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PAPER

CRIMINALISTICS

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Performance Evaluation and Optimization of Multiplex PCRs for the Highly Discriminating OSU 10-Locus Set Y-STRs*,†

ABSTRACT: In a previous study, a new set of Y-chromosome short tandem repeats, the OSU 10-locus set (MPM1 and MPM2), was shown to have a higher discrimination power when evaluated against the 10 SWGDAM loci on a common population panel. Here, we describe the optimization of the multiplex reactions using dye-labeled primers followed by performance evaluations. The loci exhibited high precision, human male specificity, reliability in different body fluids, high sensitivity, stability, and the ability to amplify nonprobative casework and mixture samples. Stutter for the all of the loci, with the exception of the highly polymorphic locus DYS688, was similar to that observed for autosomal loci. The results of the performance evaluations reinforced the utility of these loci.

KEYWORDS: forensic science, short tandem repeats, microsatellites, Y-chromosome, DYS448, DYS487, DYS488, DYS504, DYS576, DYS657, DYS685, DYS688, DYS703, DYS707

Y-chromosome short tandem repeats (Y-STRs) are highly useful in forensic casework to identify the male component in male/female body fluid mixtures and the identification of paternity even in the case of a deceased putative father. In 2005, the Scientific Working Group on DNA Analysis Methods (SWGDAM) selected 10 loci for forensic use (1). In several studies and Y-STR haplotype databases, common nonunique haplotypes have been observed with the SWGDAM set (http://www.usystrdatabase.org, accessed Sept. 16, 2010; http://YHRD.org, accessed Sept. 16, 2010) (2–4). Additional polymorphic Y-STR loci would greatly improve the discrimination power of the SWGDAM set. Several kits are currently available, which contain additional Y-STR loci to augment the SWGDAM set, Y-filer with five additional loci (5) and PowerPlex® Y with one additional locus (6,7). Recently, a new set of highly informative Y-STRs, The Ohio State University (OSU) 10-locus set, was identified from a library of 465 loci found on the Y-chromosome outside of the two concentrated regions of frequently used loci (8). In a comparative study with the SWGDAM loci on two common population panels of African Americans and Caucasians, the OSU 10-locus set displayed a greater average number of alleles, a greater average gene diversity, a lower number of nonunique haplotypes, and thus a higher power of discrimination (4). The increased power of discrimination of this set of loci is illustrated by the fact that 45 pairs of individuals with identical haplotypes, as assessed by the SWGDAM loci set, were uniquely identified with the OSU set (4). Based on several studies, it appears that many of the OSU loci are more polymorphic than the additional loci used in the kits to augment the SWGDAM set.

To further evaluate the OSU 10-locus set and test the effectiveness of the markers on forensic samples, the polymerase chain reaction (PCR) conditions for two multiplex reactions were optimized for dye-labeled primers and the loci were evaluated for precision, stutter, specificity, reliability, sensitivity, and the ability to successfully amplify nonprobative casework samples and mixture samples.

Methods

Sample Collection and Preparation of Body Fluid Stains

The human-use procedures utilized were approved by the University of Central Florida's Institutional Review Board. Venipuncture was used to draw liquid blood samples, and 50 μL aliquots were dried onto cotton swatches at room temperature. Discrete aliquots of semen were packaged in sealed plastic tubes and frozen. The semen specimens were subsequently dried onto sterile cotton swabs. Buccal samples and nonprobative casework specimens were collected using sterile swabs. Contact DNA, obtained for one of the nonprobative casework evaluations, was collected with a moist swab from the outer rim of a plastic coffee cup lid after the cup was used by a male subject. Two sets of postcoital

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cervicovaginal swabs were collected using sterile cotton-tipped applicators (Puritan, Guilford, MA), for the other nonprobative casework study, by a female participant 48 and 60 h subsequent to copulation. To test for cross-reactivity with other DNA sources, the following nonhuman blood samples were used: dog and cat (Tuscanilla Oaks Animal Hospital, Orlando, FL); sheep, horse, and bull (Hemestat Laboratories, Dixon, CA); and male and female chimpanzees, male and female gorillas, and male and female orangutans (Coriell Cell Repository, Camden, NJ). Additionally, the surface of several bacterial culture samples donated by the University of Central Florida's Molecular Biology Department (*Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Enterobacter aerogenes*, and *Pseudomonas aeruginosa*) was swabbed using a sterile cotton swab. All samples were stored at −47°C until needed.

Positive and Negative Controls

Throughout the study, positive and negative controls were employed. One male sample, 9948 (Applied Biosystems, Foster City, CA), was used as the positive control. Several negative controls were utilized including a reagent blank for each set of extractions and an amplification blank. During the specificity studies, an additional negative control containing 300 ng of female DNA was utilized.

DNA Isolation and Purification

Organic extractions were conducted to isolate DNA. Bloodstains or stained swabs were cut into small pieces and placed into a Spin-Ease tube (Gibco-BRL, Grand Island, NY). Samples were incubated in 400 μL of DNA extraction buffer (100 mM NaCl, 10 mM Tris–HCl, pH 8.0, 25 mM EDTA, 0.5% SDS) overnight in a 56°C water bath. The extraction buffer was modified for stained swabs containing semen to facilitate the extraction of DNA from sperm cells with 0.1 mg/mL Proteinase K and 40 μL of 0.39 M DTT. Following incubation, the stained pieces were removed from the extraction buffer and placed into Spin-Ease baskets (Gibco-BRL). The Spin-Ease baskets were then inserted into the original tubes and centrifuged at $14{,}000 \times g$ for 5 min. The baskets and stained pieces were removed, and a volume of phenol/chloroform/isoamyl alcohol equal to the extract volume was added to each tube, which was mixed by shaking. Subsequently, each tube was subjected to centrifugation followed by removal of the aqueous layer, containing the DNA.

Two different methods were used to purify the DNA. DNA collected with swabs was precipitated in cold absolute ethanol (two and a half times the volume of the aqueous layer extract) overnight at −20°C. The DNA was then pelleted by centrifugation, washed once in 70% ethanol, and resolubilized with 100 μL of TE^{-4} (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.5) overnight at 56°C. Extract obtained from bloodstains was purified using Centricon 100™ concentrators (Millipore, Bedford, MA), according to the manufacturer's instructions. DNA was eluted using 100 μL of TE^{-4} (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.5), and the samples were kept at 4°C until analysis.

DNA Quantification

The quantity of DNA was determined by comparison of ethidium bromide (Fisher Scientific, Norcross, GA)-induced fluorescence on a 1% agarose yield gel with a set of DNA reference standards of known concentration, 25 pg–5 ng.

Polymerase Chain Reactions

Two multiplex systems, Multiplex Maybruck 1 (MPM1; DYS576, DYS504, DYS688, DYS487, and DYS707) and Multiplex Maybruck 2 (MPM2; DYS448, DYS488, DYS471, DYS685, and DYS703), have previously been designed (8). As fluorescently labeled dNTPs were used to design the multiplexes, the amplification reactions were optimized for dye-labeled primers. Sequences of all 10 primer pairs have been published previously (4). Amplification of MPM1 was carried out in reactions of 25 μL final volume, which contained 3 ng of template DNA unless otherwise stated, 0.38–0.88 μM primers (FAM-DYS576—0.38 μM; FAM-DYS504—0.80 μM; VIC-DYS688—0.44 μM; FAM-DYS487—0.50 μM; VIC-DYS707—0.88 μM [Invitrogen, Grand Island, NY and Applied Biosystems]), 1 mM dNTPs, 1× PCR Buffer II (10 mM Tris–HCl, pH 8.3, 50 mM KCl), 2.25 mM $MgCl_2$, 10 μg of nonacetylated bovine serum albumin (Sigma-Aldrich, St. Louis, MO), and 1.25 units of AmpliTaq Gold DNA Polymerase (Applied Biosystems). The MPM2 amplification was also carried out in reactions of 25 μL final volume reactions, which contained 3 ng of template DNA unless otherwise stated, 0.25–0.44 μM primers (FAM-DYS448—0.25 μM; VIC-DYS488—0.25 μM; NED-DYS471—0.25 μM; VIC-DYS685—0.25 μM; FAM-DYS703—0.44 μM [Invitrogen and Applied Biosystems]), 1 mM dNTPs, 1× PCR Buffer II, 2.25 mM $MgCl_2$, 10 μg nonacetylated bovine serum albumin, and 1.75 units of AmpliTaq Gold DNA Polymerase. The PCR cycling conditions for both multiplex reactions were as follows: (i) 11-min heat-soak at 95°C, (ii) 28 cycles of 1 min at 94°C, 1 min at 60°C, and 30 sec at 72°C, and (iii) a final extension at 60°C for 60 min. All PCR amplifications were carried out in an Applied Biosystems 9700 Gene Amp PCR System thermal cycler.

Allelic Detection and Genotyping

The PCR product was detected in the same manner for both multiplex reactions. A 1.0-μL aliquot of the amplified product was added to 8.7 μL of deionized formamide (Applied Biosystems) and 0.3 μL GeneScan 500 LIZ internal lane standard (Applied Biosystems). Ninety-six well plates containing the prepared samples were heated at 95°C for 3 min and snap-cooled on ice for 3 min. Samples were injected using Module G5 (5 sec injection, 15 kV, 60°C). Fluorescently labeled products were separated and detected using a 3130 Genetic Analyzer (data collection v3.0; Applied Biosystems), and data were analyzed with GeneMapper Analysis Software v3.7. Alleles were noted whether the allele peak was equal or greater than the peak detection threshold of 50 relative fluorescence units (RFUs). Allele designations were determined based on variant and nonvariant repeats and comparison with the reference sequence sizes in the human genome sequence for the Y-chromosome in the GenBank database (8).

Optimization of PCR Parameters

The following PCR amplification conditions were optimized for both multiplex reactions: primer concentrations, magnesium concentrations, DNA polymerase concentrations, and thermal cycling parameters. Each empirical study was assessed for optimal amplification conditions with 3 ng of male DNA. Optimal amplification conditions are those in which artifacts and stutter products were reduced and higher peak heights with better locus balance across all loci were obtained. Primers were tested independently and subsequently in the multiplex system using male DNA as well

as 300 ng of female DNA. The magnesium concentration was evaluated utilizing 1.5–3.25 mM $MgCl_2$ in 0.25-mM increments. The DNA polymerase concentration was analyzed for 1.0–2.75 units in 0.25-unit increments.

To determine the optimal cycling conditions for co-amplification of all loci in each multiplex, the following thermal cycling conditions were tested: annealing temperature and time, extension time, and number of amplification cycles. The annealing temperatures were examined at 58, 60, and 62°C. The length of the annealing step and the extension step was evaluated for 30 sec, 1 min, and 1 min 30 sec. Finally, the optimum number of amplification cycles for both multiplexes was analyzed for 26, 28, 30, and 32 cycles.

Multiplex System Performance

All loci in both MPM1 and MPM2 were evaluated for precision and stutter. Precision was demonstrated by injecting the same sample 20 times using capillary electrophoresis and calculating the standard base pair deviation for each locus. Stutter was evaluated in 20 male samples in which the peak height ratios of stutter peak to true allele peak were calculated. Because of the diversity of the loci, these 20 samples represented 20 different haplotypes.

Two levels of specificity were evaluated for each multiplex: species specificity and human male specificity. Cross-reactivity of the following nonhuman species was analyzed with 3 ng of DNA: dog, cat, sheep, horse, bull, male and female chimpanzees, male and female gorillas, male and female orangutans, and several bacterial samples (*E. coli, S. aureus, S. pyogenes, E. aerogenes*, and *P. aeruginosa*). MPM1 and MPM2 were also assessed for human female amplification with the following quantities of female DNA: 3, 30, 300 ng, and 1 μg.

The sensitivity of each multiplex reaction was tested with different quantities of DNA from a single male individual. The quantities tested included 5, 3, 1 ng, and 500, 250, 150, 125, 100, 50, 35, 25 pg.

Stability Studies

Stability studies were conducted to evaluate the two multiplexes for haplotype reproducibility in body fluids and in environmentally challenging conditions. Blood, semen, and saliva from a single male individual were amplified with both MPM1 and MPM2 to ensure that identical Y-STR haplotypes were obtained from different body fluids.

Both multiplexes were also evaluated for Y-STR haplotype reproducibility in environmentally compromised blood samples. Human blood samples previously dried onto cotton swatches were exposed to the following environmental conditions: room temperature, envelope-dried (RTED), UV (shortwave at room temperature, ultraviolet light), heat, light, humidity, and rain (HLHR; outside exposure to HLHR), and heat, light, and humidity (HLH; covered and outside exposure to HLH). Samples from each type of exposure were collected and analyzed after the following times: 3 days, 1 week, 1 month, 3 months, 6 months, and 1 year. The 1-year UV sample was inadvertently discarded during the extraction process, but a sample exposed to UV light for 18 months was available for testing and was included in the analysis. The 18-month exposure samples for the other conditions were not available for testing.

The specimens exposed to outdoor elements, HLH and HLHR, were placed in an unwooded area in Central Florida. The weather conditions during 1 year of the study consisted of temperatures ranging from 32 to 94°F (average high of 88°F and average low of 37°F) and rain for 137 days (3-day samples—1 day of rain; 1-week samples—1 day of rain; and 6-month samples—73 days of rain).

Mixture Studies

Mixture studies were evaluated for male/female mixtures as well as male/male mixtures. Male/female mixtures consisted of 1 ng of male DNA amplified with up to 5000 times more female DNA in the following ratios: 1:1 (1 ng male DNA: 1 ng female DNA), 1:10 (1 ng male DNA: 10 ng female DNA), 1:100 (1 ng male DNA: 100 ng female DNA), 1:500 (1 ng male DNA: 500 ng female DNA), 1:1000 (1 ng male DNA: 1 μg female DNA), and 1:5000 (1 ng male DNA: 5 μg female DNA).

The male/male DNA mixtures involved two unrelated males combined in the following manner: 1:1 (1.5 ng male DNA: 1.5 ng male DNA), 1:2 (1.0 ng male DNA: 2.0 ng male DNA), 1:5 (0.5 ng male DNA: 2.5 ng male DNA), 1:11 (0.25 ng male DNA: 2.75 ng male DNA), 1:19 (0.15 ng male DNA: 2.85 ng male DNA), and 1:29 (0.1 ng male DNA: 2.9 ng male DNA). In each case, 3 ng of the admixed DNA was tested.

Statistical Analysis

The counting method was employed to evaluate the discrimination power of the OSU set. The sample population from the previous study was utilized as a database (4). The calculation of the upper 95% confidence limit for a haplotype observed in the database was $p \pm 1.96\sqrt{p(1-p)/n}$, where $p = x/n$, x = number of observations of the haplotype in the database, and n = size of the database (9). The calculation of the upper 95% confidence limit for a haplotype not observed in the database was $1 - (0.5)^{1/n}$ (9).

Results and Discussions

Optimization of PCR Parameters

During the initial study in which the loci for the OSU 10-locus set were selected, amplicons were labeled with fluorescently labeled dNTPs for visualization (8). In this study, the multiplex amplification reactions were optimized for fluorescent dye-labeled primers (Fig. 1) to obtain peak height balance across all loci. MPM1 and MPM2 were designed with a five-dye based system, using FAM (blue), VIC (green), NED (yellow), PET (red), and LIZ (orange, internal size standard). To optimize the conditions for amplification and scoring, the following parameters were considered in order: primer concentration, magnesium concentrations, polymerase concentration, annealing temperature, annealing time, extension time, and number of PCR cycles.

Various primer concentrations were evaluated to obtain peak height balance across all loci with acceptable signal intensity and limited or absent stutter or artifact. The resulting primer concentrations for MPM1 and MPM2 were as follows: DYS576 (0.38 μM), DYS504 (0.80 μM), DYS688 (0.44 μM), DYS487 (0.50 μM), DYS707 (0.88 μM), DYS448 (0.25 μM), DYS488 (0.25 μM), DYS471 (0.25 μM), DYS685 (0.25 μM), and DYS703 (0.44 μM). Lower primer concentrations generated a signal intensity that was reduced or absent. Higher primer concentrations increased the stutter levels and artifact production.

Additional PCR reagents as well as thermal cycling conditions were also evaluated to obtain the best conditions for the amplification of MPM1 and MPM2. The magnesium concentration in the initial study of these loci was 2.5 mM (8). Therefore, magnesium concentrations of 1.5–3.25 mM were tested in 0.25-mM increments.

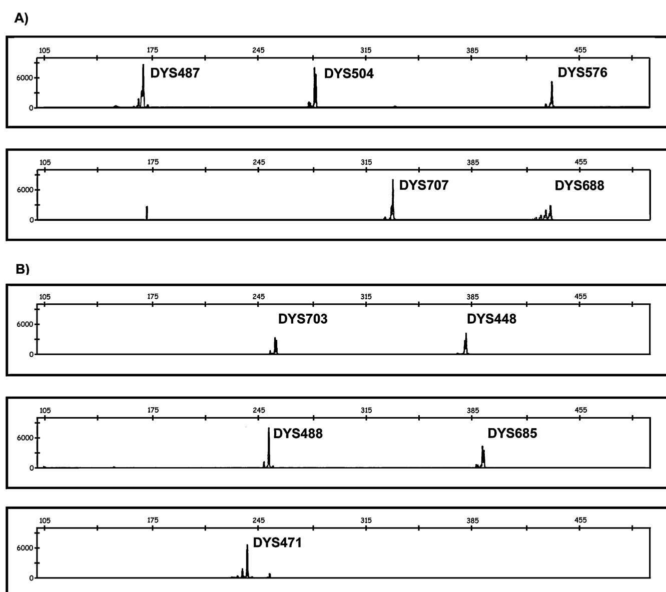

FIG. 1—*Electropherograms of the Ohio State University 10-locus set. (A) Multiplex M1—five-locus male haplotype obtained using 3 ng of input male DNA. (B) Multiplex M2—five-locus male haplotype obtained using 3 ng of input DNA. The x-axis of each electropherogram represents fragment size (bp) and the y-axis represents signal intensity (relative fluorescence units [RFU]).*

Both multiplexes performed well with magnesium concentrations from 2.0 to 2.75 mM. In MPM1, the amplification of DYS487, DYS576, and DYS707 was not optimal with concentrations below 2.0 mM. Concentrations above 2.75 mM resulted in the failure of DYS504 to amplify. Magnesium concentrations for MPM2 below 2.0 mM resulted in the poor amplification of DYS448, and concentrations above 2.75 mM resulted in an overall increase in stutter product intensity. As a result, 2.25 mM was selected for MPM1 and MPM2 which provided strong signal intensity. Additionally, peak height balance was improved for MPM1 and stutter product intensity was reduced for MPM2, compared to other concentrations of magnesium.

The concentration of DNA polymerase was also evaluated for each multiplex system. A range of 1.0–2.75 units was tested in 0.25-unit increments. The most optimal amplification of MPM1 required 1.25 units of DNA polymerase. Slightly more enzyme, 1.75 units, was necessary for the optimal amplification of MPM2.

Thermal cycling conditions were modified to improve peak height balance and to reduce stutter percentage. The annealing temperatures and times, extension times, and cycle number were adjusted to obtain the best possible amplification results for each multiplex.

A range of annealing temperatures used to optimize the MPM1 and MPM2 reactions was based on the temperature utilized in the original multiplex design, 60°C (8). Annealing temperatures of 58, 60, and 62°C were evaluated for both multiplexes. At 62°C, poor amplification was observed for DYS707 in MPM1. The most effective annealing temperature for MPM1 was determined to be 60°C, as a result of stronger signal intensity, compared to 62°C, and reduced stutter product formation for most loci, compared to 58°C. The same temperature, 60°C, was also selected as optimal for MPM2.

Three annealing times (30 sec, 1 min, and 1 min 30 sec) were assessed in both multiplexes. The 30-sec annealing time resulted in poor amplification of DYS707 in MPM1. The signal intensity for 1-min and 1-min 30-sec annealing times was similar to each other. Therefore, 1 min was selected as optimal for MPM1. The MPM2 loci were successfully amplified regardless of the annealing time. However, the annealing time of 1 min 30 sec resulted in increased stutter products, and the 30-sec annealing time caused a slight decrease in signal intensity. Therefore, 1 min was also selected for MPM2.

Several extension times were tested to ensure that sufficient time was allotted for complete amplification of MPM1 and MPM2. Extension times of 30 sec, 1 min, and 1 min 30 sec were tested. The use of 1-min and 1-min 30-sec extension times resulted in diminished peak heights. However, the 30-sec extension time resulted in increased peak heights for all loci and was therefore selected as optimal for both MPM1 and MPM2.

In the original design of the multiplexes, 30 cycles were utilized to amplify the DNA, which was visualized using an ABI Prism 310 Genetic Analyzer (8). The range of cycle numbers evaluated included several cycles fewer and greater than 30 cycles. MPM1 and MPM2 were amplified successfully with all cycle numbers tested. The validation of both MPM1 and MPM2 was performed using the ABI Prism 3130 Genetic Analyzer; however, amplification products were also run on an ABI Prism 310 Genetic Analyzer to compare the sensitivity of the two instruments. It was determined that 28 cycles was sufficient for MPM1 and MPM2 when amplifications were detected using an ABI Prism 3130 Genetic Analyzer. Any increase above 28 cycles resulted in significantly high peak heights, leading to increased stutter product and artifact production. When the products were visualized using the ABI Prism 310 Genetic Analyzer, 30 cycles was the most sufficient as result of a difference in sensitivity of the two instruments.

Once the PCR parameters were determined for dye-labeled primers, we were able to evaluate the performance of the multiplexes. Throughout the entire study including the optimization of the multiplexes, no contamination was observed in any of the negative controls.

Multiplex System Performance

We evaluated the performance of MPM1 and MPM2 for genotyping precision, stutter, specificity, and sensitivity. In any multiplex system, the sizing of the alleles should be reliable. Precision of the OSU set was evaluated; the standard deviation values ranged from 0.04 to 0.16 bases for MPM1 and from 0.05 to 0.09 bases for MPM2.

During the amplification of microsatellites, stutter may occur as a result of slippage of the DNA polymerase. In general, the true allele peak signal intensity is much greater than the stutter peak intensity. Stutter is typically one or more repeat units smaller than the true allele peak but may occur as one or more repeat units larger than the true allele peak. Amplification of DYS688 resulted in the appearance of multiple stutter products, located in the n-1, n-2, and n-3 positions, with an average percentage stutter of 57%, 22%, and 9% at each position, respectively. The average percent stutter for the remaining loci was 8–14% for the MPM1 loci (DYS576, 8%; DYS504, 13%; DYS487, 14%; DYS707, 12%) and 4–15% for the MPM2 loci (DYS448, 4%; DYS488, 11%; DYS471, 15%; DYS685, 9%; DYS703, 11%). High stutter is understandable for DYS688, as the repeat structure of the locus is a complex trinucleotide repeat, which contains several exceptionally large simple repeat stretches. The reference sequence obtained from GenBank identified 80 repeat units including two simple repeat stretches of 31 and 25 repeats. The large simple repeat stretches provide ample opportunity for slippage. This was evident in the population study

as DYS688 was the most polymorphic with a gene diversity of 0.910 for the total population (Caucasian population 0.890 and African–American population 0.899) (4). In spite of the high levels of stutter observed for DYS688, for all amplification reactions including all but one of the mixtures, the true allele was undoubtedly the band with the greatest intensity. Further discussion regarding mixture samples is found in the Mixture Studies section.

The amplification of forensic samples requires the multiplex systems to be highly sensitive because of the potential limited quantities of DNA present. The sensitivity of the OSU 10-locus set was evaluated in male DNA samples of 25 pg (~4 diploid cells) to 5 ng. The optimum amplification reactions for MPM1 and MPM2 contained 200 pg–1 ng of DNA. Alleles from all loci in the full haplotypes were detected for MPM1 and MPM2 even at 25 and 35 pg, respectively (Fig. 2), using a 50 RFU peak height detection threshold. At these small concentrations of DNA, peak heights were low and occasional rare extraneous peaks were observed that could under circumstances of an unknown sample be scored as possible alternative alleles. We did not attempt in this study to determine whether any conditions contributed to this occurrence and emphasize that this occurred rarely. Also, there did not seem to be any significant effect of lowered DNA concentration on the occurrence of stutter.

In addition to sensitivity, male specificity in humans is also important to aid in the identification of the male contributor in male/female mixtures associated with crimes such as sexual assaults. The loci selected for the OSU 10-locus set were screened through BLAST searches and empirical studies to ensure no amplification with female DNA (8). After optimization of the PCR with dye-labeled primers, both multiplex systems were assessed for male specificity using 3, 30, 300 ng, and 1 μg of female DNA. No amplification products were observed for any of the quantities tested for both MPM1 and MPM2.

The performance of the OSU 10-locus set was further tested to assess species specificity. DNA amplification was attempted with male and female primate samples, nonprimate male mammals, and five bacterial samples. There was no observed amplification for any of the bacterial samples tested with MPM1 or MPM2. The nonprimate animal species tested included a bull sample and male samples from dog, cat, sheep, and horse. Again, the OSU 10-locus set failed to produce any detectible amplification products.

Because of the high degree of sequence homology between humans and other higher primates, several male and female primate samples were also tested. The primates analyzed included male and female chimpanzees, gorillas, and orangutans. No amplification of DNA from the male orangutan was observed using either MPM1 or MPM2. However, amplification of DNA from a female orangutan resulted in the production of one product for MPM2 that did not fall within any of the allelic ranges. Amplification of the male chimpanzee DNA resulted in one out-of-range amplification product for MPM1. Additional products were observed for the male chimpanzee with MPM2, one out of range and one within the size range of the DYS488 alleles (244–259 bp). No amplification products were produced from the female chimpanzee sample. Amplification of DNA from the male gorilla using MPM1 resulted in three products within the size ranges of three loci, DYS487 (161–173 bp), DYS504 (256–286 bp), and DYS576 (405–441 bp). No amplification products were observed using MPM2 for the male gorilla. Amplification of DNA from the female gorilla using MPM1 resulted in no observed amplification products, but amplification with MPM2 resulted in one out-of-range product. Cross-reactivity with higher primate species is not unexpected. Even though the amplification products were within the size ranges of several loci, the location of the alleles observed does not allow the identification of the primers that produced the amplicons because the DNA was amplified as a multiplex.

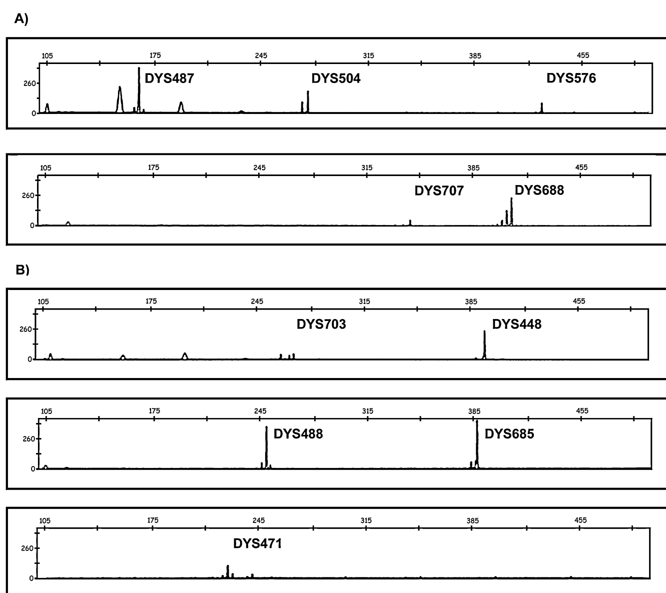

FIG. 2—*Sensitivity analysis results for the Ohio State University set. (A) Multiplex M1—A full five-locus male haplotype obtained with only 25 pg of template DNA. (B) Multiplex M2—A full five-locus male haplotype obtained with 35 pg of template DNA.*

Stability Studies

The reliability of the OSU 10-locus set to generate the same haplotypes from various body fluids of a single male individual was evaluated. As expected, identical haplotypes were obtained for MPM1 and MPM2 in blood, semen, and saliva samples from the same male individual.

Prior to evidence collection, the evidence may have been exposed to various environmental conditions. Therefore, multiplex systems should be tested to determine the effectiveness of DNA profile recovery from samples that have been exposed to various environmental insults. Dried blood exemplars from a male individual were exposed to the following conditions, and samples were collected over a period of 1 year, stored in RTED, exposed to UV light, exposed to HLH, and exposed to HLHR (Table 1).

The ability of the OSU 10-locus set to generate profiles in environmentally compromised samples was similar to four other multiplexes described by Hanson et al. (10,11). When biological samples are subjected to HLHR, DNA may become degraded. Therefore, it was not unexpected that full profiles were only observed under HLH and HLHR conditions, covered and uncovered, respectively, for up to 1 month. The exemplars stored at room temperature were successfully amplified using MPM1 and MPM2 for all six measurements. UV light prevents PCR amplification when DNA is unprotected by cell proteins. For several previous multiplexes, UV light exposure had no effect on amplification success (10,11). For MPM1 and MPM2, the evaluation of UV light exposure resulted in full profiles, even in an 18-month sample. The 1-year sample for UV exposure was inadvertently discarded during the extraction process. As the 18-month sample was available and produced a full profile, it is assumed that the 1-year sample would have similarly produced a full profile with both multiplexes.

TABLE 1—*Recovery of MPM1 and MPM2 haplotypes from environmentally compromised bloodstains.*

	3 Days	1 Week	1 Month	3 Months	6 Months	1 Year	>1 Year
HLH							
MPM1	+	+	+	−	−	−	NA
MPM2	+	+	+	−	−	−	NA
HLHR							
MPM1	+	+	+	−	−	−	NA
MPM2	+	+	+	−	−	−	NA
UV							
MPM1	+	+	+	+	+	NA	+
MPM2	+	+	+	+	+	NA	+
RTED							
MPM1	+	+	+	+	+	+	NA
MPM2	+	+	+	+	+	+	NA

+, full profile obtained; −, no alleles >50 RFU observed; NA, not available for testing; HLH, heat, light, and humidity; HLHR, heat, light, humidity, and rain; MPM, Multiplex Maybruck; RTED, room temperature, envelope-dried.

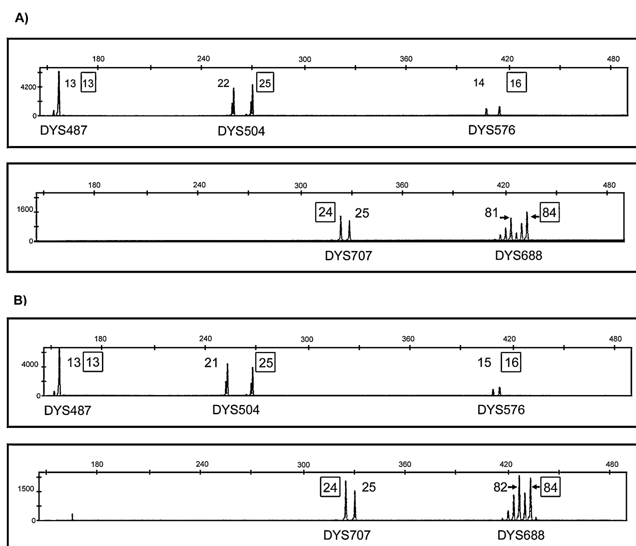

FIG. 3—*The DYS688 alleles in these mixtures overlap with stutter product. The alleles of the contributor with allele 84 at locus DYS688 are denoted with square borders. (A) Two-male mixture with DYS688 alleles 81 and 84. (B) Two-male mixture with DYS688 alleles 82 and 84.*

Mixture Studies

Forensic evidence commonly contains a mixture of more than one individual. Therefore, mixture studies were performed to determine the capability of the OSU 10-locus set to obtain a full profile for the male contributor(s). In the first mixture study, full male haplotypes were obtained for male-to-female DNA ratios of 1:1, 1:10, 1:100, 1:500, 1:1000, and 1:5000. This was expected because of the specificity observed in the initial screening of the OSU loci (8).

An item of evidence may contain multiple male contributors. Therefore, several ratios of male/male DNA mixtures from two unrelated individuals were examined in MPM1 and MPM2. In every mixture, full profiles of the individual contributors were observed in the mixture. The two male samples selected for this portion of the study had significantly different-sized alleles for DYS688 with no overlap of allele and stutter products. The following ratios were evaluated: 1:1, 1:2, 1:5, 1:11, 1:19, and 1:29. In all samples tested with the OSU 10-locus set, the major and minor contributors amplified, including the sample where the minor contributor comprised only 1/30th of the total DNA. Owing to the high percentage of stutter apparent for DYS688, we also evaluated the male/male mixtures for samples in which the alleles and stutter products overlapped (Fig. 3). Based on the number of alleles observed per locus, two individuals contributed to the mixture. In each 1:1 male/male mixture for DYS688, the true alleles were readily identified regardless of whether the two individual alleles were separated by one (not shown) or three repeat units. Ratios of 1:3 and 1:6 were also evaluated, and it is evident that a mixture is present. In one of the samples with a 1:6 male/male ratio, the true allele of the minor component for DYS688 was not readily apparent in the mixture. However, comparisons with the known samples will not permit the exclusion of the source of the minor contribution to the sample. Also, DYS688 is one of the largest loci, and if two or more individuals were present even in limited quantities, the additional alleles would most likely be observed for the smaller loci.

Another potential problem with the stutter observed for the DYS688 locus involves the statistical calculation of a mixture. We utilized the population study results from the study of Maybruck et al. (4) as a database for comparison. To account for all possible combinations, the haplotypes that include the n-1 and n-2 alleles were also included in the database search for the haplotypes, which could account for the mixture. No haplotypes in the database could account for the patterns observed for three male/male mixtures, even two mixtures in which only MPM1 was amplified. Therefore, the probability of a random match would use the upper 95% probability limit of the frequency of haplotypes unobserved in the database. The statistical result for such a random match is the same for each mixture, with a likelihood ratio of the probability of a true match versus a random match being one in 36.7 for the African–American sample population, one in 38 for Caucasian sample population, and one in 74.7 for the total population. Three potential haplotype matches were found in the database for the remaining two MPM1 mixtures. One male/male mixture potentially matched three African–American haplotypes, yielding a random match probability of one in 35.2 for the total population, one in 17.3 for the African–American population, and, as no potential haplotypes were observed in the Caucasian population, the probability is one in 38. The final male/male MPM1 mixture potentially matched two African–American and one Caucasian haplotype. The random match probability was one in 23.2 for the African–American population, one in 38.6 for the Caucasian population, and one in 35.2 for the total population. Because of the small size of the OSU database utilized for this comparison, the likelihood ratios, even for haplotypes not observed, are low. This would obviously change drastically as the database is expanded.

To directly compare the powers of the SWGDAM and OSU sets in the same population panel, the results of the previous population study were utilized to calculate the likelihoods of random matches from the population for the most common haplotypes for each population. In the population sample as a whole, for the SWGDAM set seven individuals were not unique, resulting in a likelihood ratio of one in 18 for an identification versus a random match from the population, versus a ratio of one in 47 for the OSU set. Evaluation of the Caucasian population showed a likelihood ratio of one in 12 for the SWGDAM set and one in 38 for the OSU set. The likelihood of an identification versus a random match from the population for both the SWGDAM and OSU locus set values for the African–American population was identical, one in 23.

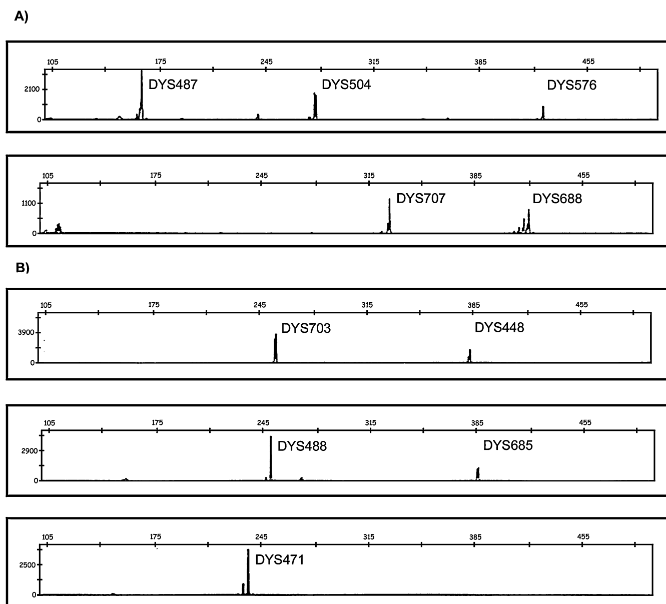

FIG. 4—*Results of nonprobative casework postcoital cervicovaginal sample. Full MPM1 (A) and MPM2 (B) profiles recovered 48 h after copulation, using a nondifferential extraction process.*

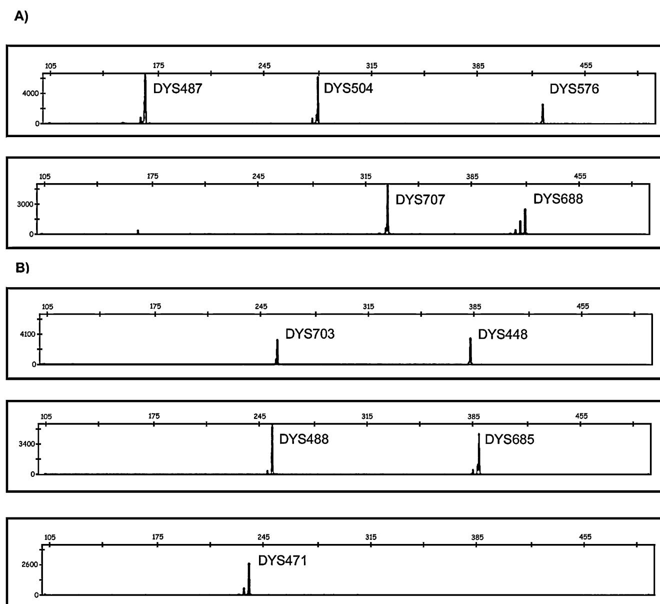

FIG. 5—*Results of non probative casework limited quantity sample. Full MPM1 (A) and MPM2 (B) profiles obtained from male DNA recovered from a coffee cup.*

Inheritance

A family study was used to assess the inheritance of the OSU 10-locus set. Typically, markers on the Y-chromosome are inherited unchanged from father to son. However, mutations in the number of repeats may occur. Additionally, the Y-chromosome may undergo intrachromosomal recombination or conversion (12). Five males from the same lineage (one grandfather, two sons, and two grandsons) were tested, and the 10-locus haplotypes were identical.

Nonprobative Casework Samples

As Y-STRs can have a significant impact on the analysis of samples collected in a sexual assault investigation, postcoital cervicovaginal samples were collected from a female donor 48 and 60 h after copulation. A nondifferential extraction was used to co-extract the male and female DNA, to eliminate the possibility of losing the small amount of male DNA. Eight percent of the total extract was used for amplification with MPM1 and MPM2. Amplification of the 48-h sample using both MPM1 and MPM2 resulted in the detection of a full male profile (Fig. 4). The 60-h sample resulted in no recovery of alleles for MPM1 or MPM2, as is typically encountered with such extended postcoital samples (13).

As stated earlier, the sensitivity of a multiplex to produce full haplotypes from limited DNA quantities is essential for the analysis of forensic evidence. Therefore, a sample was collected from the opening of a plastic coffee cup lid previously used by a male participant. Full male profiles for both MPM1 and MPM2 were detected (Fig. 5).

Conclusion

The OSU 10-locus set was evaluated in this developmental validation study, the population study (4), and the initial identification study (8). The studies have presented the higher discrimination power observed for the loci in the OSU 10-locus set when compared with the SWGDAM set and the successful performance of the set following the evaluation of precision, specificity, sensitivity, stability, and mixture and nonprobative casework analyses. The results of the stutter evaluation showed all of the loci with acceptable stutter percentages except one locus, DYS688. Even with the increased stutter observed for DYS688, the ability to determine the number of contributors and to identify a match with a known sample was not affected by the stutter even for mixture samples. The uniqueness of the haplotypes obtained from the OSU set was readily apparent when the mixture profiles, even from MPM1 only, were not found in the database. Even though the database is relatively small, in an earlier study (4), the SWGDAM loci showed 12 nonunique haplotypes overall (eight within the Caucasian population, four within the African–American population, and five shared across both populations). This resulted in 45 pairs of nondifferentiated individuals for the total population, in which several of the haplotypes were observed in other studies. In the same population panel, the OSU set revealed two nonunique haplotypes (one in the African–American population and one across both populations) in which two pairs of nondifferentiated individuals were observed (4). The loci present in OSU 10-locus set would therefore provide additional power to the currently utilized set to differentiate individuals even for partial matches. Larger population studies should be conducted to further evaluate the discrimination power of the OSU set, and this study should be reproduced in another laboratory.

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