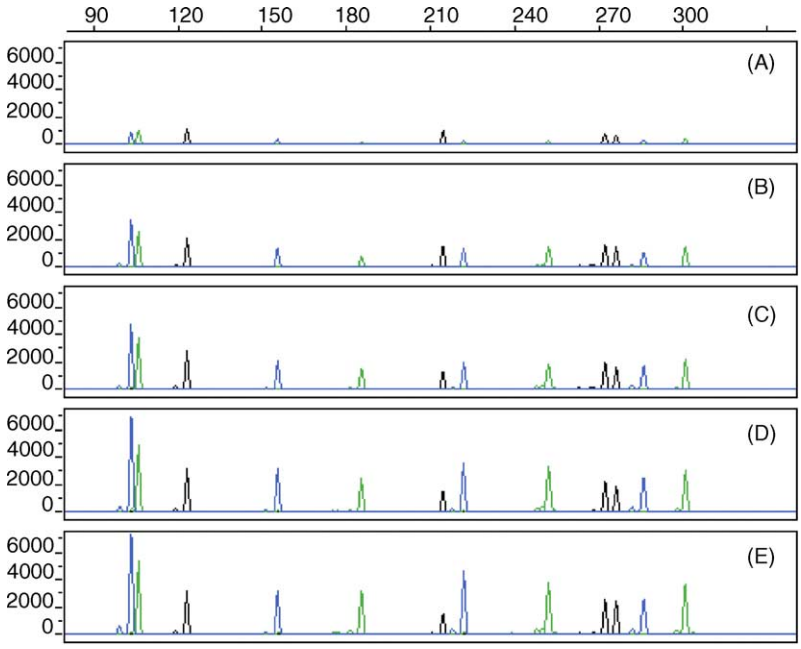

Fig. 4. Variation of primer concentration. Amplifications of 0.5 ng DNA are shown using 0.5× primer (panel A), 0.75× primer (panel B), 1× primer (panel C), 1.5× primer (panel D) and 2× primer (panel E) analyzed on the ABI PRISM® 310 Genetic Analyzer.

ability to produce complete haplotypes and the potential for creating amplification artifacts (Fig. 6). The PowerPlex® Y System's recommended annealing temperature is 60 °C for the first 10 cycles and 58 °C for the remaining cycles (60 °C/58 °C). Laboratories tested ±2 °C and ±4 °C (56 °C/54 °C, 58 °C/56 °C, 60 °C/58 °C, 62 °C/60 °C and 64 °C/62 °C) using 0.5–1 ng of template to simulate the effect of calibration drift sometimes encountered with thermal cyclers. This assay also evaluates primer design for cross reactivity and allele drop-out. Most laboratories observed considerable locus dropout when the annealing temperature was raised 4 °C; only two laboratories noted any locus dropout when the annealing temperature was raised 2 °C. The most commonly observed loci to drop-out with higher annealing temperatures were DYS19, DYS385a/b, DYS389I, DYS390, DYS391, DYS392 and DYS439 while the loci DYS393 and DYS437 were the most robust. New artifacts were not reported with increased annealing temperature. No locus drop-out was observed at or below the recommended annealing temperature. Additionally, no amplification artifacts were observed as a result of reducing the annealing temperature by 2 °C or 4 °C. The PowerPlex® Y System technical manual allows a small decrease in the annealing temperature to compensate for differences in cycler performance as part of a laboratories' internal validation. However, a maximum annealing temperature (without locus drop-out) was chosen for the PowerPlex® Y System to favor male-specificity in male/female mixtures.

3.8. Comparison of thermal cyclers

Four thermal cycler models were tested to ensure amplification success with different instruments. The GeneAmp® PCR System 2400, 9600 and 9700 and Thermal Cycler Model 480 were tested generally using 0.5–1 ng of template. All produced interpretable profiles using the provided protocols (data not shown). For the GeneAmp® PCR System 2400, 9600 and 9700, three identical test sets were amplified with each instrument to evaluate consistency of yield across loci (locus-to-locus balance) on different thermal cyclers. Each test set consisted of 10 different non-cell-line male samples, male cell-line 9948 and female cell-line 9947A (1 ng of each). No amplification was observed for the female negative control. The signal intensity of each locus (RFU) was compared to the total signal across all loci for a sample. Minimal variation in the balance across loci was observed between cycler models. The standard deviation associated with the relative balance of all samples within a test set and across test sets that successfully amplified was 1–3%.

3.9. Genotype consistency/reproducibility and standard specimens

The PowerPlex® Y System was used to type the six male individuals included in the U.S. National Institute of Standards and Technology's (NIST, Gaithersburg, MD) Standard Reference Material (SRM) 2395 kit. Both the authors and NIST (personal communication, John Butler) observed that

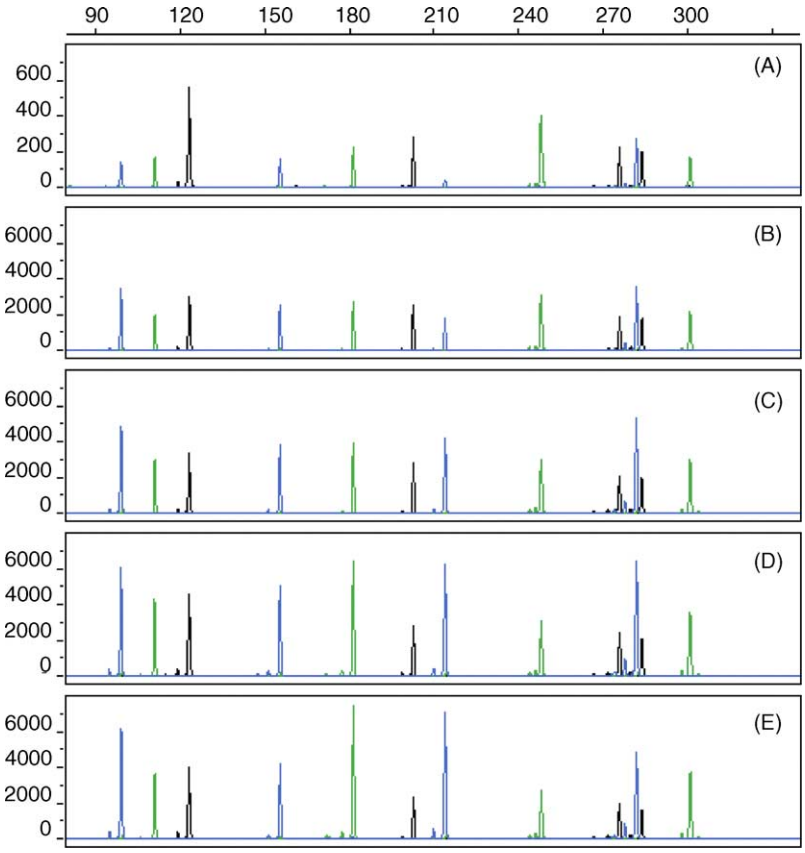

Fig. 5. Variation of magnesium concentration. Amplifications of 0.6 ng of DNA are shown using 1 mM magnesium (panel A), 1.25 mM magnesium (panel B), the standard 1.5 mM magnesium (panel C), 1.75 mM magnesium (panel D) and 2 mM magnesium (panel E) analyzed on the ABI PRISM® 3100 Genetic Analyzer.

all typings were found to be concordant with those indicated in the supplied documentation. In addition to the 9948 male DNA that is provided as a positive control (see Section 3.1), the NIST SRM 2395 samples can be used as a basic performance test.

The primers used in the PowerPlex® Y System were tested as individual primer pairs using 0.25–1 ng RAJI cell-line DNA. All monoplex amplifications were shown to type identical to the corresponding marker in the multiplex as follows: DYS19 allele 15, DYS385a/b alleles 16 and 19,

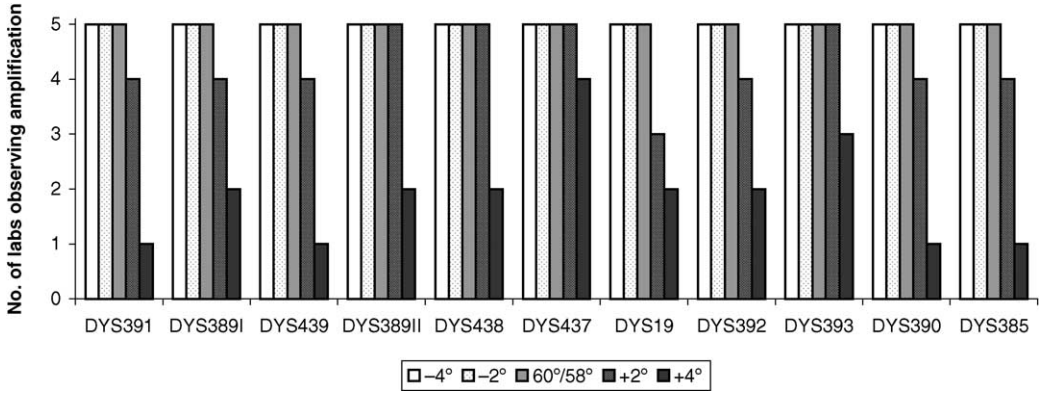

Fig. 6. Variation in annealing temperature. The ability to amplify each locus at 2 and 4 °C above and below the recommended annealing temperature (60 °C/58 °C) was evaluated by five laboratories.

DYS389I allele 14, DYS389II allele 31, DYS390 allele 21, DYS391 allele 10, DYS392 allele 11, DYS393 allele 14, DYS437 allele 14, DYS438 allele 12 and DYS439 allele 11. Liquid blood, dried blood stains stored on FTA® paper, buccal swabs and buccal swabs transferred to FTA® paper, collected from the same individual, amplified and produced identical profiles (data not shown).

3.10. Sensitivity

To evaluate the minimum required input template, two titrations of male DNA (0.03–1 ng) were tested by seven laboratories. Each laboratory obtained full profiles with ≥0.125 ng of template (Fig. 7). The most commonly observed loci to drop-out at the lowest concentrations were DYS19, DYS385 and DYS390 while the loci DYS389II, DYS391 and DYS439 were the most robust. Most laboratories reported partial profiles could be observed at both 62.5 and 30 pg. This result suggests that so-called ''low-copy number'' (LCN) analysis is possible using the standard protocol. Flexibility in the analysis process should be considered (injection time/loading volume, volume of amplified sample prepared, etc.) during internal validation to allow signal attenuation or maximization based on what is appropriate for the sample. A benefit of Y-STR analysis with low quantities of template can be the reduced risk of misinterpretation from stochastic effects. Drop-out for Y-STR loci will produce no information (locus drop-out) except in cases of male/male mixtures and the DYS385a/b marker. In autosomal markers, allelic drop-out creates potential for misinterpretation at every heterozygous locus [20]. However, as with autosomal STR analysis, excess target template DNA (male DNA for Y-STRs) should be avoided to minimize excess stutter, signal saturation, spectral bleed-through and artifacts [10].

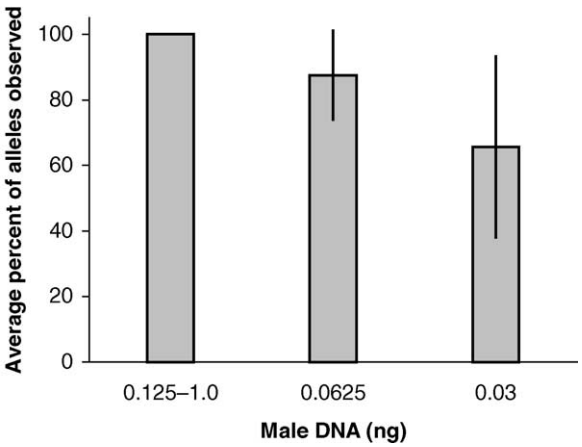

Fig. 7. Template sensitivity. Seven laboratories assessed two titrations of male DNA to determine where locus drop-out may begin to be observed. The bar indicates one standard deviation from the average percent of alleles observed.

3.11. Male-specificity

A titration of two separate female DNA samples (0–500 ng) were tested to evaluate the Y chromosome-specificity of the PowerPlex® Y System. No reproducible peaks were observed over minimum signal threshold with any quantity of female DNA (Fig. 8A and B). A male DNA sample was amplified in the presence of these female DNA samples to demonstrate PCR inhibitors were absent from the female DNA preparations (Fig. 8C and D).

3.12. Male/female mixture analysis

Six laboratories tested two different male/female mixture series including the following male to female input DNA: 1:0 ng, 1:1 ng, 1:10 ng, 1:100 ng, 1:300 ng, 1:1000 ng, 0.5:300 ng, 0.25:300 ng, 0.125:300 ng, 0.0625:300 ng and 0.03:300 ng (Fig. 9). This series tested mixtures using: (1) a constant, recommended amount of male DNA (i.e., 1 ng) with the quantity of female DNA increasing (i.e., 0–1000 ng), and (2) decreasing quantities of male DNA (i.e., 0.03–0.5 ng) with a constant and significant quantity of female DNA (i.e., 300 ng). Even with a microgram quantity of female DNA, all alleles were observable using the recommended quantity of male DNA. Only as male quantities decreased with mixture ratios exceeding 1200-fold more female DNA did some laboratories begin to observe locus drop-out. The DYS19, DYS385, DYS392 and DYS438 loci were most commonly observed to drop-out at the lowest concentrations of male DNA while the loci DYS391, DYS393 and DYS437 were the most robust. The average number of expected alleles observed by laboratories was 66% with 10,000-fold mixtures. These results demonstrate that the assay can tolerate significant amounts of a human DNA mixture (mostly female in origin) and maintain specificity and performance when a sufficient quantity of male DNA (0.25–1 ng) is present, contrary to autosomal analysis. These data suggest that this multiplex has the level of male-specificity and sensitivity to reliably produce results for the levels of male/female mixtures encountered in many forensic DNA samples.

3.13. Male/male mixture analysis

In addition to evaluation of male/female mixtures, the effect of two reactive individuals was evaluated with two series of male/male mixtures. Using 1 ng total DNA, two series of two males were mixed at the following ratios: 1:0, 19:1, 9:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:9, 1:19 and 0:1. Drop-out of unshared minor contributor alleles began to be observed for the minor contributor when it constituted 5–10% or less of the total sample (Fig. 10). This is similar to results obtained with autosomal STR analyses [20] and is influenced greatly by overall signal intensity and the absolute quantity of DNA from either contributor in addition to the ratio of mixture.

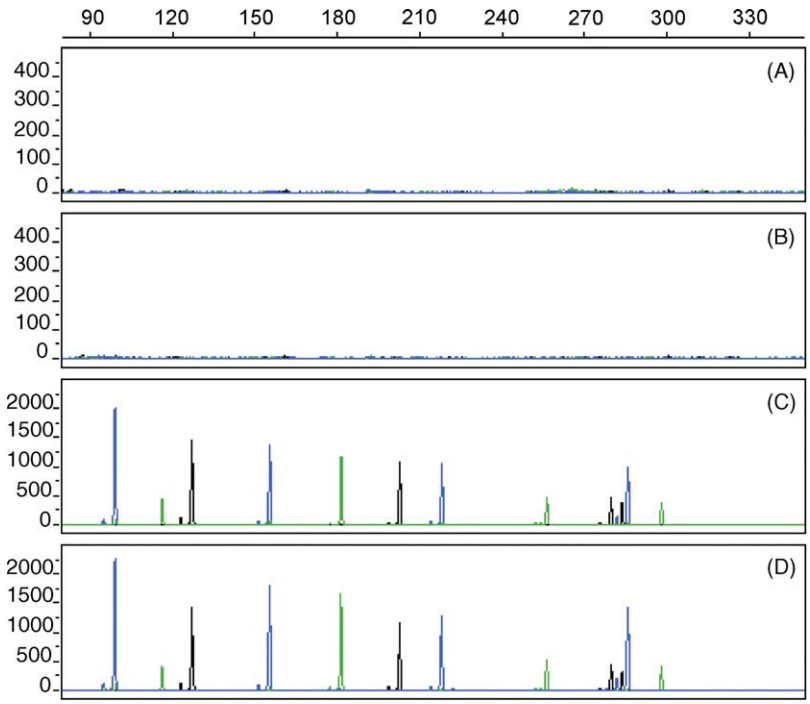

Fig. 8. Male-specificity. Two female DNA samples were analyzed with the PowerPlex® Y System. Female #1 (A) was amplified using ~390 ng of DNA and female #2 (B) was amplified using ~460 ng of DNA without male DNA. These DNA samples were also amplified using 500 ng of female #1 (C) and female #2 (D) in the presence of 0.5 ng of male DNA. Analysis was performed using the ABI PRISM® 3100 Genetic Analyzer.

3.14. Average stutter

"Stutter" is a common artifact inherent in STR amplification. These products can be observed migrating at positions usually one repeat less than the true allele (and less likely one repeat larger than the true allele). The most accepted mechanism for stutter generation is that of repeat slippage during the primer extension stage of PCR [18,19]. The repeat sequence itself, along with strand directionality of the labeled product, affect the degree of stutter commonly observed relative to the true allele. For all loci, stutter was observed migrating one repeat unit smaller then the true allele: i.e., three bases smaller ($N - 3$) for the trinucleotide marker (DYS392), four bases smaller ($N - 4$) for the tetranucleotide markers (DYS19,

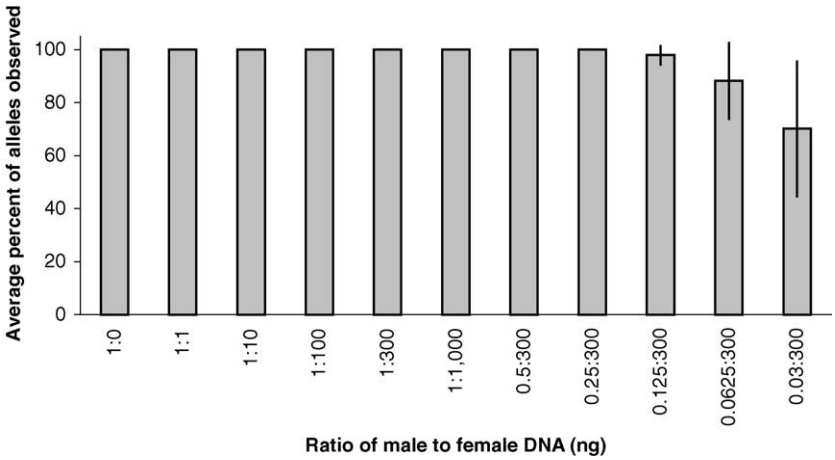

Fig. 9. Male/female DNA mixture analysis. Seven laboratories tested two different male/female mixture series. Each mixture ratio presented 24 expected alleles between the two series. The average percent of observed alleles for all laboratories is indicated for each mixture ratio. The bar indicates one standard deviation.

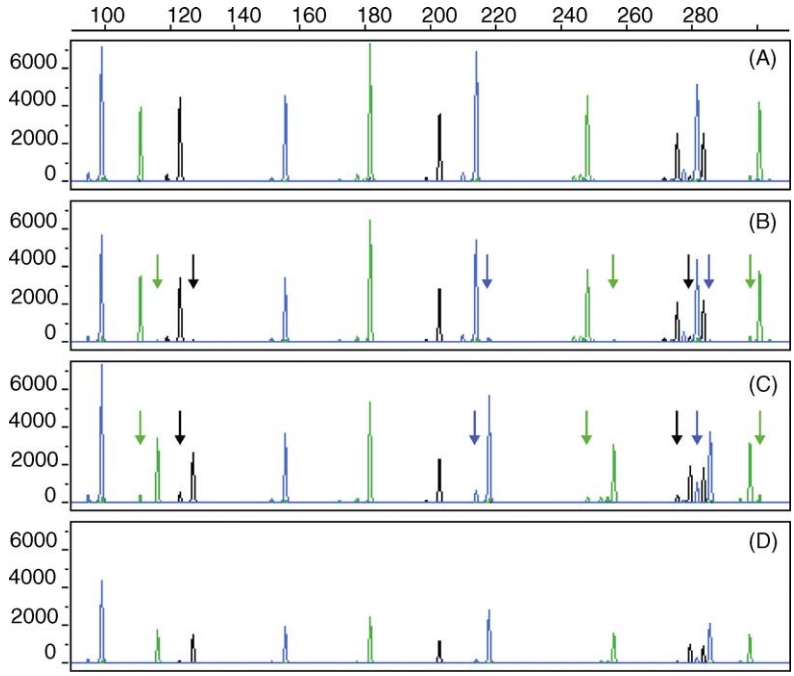

Fig. 10. Male/male mixture analysis. Amplifications of 1 ng of total template from a series of male/male mixtures. Shown are 1 ng male #1 (panel A), 0.95 ng male #1 with 0.05 ng male #2 (panel B), 0.05 ng male #1 with 0.95 ng male #2 (panel C) and 1 ng male #2 (panel D) analyzed on the ABI PRISM® 3100 Genetic Analyzer. Arrows indicate unshared minor contributor alleles.

DYS385, DYS389I/II, DYS390, DYS391, DYS393, DYS437 and DYS439) and five bases smaller ($N - 5$) for the pentanucleotide marker (DYS438, Fig. 11). In addition to stutter product, one repeat unit smaller in length than the true allele, stutter was observed at the following positions: DYS19, $N - 2$ [6]; DYS392, $N + 3$; and DYS385, $N - 9$ (two bases smaller, three bases larger and nine bases smaller, respectively). Within each locus, the relative intensity of stutter and/or rate of observed stutter generally increased with the allele length (data not shown). Stutter products were found to be predictable and did not impact interpretation more than previously studied STR systems.

3.15. Sizing precision

Central to the typing of length-based polymorphisms is defining the precision of measurements based on migration during electrophoresis. To evaluate the consistency of frag-

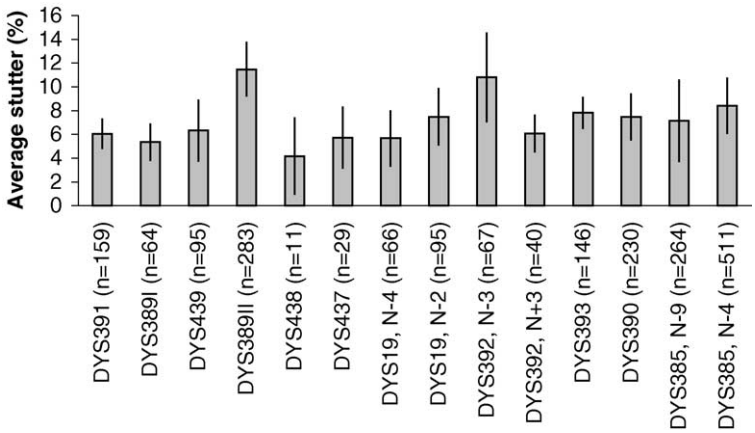

Fig. 11. Average stutter. The average intensity of the common stutter products was calculated. The average stutter listed is for the product observed one repeat unit smaller then the true allele, unless indicated by "$N - x$". The bar indicates one standard deviation from the mean. The number of samples is listed as "n".

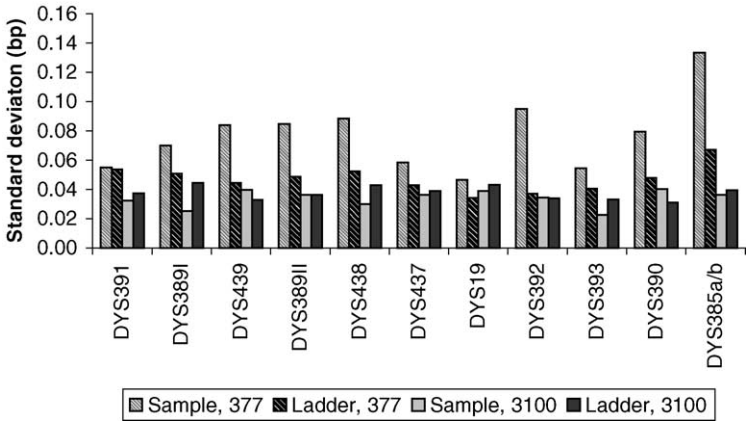

Fig. 12. Sizing precision. The standard deviation from the average allele size(s) is charted for each locus. Ten replicates from a single injection were used from the ABI PRISM® 3100 Genetic Analyzer and eight replicates from separate gel runs were used from the ABI PRISM® 377 DNA Sequencer.

ment sizing for the PowerPlex® Y System, 10 replicate analyses of the PowerPlex® Y Allelic Ladder Mix and an amplified sample were analyzed on the ABI PRISM® 3100 Genetic Analyzer and eight replicate analyses were analyzed on the ABI PRISM® 377 DNA Sequencer. The average allele size(s) were calculated for each locus and the standard deviation (sample) or average standard deviation (ladder) from that mean is shown in Fig. 12. The PowerPlex® Y System demonstrated a high degree of precision (S.D. $<$ 0.15 bp) that, in part, could be attributed to the consistently performing size standard, ILS 600 [22]. The true product length based on sequencing data has been reported in the product's technical manual for the range of alleles in each locus' allelic ladder [10]. Because the true allele length may not always be the value obtained on different electrophoretic instruments, allelic ladders are used to normalize data.

3.16. Non-human studies

In forensic DNA analyses, an assay with a high degree of human-specificity can reduce interpretation complexity regarding the impact of contaminant non-human DNA. The PowerPlex® Y System was tested with 24 different non-human DNA samples. Table 2 describes the gender (if known), quantity of DNA (1–50 ng) and amplification results for each sample. Sources included mammal, bird, fish, reptile, amphibian and yeast DNA. With the exception of the two chimpanzees, the entire animal DNA panel did not yield results with the PowerPlex® Y System. Chimpanzee #1 showed amplicons in and/or near the DYS389I, DYS392, DYS393, DYS437 and DYS439 assay ranges, while chimpanzee #2 showed one amplicon in the DYS393 assay range.

Table 2
Non-human DNA analysis

Individual sources	Gender	Quantity tested (ng)	Amplification result
Dog #1 (German Shepard), dog #2 (mixed-breed), duck, cat #1	Male	50	Negative
Macaque	Male	1, 10	Negative
Chimpanzee #1	Male	1, 10	Within and/or near DYS389I, DYS392, DYS393, DYS437 and DYS439 assay range
Chimpanzee #2	Male	1, 5	Within DYS393 assay range
Candida albicans	Not applicable	50	Negative
Pig, bovine, galliform, dog #5, rabbit, mouse, sheep, fish, frog, rat	Unknown	1, 10	Negative
Ferret	Unknown	50	Negative
Iguana, cat #2, dog #3 (German Shepard), dog #4 (mixed-breed)	Female	50	Negative
Gorilla	Female	1, 10	Negative

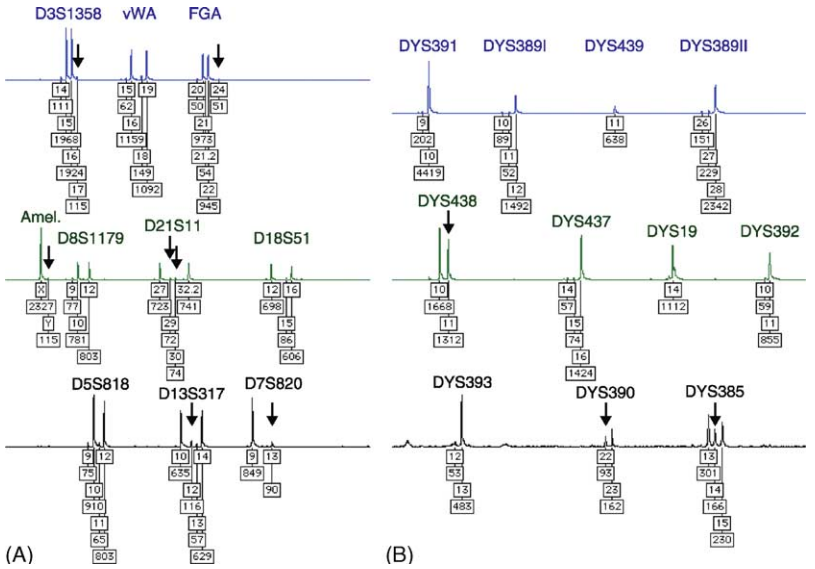

Fig. 13. Non-probative case sample analysis. DNA extracted from an acid phosphatase/P30-positive bed sheet stain was quantified for total human DNA using quantitative, real-time PCR. Amplifications of 1 ng total human template (male/female mixture) with the Ampf*l*STR® Profiler® Plus Amplification Kit (A) and the PowerPlex® Y System (B) were analyzed on the ABI PRISM® 377 DNA Sequencer. Arrows in the autosomal analysis (A) indicate presence of a male minor contributor. Arrows in the Y-STR analysis (B) indicate the presence of a second male contributor.

3.17. Non-probative case studies

One of the informative tests of a new assay for human identification is the reanalysis of case samples to ensure that the system can analyze these samples and that the outcome of the new method is consistent with findings generated by previous methods. Three laboratories used the PowerPlex® Y System to reanalyze 102 samples from 65 adjudicated cases, collectively (data not shown). Previous analysis methods included the AmpF*l*P® D1S80, Ampf*l*STR® Profiler® Plus and COfiler® Amplification Kits. Sample types included fabric/clothing stains, cigarette butts, condom swabbing, fingernail cuttings, common sexual assault swabs and miscellaneous skin swabs. Both fractions of differentially extracted material [17] were tested. Samples included single-source male and female samples, multiple male mixtures and male/female mixtures. A number of male/female mixture samples were notably limited in male template (i.e., 0.5–1 ng total mixture DNA were used rather than 0.5–1 ng male DNA). Many samples were estimated to contain picogram quantities of male DNA. Of the 102 samples tested, 76 samples generated complete Y-STR profiles, 23 samples generated partial profiles and three samples did not produce results. The three failed amplifications were likely due to inadequate male DNA or potential degradation of the Chelex-extracted sample during storage. All positive PowerPlex® Y System results were consistent with the previous findings of these cases. In a number of mixture samples, additional information was obtained from the minor contributor beyond the initial autosomal analysis using the PowerPlex® Y System (Fig. 13).

4. Conclusion

The results of this collaboration demonstrate the reliability and robustness of the PowerPlex® Y System as a genotyping tool for forensic DNA samples. In addition to the population study reported by Budowle and colleagues using the PowerPlex® Y System [12], these findings represent considerable support for the use of this system in human identification. This developmental validation is intended to not only provide results with which individual laboratories can compare for concordance but also to reduce the number of studies each laboratory needs to repeat to implement this assay. The developmental studies presented here allow laboratories to focus on optimization of protocols to best integrate the PowerPlex® Y System with existing procedures and instrumentation.

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