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# Development and Validation of the Y-PLEX™5, a Y-Chromosome STR Genotyping System, for Forensic Casework*

**ABSTRACT:** A genotyping system, Y-PLEX™5, has been developed for use in human identification. The Y-PLEX™5 enables simultaneous amplification of five polymorphic short tandem repeat (STR) loci residing on the Y-chromosome, which are DYS389I, DYS389II, DYS439, DYS438, and DYS392. As little as 0.1 ng of template DNA can be used for analysis. The specificity of the amplification reaction enabled analysis of male DNA in a male: female DNA mixture at a ratio of 1:600. Mean stutter values ranged from 3.60–10.97%. Among the different primates investigated, the DNA from orangutan exhibited amplification at DYS438 locus and from gorilla at DYS439 and DYS438 loci. The DNA from cat, dog, and horse did not yield any amplified product. Studies on development of the genotyping system, generation and description of the allelic ladder, and validation of the multiplex PCR as per the FBI Director's Quality Assurance Standards were carried out. Y-STR allele and haplotype frequencies in two populations were generated. The data indicate that the Y-PLEX™5 genotyping system is sensitive and reliable, and can be used in human forensic and male lineage identification cases.

**KEYWORDS:** forensic science, Y-Chromosome, short tandem repeats, DNA typing, human identification, multiplex, polymerase chain reaction, Y-STR, Y-PLEX, DYS389I, DYS389II, DYS439, DYS438, DYS392

Short tandem repeat (STR) loci that are distributed ubiquitously throughout the genome are now routinely used in forensic casework and paternity evaluations (1–3). In sexual assault cases the evidence sample such as fingernail scrapings may have minimal amounts of foreign DNA from a male assailant. In some cases, where autosomal STR results contain predominantly female alleles mixed with alleles from the male suspect at minimal levels makes the interpretation of the results difficult, if not impossible. In such cases, where male and female biological material is mixed together, polymorphic STR loci residing on the non-recombining region of the Y-chromosome may provide very useful information. Thus, in forensic DNA analysis, Y-chromosome specific STR (Y-STR) loci have become increasingly important in investigating difficult cases (4–7). Further, the haploid nature of Y-specific genetic markers may aid in genetic characterization of male contributors in a multiple source DNA sample.

A core set of nine Y-STR loci including DYS19, DYS385, DYS389I, DYS389II, DYS390, DYS391, DYS392, and DYS393 provide high discrimination and the generated profiles are termed as minimal haplotypes (5). Of these loci, the DYS385 locus demonstrates variation at two loci due to gene duplication; hence nine loci (8). Sinha et al. (9) reported on the validation and forensic application of the Y-PLEX™6 system, comprising the DYS393, DYS19, DYS389II, DYS390, DYS391 and DYS385 loci. A Y-PLEX™5 genotyping system that enables simultaneous amplification of the DYS389I, DYS389II, DYS439, DYS438, and DYS392 loci has been developed. The Y-PLEX™5 and Y-PLEX™6 systems together provide results for 11 Y-STR loci including the nine minimal haplotype loci. This report describes the validation and application of the Y-PLEX™5 system. The studies show that the Y-PLEX™5 genotyping system can be used for forensic casework and male lineage studies.

## Materials and Methods

The custom primers, fluorescent labeled and unlabeled, were synthesized and obtained from commercial sources (Proligo, Boulder, CO; MWG Biotech, High Point, NC; Operon, Alameda, CA; Biosource International, Camarillo, CA). AmpliTaq Gold™, performance optimized polymer POP 4, matrix standards (FAM, ROX, TAMRA and HEX), GS500ROX, formamide and other supplies for use of the 310 Genetic Analyzer and 377 DNA Sequencer were obtained from Applied Biosystems (Foster City, CA). TBE buffer (100×) was obtained from Life Technologies, (Rockville, MD). Long Ranger® gel packs were from BioWhittaker Molecular Applications ApS (Denmark). All other chemicals used in this study were of analytical grade. A male cell line DNA (ATCC#45514) and a female cell line DNA (ATCC#45510) were used as positive and negative controls, respectively. The samples for database studies were obtained from unrelated males from the indicated population groups and were anonymized before analysis.

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*Extraction and Quantitation of DNA*

The DNA from anonymous donor samples (60 male and 30 female) was obtained from blood drawn in EDTA vacutainer tubes or buccal swabs and was extracted either by phenol-chloroform (10), Chelex® (10) or QIAamp® MiniKit (Qiagen, Valencia, CA) procedures. The quantity of human DNA was determined by slot blot hybridization using the Quantiblot kit (Applied Biosystems, Foster City, CA). DNA in the samples used for sensitivity and mixture studies was quantitated by using serial dilutions in triplicate.

*Amplification*

The 5X Y-PLEX™ 5 Primer Mix was prepared by combining the forward and reverse primers for all the five loci, dNTPs, buffer, and salts. The primers were selected and designed based on the published sequences (11–14). Amplification reactions contained 5.0 μL of 5X Y-PLEX™ 5 Primer Mix, 0.5 μL of AmpliTaq Gold™ (5 units/μL), 1–3 ng of DNA template (unless otherwise stated) and sterile water to raise the volume to 25 μL. Final concentration of the buffer in amplification reaction was 1.4X. Amplification reactions were performed in a GeneAmp® PCR systems 9600 or 9700 (Applied Biosystems, Foster City, CA) or a PTC-200 Peltier Thermal Cycler (M J Research, Waltham, MA) with conditions as follows: 95°C, 10 min; 32 cycles of 94°C, 30 sec; 56°C, 1 min and 70°C, 45 sec; 60°C, 60 min and 4°C until the samples were removed from the thermal cycler. The positive control (1–3 ng of male DNA, ATCC#45514) and the negative control (1–3 ng of female DNA, ATCC#45510) were amplified with every batch of amplification reaction.

*Analysis on a 310 Genetic Analyzer*

The samples for analysis were prepared by combining 1.0 μL of PCR product and 24.0 μL Hi-Di formamide containing 0.5 μL GeneScan® - 500[ROX] Size Standard in a 200 μL tube followed by denaturation at 95°C for 3 min using a 9700 Thermal Cycler; or by combining 1.5 μL of PCR product and 25.0 μL deionized formamide containing 1.0 μL of GeneScan® - 500[ROX] Size Standard in a 0.5 mL tube followed by denaturation at 95°C for 3 min using a 480 Thermal Cycler. The denatured products were subjected to electrophoresis on the 310 Genetic Analyzer using POP-4 with an injection time of 5 sec and using filter set A. The run time was approximately 26 min (or sufficient time necessary to elute the 450 base pair size standard peak in GS500 ROX). A matrix file using the matrix standards FAM, HEX, ROX, and TAMRA was generated and used. A minimum value of 75 rfu was used for interpretation of the data.

*Analysis on a 377 DNA Sequencer*

The samples for analysis were prepared by combining 1.0 μL of PCR product and 2.1 μL Hi-Di formamide containing 0.75 μL GeneScan® - 500[ROX] Size Standard and 0.75 μL of loading buffer in a 200 μL tube followed by denaturation at 95°C for 3 min using a 9700 Thermal Cycler. The denatured products were subjected to electrophoresis on the 377 DNA Sequencer using filter set A. The run time was approximately 3–4 h (time necessary to elute the 450 base pair size standard peak in GS500 ROX). A matrix file using the matrix standards FAM, HEX, ROX, and TAMRA was generated and used.

*Sequencing of Alleles*

Individual alleles, after amplification, were sequenced by using BigDye™ terminator cycle sequencing using 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

*Validation Studies*

The FBI Director's Quality Assurance Standards were followed for conducting validation studies (15). The methods used in validation studies are described in the text.

*Contamination and Environmental Insult Analysis*

Stains were prepared on freshly laundered 100% cotton cloth. Bloodstains from a male donor were prepared using 50 μL of freshly drawn blood without any preservatives or anticoagulants. Semen stains were prepared from pooled ejaculates. The pooled semen, stored at −20°C, was thawed and mixed and stains were prepared by using 25 μL. The contaminant study was divided into three sections: contaminants–wet; contaminants–dry; and environmental. Samples were stored at room temperature in the dark until processed unless otherwise noted. DNA was organically extracted (16) from the stains at intervals of 1, 4, 8, 14, and 28 days. Extracts were stored at −4° to −20°C until analysis. Extracts from day 1 and day 28 were used for this study except for the bleach/blood mixture, for which day 14 was used since the extract from day 28 was consumed for RFLP testing. Yield gel analysis as well as slot blot analysis was used to determine the quality and quantity of the DNA obtained.

*Contaminants (Wet)*—Clean pieces of 100% cotton cloth were saturated with the contaminants and allowed to air-dry overnight. The contaminants used in this part of the study were soil solution, liquid hand soap, unleaded gasoline, and 10% bleach. The blood or semen was then applied to the dry contaminated cloth.

*Contaminants (Dry)*—Blood or semen was applied to 100% cotton cloth and allowed to air-dry overnight. The cloth pieces were then saturated with either a soil solution or unleaded gas.

*Environmental*—Blood and semen samples were applied to clean 100% cotton cloth and dried overnight at room temperature. The stains were placed outdoors where they were exposed to a range of temperatures (10–35°C). One set was subjected to direct sunlight and the second kept in the dark.

*Escherichia Coli*—Clean pieces of 100% cotton cloth were saturated with *E. coli* strain HB101 and allowed to air dry overnight. The blood or semen was then applied to the dry contaminated cloth.

*Sample Clean-up*—Samples, which did not initially amplify using the Y-PLEX™5 kit were subjected to clean-up using two procedures: Chelex® or DNA IQ™. For the Chelex procedure, a portion of the DNA extract (5 ng) was transferred to a new microfuge tube containing 200 μL of 5% Chelex® and incubated at 56°C for 2 h, followed by 100°C for 8 min. Samples were microfuged and the supernatant was then filtered using a microcon-100 to a final volume of 25 μL (0.2 ng/μL). For the DNA IQ™ procedure, a portion (5 ng) of the DNA extract was transferred to a new microfuge tube and 7 μL of the DNA IQ™ resin beads were added and allowed to incubate at room temperature for 5 min. Samples were briefly vortexed, placed on a magnetic stand, and the liq-

uid removed. Lysis buffer (100 μL) was added to each sample, briefly vortexed, placed on the magnetic stand and the liquid removed. This was repeated three times with 100 μL of wash buffer. After the third wash, the samples were allowed to air dry for 5 min. To each sample, 25 μL of elution buffer was added and the samples placed in a 65°C heat block for 5 min. The samples were then placed on the magnetic stand and the DNA transferred to a new tube. After both clean-up procedures, 5 μL of DNA (1 ng) were amplified adding bovine serum albumin (BSA) (8 μg) and an additional 0.5 μL (2.5 units) of AmpliTaq Gold™ to the reaction mix.

*Statistical Analyses*

Allele frequencies for each marker were determined by the gene-count method (17). Genetic diversity was calculated according to the method of Tajima (18) and haplotype random match probability was calculated according to the method of Stoneking et al. (19).

## Results

*Development of Multiplex System*

The GenBank accession numbers, repeat motifs and size of PCR products for the five Y-STR loci are summarized in Table 1. The repeat motifs for the loci DYS389I, DYS389II and DYS392 are according to the recommendations of International Society of Forensic Haemogenetics (ISFH) (20) and for DYS438 and DYS439 are as described by Ayub et al. (11) and supported by recent studies (21). Further, the repeat unit is 5 bases at locus DYS438; 4 bases at DYS389I, DYS389II, and DYS439 loci; and 3 bases at DYS392 locus. The Y-PLEX™5 primer mix contains locus specific fluorescent labeled and unlabeled primers in a 1.4X GeneAmp® PCR Gold buffer (Applied Biosystems, Foster City, CA), 0.8 mM dNTPs and salts. Primers for the loci DYS389I and DYS389II were labeled with FAM, DYS439 with HEX, and DYS438 and DYS392 with TAMRA.

Twenty-five male and ten female DNA samples (1–3 ng) were first amplified for each locus in a singleplex reaction. All male samples yielded amplified product for every locus and none of the female samples exhibited nonspecific or locus specific amplified product.

The amplification of the DYS389I and DYS389II loci was achieved by using a single set of primers. When the forward and reverse primers were used at 1:1 ratio, the extent of amplification of DYS389II locus, as judged by the peak area, was 40% of DYS389I locus (Fig. 1). The extent of amplification for DYS389II increased to 70% of the DYS389I locus when the forward and reverse primers were used in the ratio of 2:1. This observation is likely due to the forward primer binding at two regions (Fig. 2). Higher concentration of the forward primer for the DYS389 locus also enhanced the amplification at DYS389I, DYS389II, and DYS439 as revealed by the allele peak height. The primer pair titration was tested at 0.25X, 0.5X, 1X, 1.5X, 2X, and 3X. The concentration of primers in the 1X primer mix was as follows: DYS389 forward, 0.5 μM; DYS389 reverse, 0.25 μM; DYS439 forward and reverse, 0.57 μM; DYS438 forward and reverse, 0.5 μM and DYS392 forward and reverse, 1.5 μM. The amplification of the DYS439 and DYS392 loci at 0.25X concentration of primers was 11 and 75%, respectively, as compared to a 1X concentration (data not shown). At 2X and 3X concentration of primers, the locus DYS389I amplified to a greater extent, whereas the locus DYS389II amplified poorly (data not shown). At primer concentrations of 2X and higher, -A product was predominant at all loci except the DYS438 locus (data not shown). The final concentration (1X) of primers in the Y-PLEX™5 primer mix, which provided locus-to-locus balance, was selected. A TAMRA labeled nonspecific amplification product of 128 bases was observed in some amplification reactions. The extent of this peak was lower and did not interfere in the analysis.

TABLE 1—*General criteria of loci in the Y-PLEX™5 system.*

| Y-STR Locus | Dye | Color | PCR Product (~bases)* | Repeat Motif | GenBank Accession # |
|---|---|---|---|---|---|
| DYS389I | FAM | Blue | 243–260 | TCTG/TCTA | G09600 |
| DYS389II | FAM | Blue | 361–385 | TCTG/TCTA | G09600 |
| DYS439 | HEX | Green | 238–254 | GATA | AC002992 |
| DYS438 | TAMRA | Yellow | 131–158 | TTTTC | AC002531 |
| DYS392 | TAMRA | Yellow | 247–262 | TAT | G09867 |

* The sizes are on 310 Genetic Analyzer.

The concentration of $MgCl_2$ in the PCR was varied from 1.15–1.73 mM. At 1.15 mM $MgCl_2$, allele drop out at the DYS389II locus was observed (data not shown). The primer mix used in the present investigation contained 1.7 mM of $MgCl_2$.

Different DNA polymerase preparations (2.5 units) were investigated for the amplification with the Y-PLEX™5 reagents. These were: AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Foster City, CA); Platinum® *Taq* DNA Polymerase High Fidelity, Platinum® *Pfx* DNA Polymerase, Platinum® *Taq* DNA Polymerase and *Taq* DNA Polymerase, recombinent (all from Invitrogen Corporation, Carlsbad, CA). AmpliTaq Gold® DNA Polymerase provided amplification of all five loci without any non-specific amplified products observed. Among the enzymes from Invitrogen, a good amplification of all five loci was obtained with Platinum® *Taq* DNA Polymerase High Fidelity and Platinum® *Taq* DNA Polymerase. However, HEX labeled nonspecific amplification products were obtained (data not shown). Amplification products for all five loci were not observed when Platinum® *Pfx* DNA Polymerase or *Taq* DNA Polymerase, recombinant was used. Instead, TAMRA and FAM labeled nonspecific amplified products, respectively, were observed when using these enzymes (data not shown). The amount of AmpliTaq Gold® was varied, at regular intervals, from 0.625–3.75 units in the PCR reaction using five known DNA samples (2 ng). When 0.625 and 1.25 units of AmpliTaq Gold® DNA Polymerase were used, the amplification of DYS392 and DYS389II was reduced notably (data not shown). At greater amounts of polymerase, all loci amplified well. Therefore, 2.5 units of AmpliTaq Gold® DNA Polymerase is used for amplification using the Y-PLEX™5 system.

*Annealing Temperature*

The calculated $T_m$ for the primers for the five loci is between 49°C and 54°C. The annealing temperature for the multiplex PCR was determined empirically by amplification of five known male DNA samples (2.0 ng) at different annealing temperatures ranging from 48–65°C using a PTC200 thermal cycler (MJ Research) and 30 cycles of PCR. The well temperatures were recorded. An annealing temperature of 56°C was suitable for amplification of all 5 loci (Fig. 3). At 54°C and lower temperatures, nonspecific amplification was observed at the region where the DYS439 locus migrates. The amplification yield for the DYS389II locus was poor at 51°C and lower temperatures. Nonspecific amplification at the region where the DYS439 locus migrates was also observed at 58°C or higher temperature (data not shown).

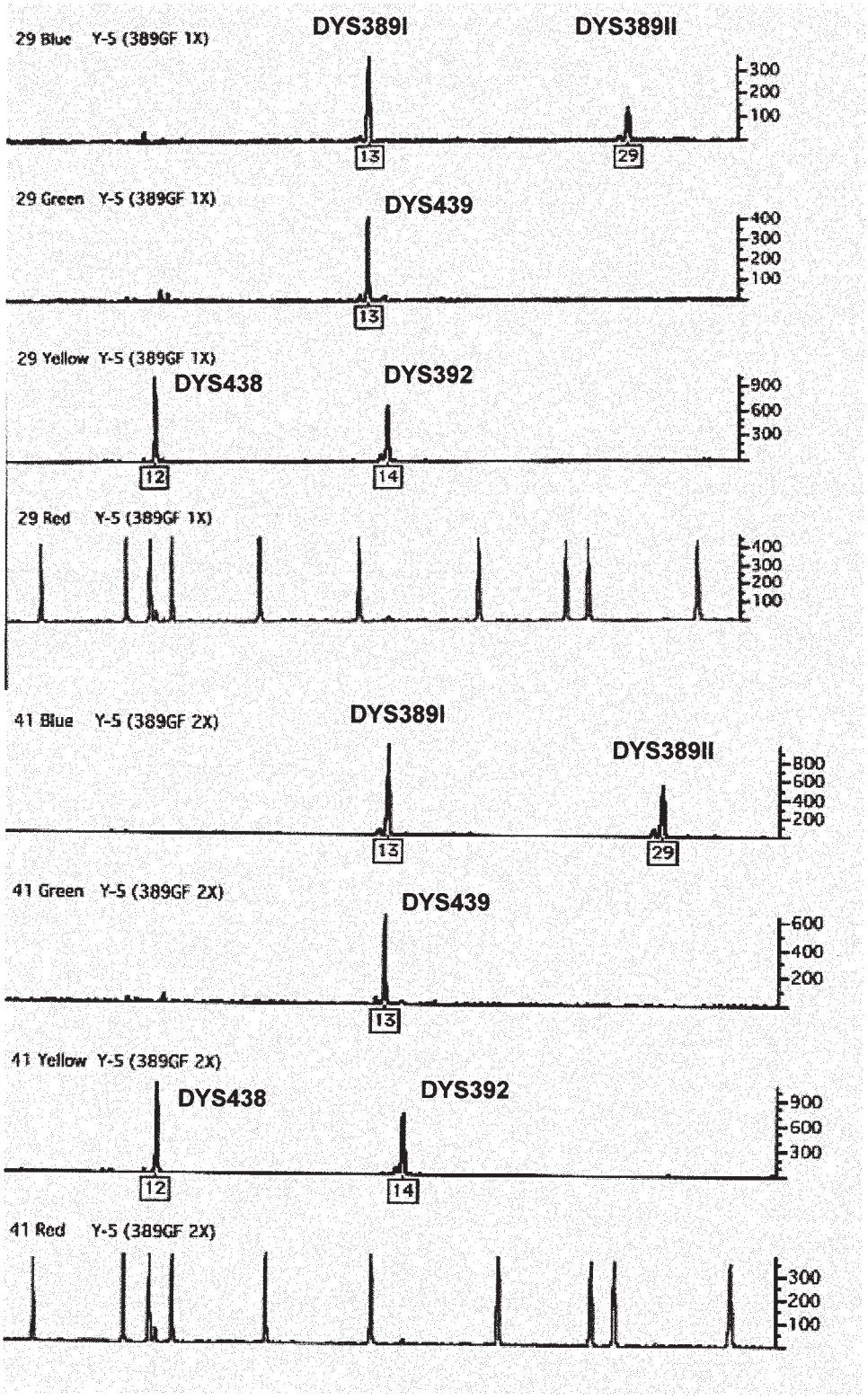


FIG. 1—*Amplification of 0.2 ng of male DNA using 1:1 (Top) and 1:2 (Bottom) ratio of the DYS389 forward and reverse primers in the Y-Plex™5 primer mix.*

*Number of Cycles*

DNA from five male samples (0.5 ng) was amplified using 26, 28, 30, 32, and 34 cycles and annealing temperature of 56°C. The profiles obtained at 28 and 32 cycles are presented in Fig. 4. At 26 and 28 cycles, the height of allele peaks was less than 100 rfu for all five loci amplified. The height of the allele peaks for the DYS389I, DYS389II, DYS439, DYS438, and DYS392 loci obtained at 32 cycles was 1.86, 1.8, 3.0, 2.38, and 1.9 fold, respectively, than that obtained at 30 cycles. At 34 cycles, there was a tendency of accumulation for–A allele peaks to be produced. Thus, 32 cycles were selected for amplification. The female DNA (2 ng) did not exhibit any products when amplified for 26, 28, 30, 32, or 34 cycles (data not shown).

*PCR Volume*

Five male samples (1.5 ng of DNA), were amplified using 12.5, 25, and 50 μL reaction volumes in a 9700 Thermal Cycler. The final concentration of all components in the PCR mixture was not altered. The amplified products were analyzed on a 310 Genetic Analyzer. The correct haplotype and similar extent of amplification was obtained for all the samples at all reaction volumes (data not shown). Thus, any of these volumes can be used for single source samples. The nonprobative casework samples were amplified using 25 μL reaction volume.

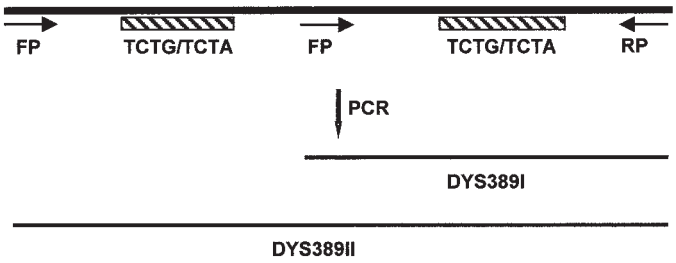


FIG. 2—*Schematic presentation of the binding of forward and reverse primers at the DYS389I and DYS389II loci.*

*Thermal Cycler*

Amplification using the Y-PLEX™5 kit was performed in the GeneAmp® PCR Systems 9600 and 9700 (Applied Biosystems, Foster City, CA) and the PTC-200 Peltier Thermal Cycler (M J Research, Waltham, MA). All five loci amplified well using any of the three thermal cyclers (data not shown). General peak balance was observed and there was no allele drop out. A comparison of peak heights of alleles at all five loci suggests that amplification yield was greatest when using PTC 200 followed by 9600 and 9700, although there was no substantial difference among the thermal cyclers.

*Denaturation, Annealing, Cycle Extension, and Final Extension Time*

The different time intervals investigated for denaturation and annealing were 30 and 60 sec; for cycle extension 30, 45, and 60 sec; and for final extension 30, 45, and 60 min. Five male samples (1 ng of DNA) samples were amplified at these time intervals. Based on the amplification yield, inter-locus balance of alleles and extent of–A product formation, the optimal conditions for the simultaneous amplification of all five loci were denaturation for 30 sec, annealing for 1 min, cycle extension for 45 sec, and final extension for 60 min (data not shown).

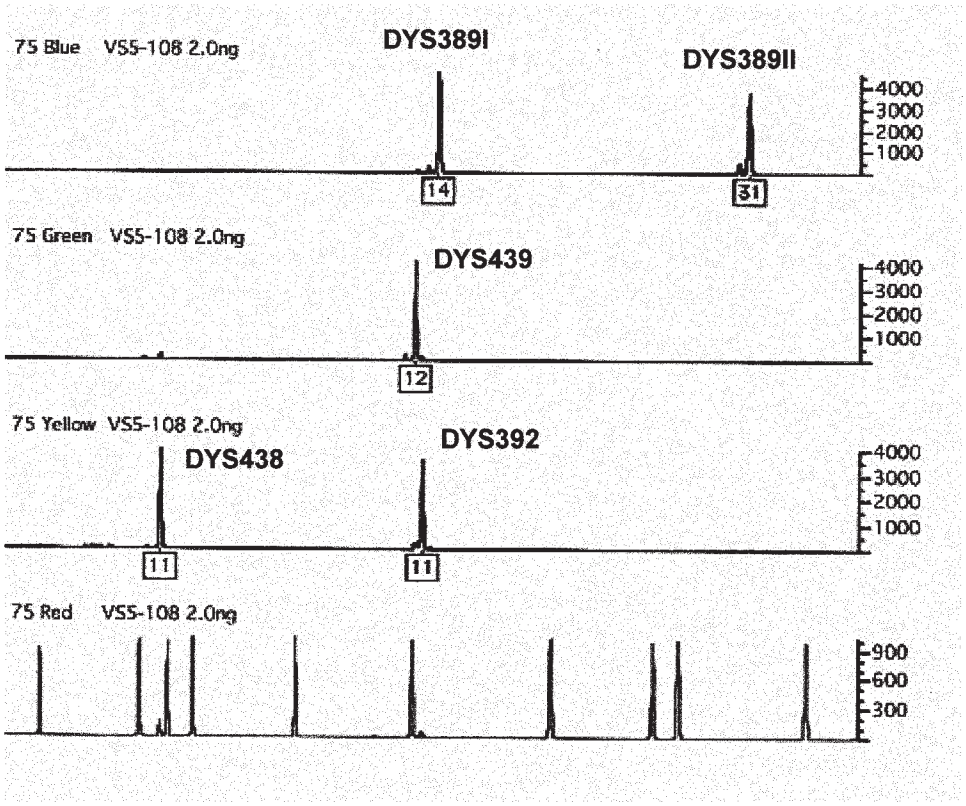


FIG. 3—*Amplification profile obtained at annealing temperature of 56°C during the PCR. 2.0 ng of DNA template were used.*

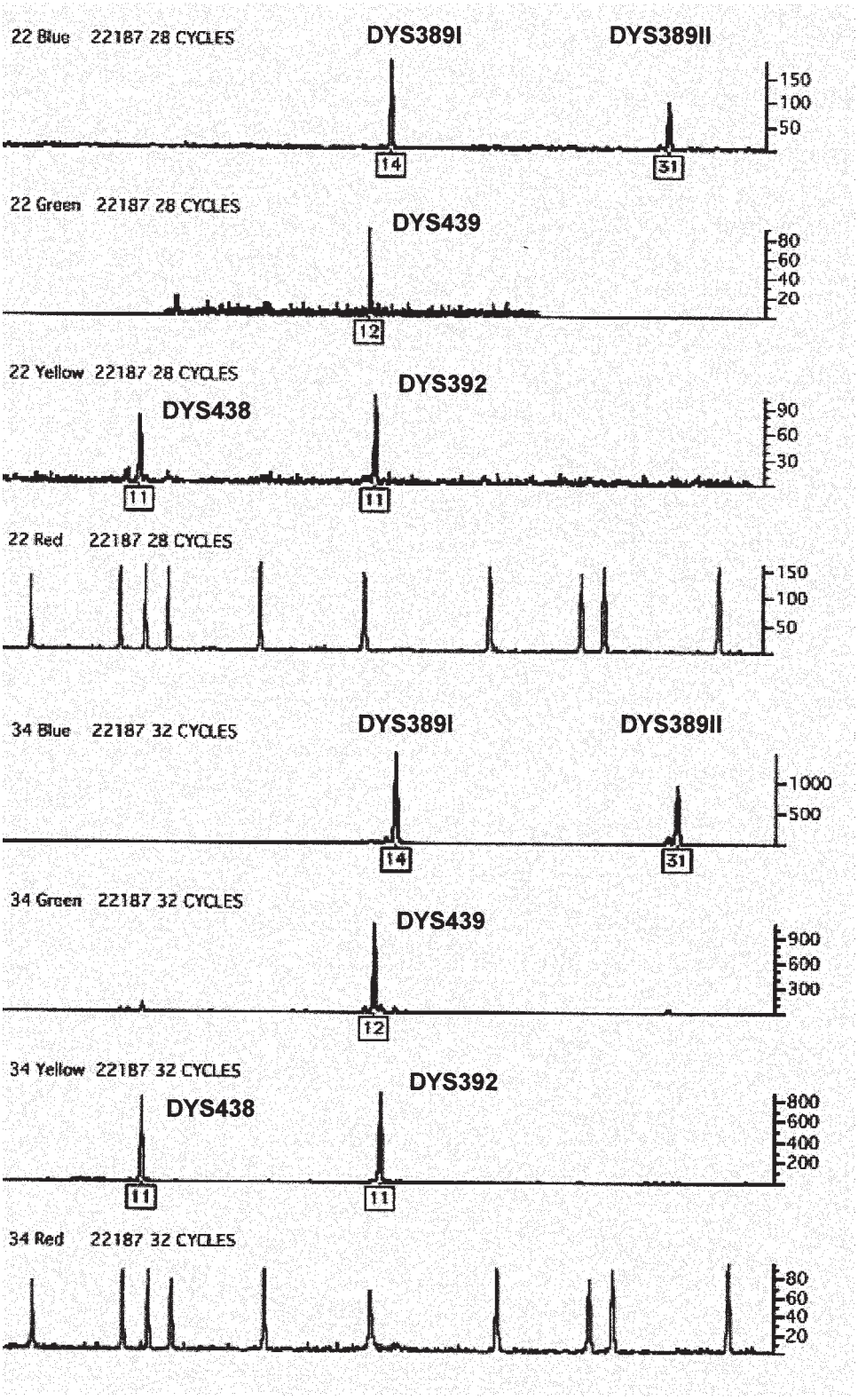


FIG. 4—*Profiles obtained from amplification of 28 (Top) and 32 (Bottom) cycles.*

*Generation of Allelic Ladder*

The reference allelic ladder for genotyping was generated from genomic DNA and by selecting common alleles present in the population. Allele distribution for each locus in 500 individuals (African American and Caucasian) was investigated. Subsequently, DNA samples for individual alleles were chosen and combined so that the desired combination of alleles was obtained for generation of the Y-PLEX™5 allelic ladder. The locus-mix was prepared from amplified products for individual alleles. The amplified products were pooled so that the ladder alleles at all loci provided peaks of similar height. A typical Genotyper® profile of the Y-PLEX™5 ladder is provided in Fig. 5. The repeat motifs and nucleotide sequence of the alleles in the allelic ladder were confirmed by sequencing all alleles individually (data not shown).

*Minimum Sensitivity*

The minimum sensitivity, i.e., minimum quantity of male DNA required for obtaining a complete profile, of the Y-PLEX™5 was determined by amplification of DNA samples from 15 individuals. The amount of template DNA in the reactions was: 0.05, 0.075, 0.1, 0.2, 0.3, 0.5, 1.0, and 2.0 ng. The profiles obtained by using 0.05 ng of DNA exhibited allele drop out at one or more loci (data not shown). When 0.075 ng of DNA was amplified, allele drop out at the DYS439 locus in one sample was observed (Fig. 6). When 0.1 ng and higher quantities of DNA were used, the alleles at all five loci were amplified well in all samples; the alleles had peak heights between 300 to 800 RFU (Fig. 6). Based solely on the quantities tested, minimum sensitivity for the Y-PLEX™5 kit was 0.1 ng of male DNA. When 2.0 ng of male DNA were amplified, the height of the alleles was between 4000–4500 rfu (data not shown), and the profiles were devoid of any amplification artifacts.

*Male–Female Mixture Studies*

The male–female mixture samples were prepared in the proportions 1:0, 1:10, 1:50, 1:100, 1:200, 1:300, 1:400, 1:500, and 1:600 and were analyzed in duplicate under standard conditions. The quantities of DNA used in preparing mixtures and results of mixture studies are summarized in Table 2. A complete male profile was detected in the male–female mixture samples up to the 1:600 ratio, which contained 0.5 ng of male DNA and 300 ng of female DNA. Thus, it was possible to obtain a male profile in presence of excess amounts of female DNA using the Y-PLEX™5. A HEX labeled nonspecific amplification product of size 136 bases long, which does not fall within the allelic ladder for the DYS439 locus, was observed in mixture samples containing 50 ng or higher amounts of female DNA. This HEX labeled nonspecific amplification product was also present when 50 ng or higher quantities of male DNA was used as a template (data not shown). Although this artifact does not affect interpretation, further studies are needed to determine the nature of this amplification product.

*Two Male Mixtures*

The two male samples, male-1 and male-2 were selected so that the allele profile for all five loci was distinct. The allelic profile of male-1 for loci DYS389I, DYS389II, DYS439, DYS438 and DYS392 was 12, 28, 14, 8, and 11 and of male-2 was 14, 30, 12, 12 and 13, respectively. The male–male mixtures were prepared by combining the DNA from male-1 and male-2 in the proportions 1:0, 1:1, 1:2, 1:5, 1:10, 1:20, 1:30, 1:40, 0:1, 2:1, 5:1, 10:1, 20:1, 30:1, and 40:1. The samples were amplified and analyzed in duplicate under standard conditions. The quantities of DNA used in preparing mixtures and results of mixture studies are summarized in Table 3. The complete DNA profile of male-1 was detected in mixtures up to a ratio of 1:5 (male-1: male-2), whereas the complete DNA profile of male-2 was detected up to a ratio of 10:1 (male-1: male-2). At greater proportions, loss of some alleles was observed for the minor component. Some variation in the amplification, reflected by RFU values, was observed (Table 3). This may be, in part, due to stochastic effects during PCR.

*Precision of Allele Sizing and Reproducibility*

A total of 129 injections of the Y-PLEX™5 allelic ladder were made on five different 310 Genetic Analyzers at different times and at two laboratories. Similarly, the Y-PLEX™5 allelic ladder was subjected to electrophoresis on the 377 DNA Sequencer for 38 times on three instruments. The precision of migration of each allele was calculated. The sizes of the alleles from sequence studies, observed size range, mean size, and standard deviation for each allele in the allelic ladder are summarized in Table 4. In general, the range for migration and standard deviation values for alleles were higher for 310 Genetic Analyzer than on 377 DNA Sequencer. The standard deviation values obtained ranged from 0.137–0.229 and 0.048–0.187 for these instruments, respectively. Estimated size differences of up to 4 bases have been observed for some alleles for Y-PLEX™5 when subjected to electrophoresis on a 310 Genetic Analyzer and a 377 DNA Sequencer (Table 4).

The reproducibility of the amplification was demonstrated by amplification of seven male samples (1 ng) at three different times on a 9700 thermal cycler and analysis on a 310 Genetic Analyzer. Identical and conclusive allelic profiles were obtained for all samples in the three amplification reactions (data not shown).

*Stutter Studies*

The extent of stutter for the alleles at all five loci was measured by analyzing 55 male samples. The height of the stutter peak was compared with the corresponding allele at each locus. The values for stutter range, mean stutter, standard deviation and upper range stutter for the loci DYS389I, DYS389II, DYS439, DYS438, and DYS392 are summarized in Table 5.

*Nonhuman Studies*

DNA (2–4 ng) from male cat, dog, horse, chimpanzee, mandrill (baboon), pygmy, orangutan, and gorilla was amplified using the Y-PLEX™5 reagents. The DNA from cat, dog, horse, chimpanzee, mandrill, and pygmy did not yield amplified product at any loci (data not shown). When a positive control (1 ng) was co-amplified with these samples, the complete profile for the positive control was obtained (data not shown), which demonstrates that DNA extracts from these samples did not contain PCR inhibitors affecting the amplification. A TAMRA labeled product of 252 bases in length was observed at the DYS438 locus when the DNA from orangutan was amplified (Fig. 7). Similarly, a HEX labeled product of 223 bases in length at the DYS439 locus and a TAMRA labeled product of 237 bases in length at the DYS438 locus were obtained with the DNA from gorilla (Fig. 7). Thus, the primers present in the Y-PLEX™5 are specific for human DNA and some higher primates.

*Interlaboratory Comparison and NIST Standards*

Ten male samples each from the Institute for Pathology and Molecular Immunology, University of Porto (IPATIMUP), Porto,

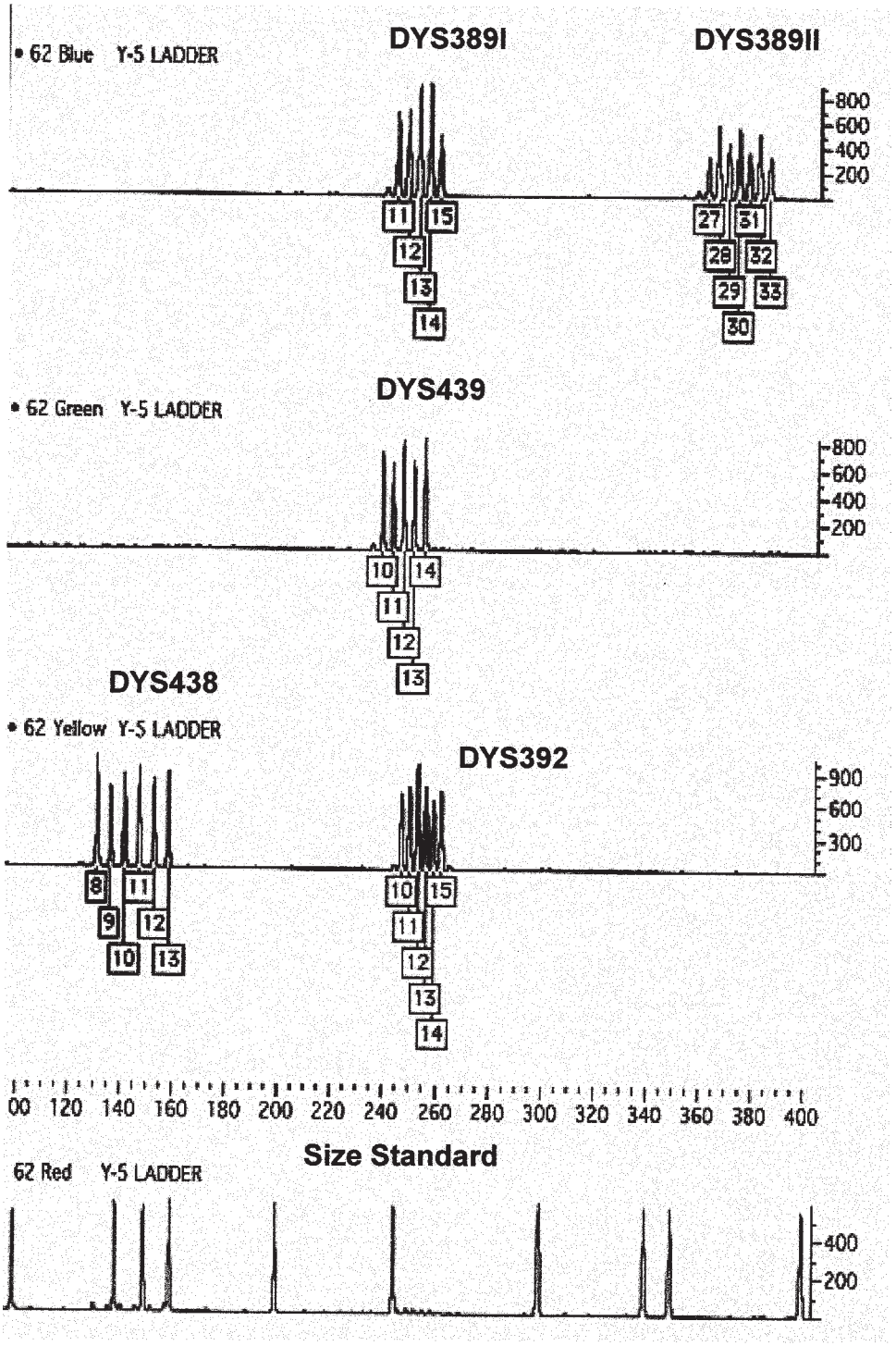


FIG. 5—*Y-PLEX™5 allelic ladder used for genotyping.*

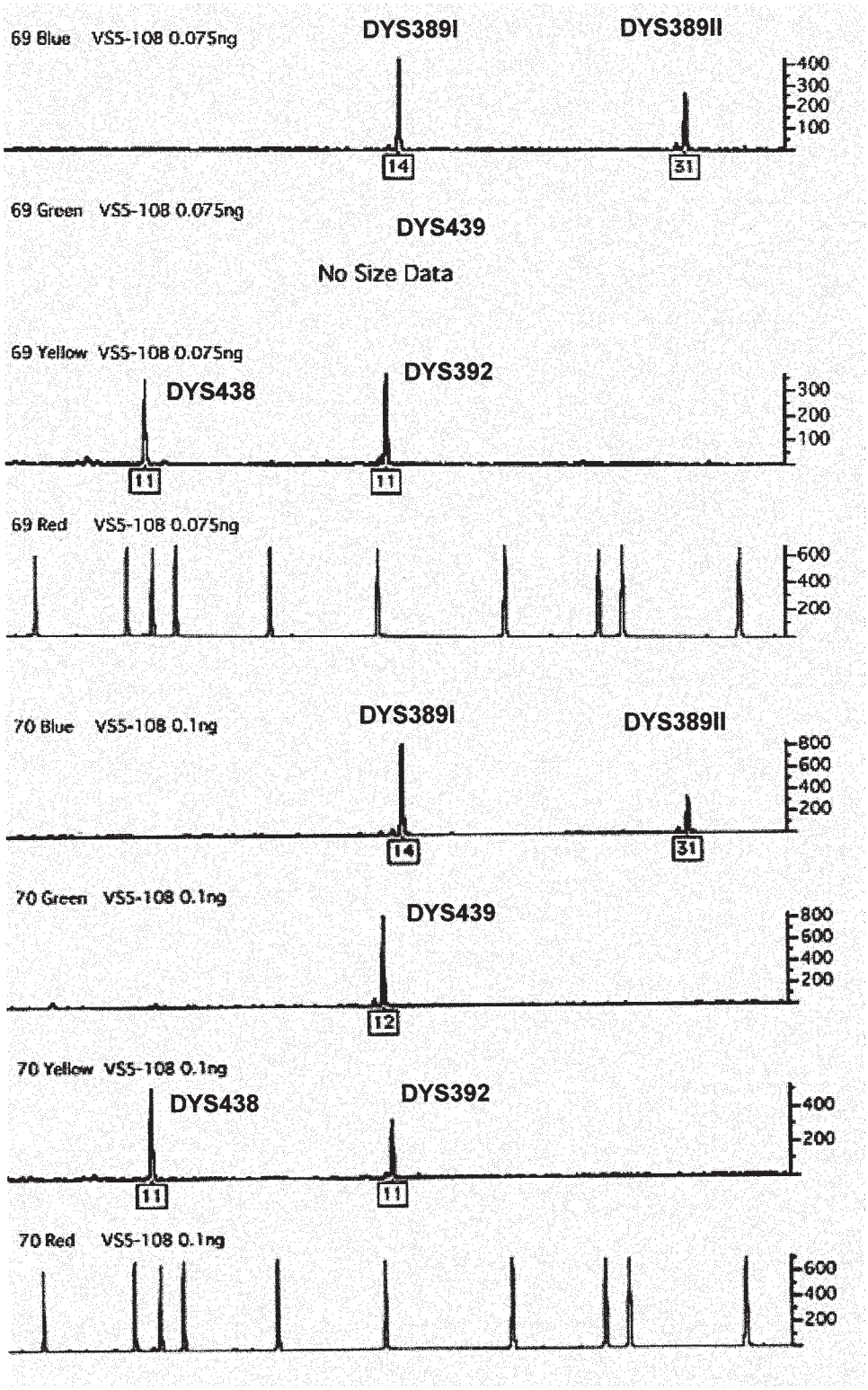


FIG. 6—*Profile of a male sample from 0.075 ng (Top) and 0.1 ng (Bottom) of template DNA using the Y-PLEX™5 system.*

TABLE 2—*Amplification of male and female mixtures by using the Y-PLEX™5 reagents.*

| Male:Female DNA Ratio | Male DNA (ng) | Female DNA (ng) | Peak Height (rfu) of the Alleles in Male Profile in a Mixture Sample | | | | |
|---|---|---|---|---|---|---|---|
| | | | DYS389I | DYS389II | DYS439 | DYS438 | DYS392 |
| 1:0 | 0.5 | 0 | 2020 | 1509 | 1124 | 1538 | 2522 |
| 1:10 | 0.5 | 5 | 1114 | 605 | 593 | 1802 | 916 |
| 1:50 | 0.5 | 25 | 714 | 297 | 1016 | 1510 | 1255 |
| 1:100 | 0.5 | 50 | 1640 | 637 | 1050 | 1371 | 865 |
| 1:200 | 0.5 | 100 | 533 | 179 | 496 | 1286 | 553 |
| 1:300 | 0.5 | 150 | 393 | 147 | 558 | 1000 | 504 |
| 1:400 | 0.5 | 200 | 835 | 250 | 616 | 641 | 280 |
| 1:500 | 0.5 | 250 | 846 | 221 | 806 | 714 | 248 |
| 1:600 | 0.5 | 300 | 820 | 208 | 754 | 641 | 224 |

TABLE 3—*Amplification of two male mixtures using the Y-PLEX™5 reagents.*

| Male-1: Male-2 DNA Ratio | Male-1 DNA (ng) | Male-2 DNA (ng) | Peak Height (rfu) of the Alleles | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | DYS389I | | DYS389II | | DYS439 | | DYS438 | | DYS392 | |
| | | | Male-1 | Male-2 | Male-1 | Male-2 | Male-1 | Male-2 | Male-1 | Male-2 | Male-1 | Male-2 |
| 1:0 | 0.2 | 0 | 334 | … | 216 | … | 131 | … | 269 | … | 382 | … |
| 1:1 | 0.2 | 0.2 | 221 | 257 | 128 | 160 | 124 | 256 | 223 | 391 | 406 | 196 |
| 1:2 | 0.2 | 0.4 | 175 | 581 | 85 | 338 | 119 | 397 | 248 | 1142 | 338 | 570 |
| 1:5 | 0.2 | 1 | 131 | 1778 | 80 | 1241 | 197 | 1676 | 274 | 2024 | 374 | 2131 |
| 1:10 | 0.2 | 2 | 171 | 2716 | <75 | 1476 | 168 | 4139 | 276 | 4077 | 157 | 3360 |
| 1:20 | 0.2 | 4 | 158 | 2902 | NR* | 1239 | 155 | 4957 | 80 | 5149 | 78 | 3309 |
| 1:30 | 0.2 | 6 | 263 | 3784 | NR | 1709 | 228 | 6987 | 99 | 6548 | 76 | 4101 |
| 1:40 | 0.2 | 8 | 222 | 5526 | NR | 2152 | 219 | 6103 | NR | 6441 | NR | 4967 |
| 0:1 | 0 | 0.2 | … | 309 | … | 192 | … | 157 | … | 210 | … | 313 |
| 2:1 | 0.4 | 0.2 | 811 | 306 | 522 | 182 | 646 | 90 | 478 | 164 | 1151 | 186 |
| 5:1 | 1 | 0.2 | 1602 | 223 | 1113 | 133 | 1423 | 310 | 1353 | 205 | 2187 | 230 |
| 10:1 | 2 | 0.2 | 2488 | 192 | 1420 | 97 | 2890 | 144 | 2510 | 243 | 3512 | 188 |
| 20:1 | 4 | 0.2 | 4417 | 149 | 2703 | NR | 6047 | 348 | 4455 | 173 | 5500 | 87 |
| 30:1 | 6 | 0.2 | 4346 | 226 | 2784 | NR | 6677 | 196 | 5985 | 112 | 6125 | 83 |
| 40:1 | 8 | 0.2 | 4601 | 221 | 2376 | NR | 7072 | 117 | 5411 | 80 | 4640 | NR |

* NR = No results

TABLE 4—*Precision of migration of alleles in the Y-PLEX™5 allelic ladder on the 310 Genetic Analyzer and the 377 DNA Sequencer.*

| | | | Size on the 310 (bases; $n = 129$) | | | Size on the 377 (bases; $n = 38$) | | |
|---|---|---|---|---|---|---|---|---|
| Locus | Allele | Sequence Size (bases) | Observed Range | Mean | S.D.* | Observed Range | Mean | S.D. |
| DYS389I | 11 | 244 | 242.96–243.79 | 243.40 | 0.214 | 245.89–246.23 | 246.00 | 0.090 |
| | 12 | 248 | 246.97–247.81 | 247.43 | 0.222 | 249.91–250.20 | 250.04 | 0.067 |
| | 13 | 252 | 250.97–251.89 | 251.47 | 0.226 | 253.84–254.06 | 253.94 | 0.061 |
| | 14 | 256 | 254.99–255.92 | 255.47 | 0.229 | 257.69–257.94 | 257.82 | 0.056 |
| | 15 | 260 | 259.15–259.97 | 259.60 | 0.231 | 261.60–261.87 | 261.72 | 0.058 |
| DYS389II | 27 | 360 | 361.24–362.03 | 361.63 | 0.164 | 362.45–362.95 | 362.64 | 0.108 |
| | 28 | 364 | 365.00–365.83 | 365.42 | 0.167 | 366.48–367.04 | 366.72 | 0.119 |
| | 29 | 368 | 368.82–369.64 | 369.23 | 0.182 | 370.53–371.05 | 370.73 | 0.114 |
| | 30 | 372 | 372.70–373.93 | 373.11 | 0.205 | 374.49–375.03 | 374.71 | 0.119 |
| | 31 | 376 | 376.64–377.52 | 377.09 | 0.191 | 378.46–379.00 | 378.66 | 0.128 |
| | 32 | 380 | 380.65–381.51 | 381.07 | 0.181 | 382.33–382.86 | 382.55 | 0.121 |
| | 33 | 384 | 384.73–385.44 | 385.07 | 0.161 | 386.21–386.70 | 386.39 | 0.116 |
| DYS439 | 10 | 241 | 238.15–238.82 | 238.50 | 0.163 | 242.14–242.43 | 242.24 | 0.069 |
| | 11 | 245 | 242.10–242.79 | 242.49 | 0.163 | 246.19–246.47 | 246.28 | 0.066 |
| | 12 | 249 | 246.10–246.78 | 246.49 | 0.161 | 250.24–250.43 | 250.29 | 0.048 |
| | 13 | 253 | 250.16–250.80 | 250.51 | 0.168 | 254.08–254.33 | 254.16 | 0.051 |
| | 14 | 257 | 254.16–254.83 | 254.53 | 0.153 | 257.90–258.21 | 258.02 | 0.065 |
| DYS438 | 8 | 131 | 130.71–131.32 | 131.04 | 0.149 | 130.41–131.53 | 131.00 | 0.181 |
| | 9 | 136 | 135.94–136.45 | 136.23 | 0.137 | 136.07–136.85 | 136.29 | 0.121 |
| | 10 | 141 | 141.16–141.81 | 141.58 | 0.143 | 141.74–142.70 | 141.84 | 0.187 |
| | 11 | 146 | 146.78–147.48 | 147.17 | 0.160 | 147.38–148.29 | 147.52 | 0.181 |
| | 12 | 151 | 152.27–153.06 | 152.74 | 0.182 | 152.64–153.27 | 152.78 | 0.123 |
| | 13 | 156 | 157.64–158.55 | 158.23 | 0.218 | 157.59–157.88 | 157.70 | 0.067 |
| DYS392 | 10 | 247 | 246.38–247.07 | 246.78 | 0.176 | 248.20–248.49 | 248.32 | 0.073 |
| | 11 | 250 | 249.41–250.10 | 249.82 | 0.176 | 251.15–251.44 | 251.30 | 0.070 |
| | 12 | 253 | 252.42–253.16 | 252.86 | 0.179 | 254.10–254.39 | 254.25 | 0.073 |
| | 13 | 256 | 255.43–256.18 | 255.89 | 0.185 | 257.05–257.30 | 257.20 | 0.061 |
| | 14 | 259 | 258.48–259.21 | 258.93 | 0.192 | 260.01–260.27 | 260.13 | 0.061 |
| | 15 | 262 | 261.51–263.13 | 261.99 | 0.221 | 262.94–263.18 | 263.07 | 0.061 |

* S. D. = Standard deviation.

TABLE 5—*Observed stutter values for the loci amplified using the Y-PLEX™5 system.*

| Locus | Stutter Range % | Mean Stutter % | S.D.* | Upper Range Stutter %** |
|---|---|---|---|---|
| DYS389I | 4.2–8.8 | 5.40 | 0.80 | 7.80 |
| DYS389II | 9.2–13.6 | 10.97 | 0.93 | 13.76 |
| DYS439 | 3.9–10.1 | 6.06 | 1.23 | 9.75 |
| DYS438 | 2.8–5.1 | 3.60 | 0.90 | 6.30 |
| DYS392 | 6.2–16.0 | 8.19 | 1.63 | 13.08 |

* S.D. = Standard deviation.
** Upper range stutter % = Mean + 3 S.D.

Portugal and from the Institute of Legal Medicine, Humboldt-University, Berlin, Germany and six samples (five male and one female) present in SRM 2395 provided by the National Institute for Standards and Technology (NIST) were amplified by using Y-PLEX™5 reagents (data not shown). The amplified products were analyzed on 310 and 377 instruments and the alleles were typed by using the Y5-Typer310v1.0 and Y5-Typer377v1.0 softwares. The results for all male samples obtained from IPATIMUP and NIST at all five loci were typed correctly (data not shown). The results for all samples obtained from the Institute of Legal Medicine, Humboldt-University, Berlin, Germany were typed correctly for the DYS389I, DYS389II, and DYS392 loci; this Institute did not confirm the results for DYS438 and DYS439 since these loci are not components of nine loci generating minimal haplotype. The allele designations obtained using Y-PLEX™5 genotyping system are concordant with the published nomenclature and the ISFH guidelines for STR analysis (20). Thus, the European database can be used for comparison of the results.

### *Contamination and Environmental Insult Analysis*

The allelic profile of the male donor whose DNA was used in this study was 14, 27, 12, 10, and 11 for the DYS389I, DYS389II, DYS439, DYS438, and DYS392 loci, respectively. The DNA obtained from the blood and semen stains exposed to the different environmental insults gave the same haplotype as the known blood for the donor except for the blood and semen samples treated with soil suspension (both wet and dry at both time intervals) (data not shown). For these samples no results were obtained with the initial amplification. The samples were then treated with the clean-up procedures as described under Materials and Methods. After Chelex® treatment, a complete or partial profile that matched the profile from the donor was obtained for all samples except the Day 1 bloodstain (prepared on the cloth saturated with the soil suspension). Similar conclusions were obtained when these samples were analyzed using the Y-PLEX™6 (9). After DNAIQ™ treatment, a partial profile that matched the profile from the donor was obtained from the Day 1 bloodstain prepared on the cloth saturated with the soil suspension and the Day 28 semen stain (prepared by saturating the semen stain with a soil suspension). No results were obtained for the other blood and semen stains subjected to the soil suspension.

Soil contamination had the greatest effect on the ability to amplify loci in the Y-PLEX™5 kit, with the DYS438 locus being af-

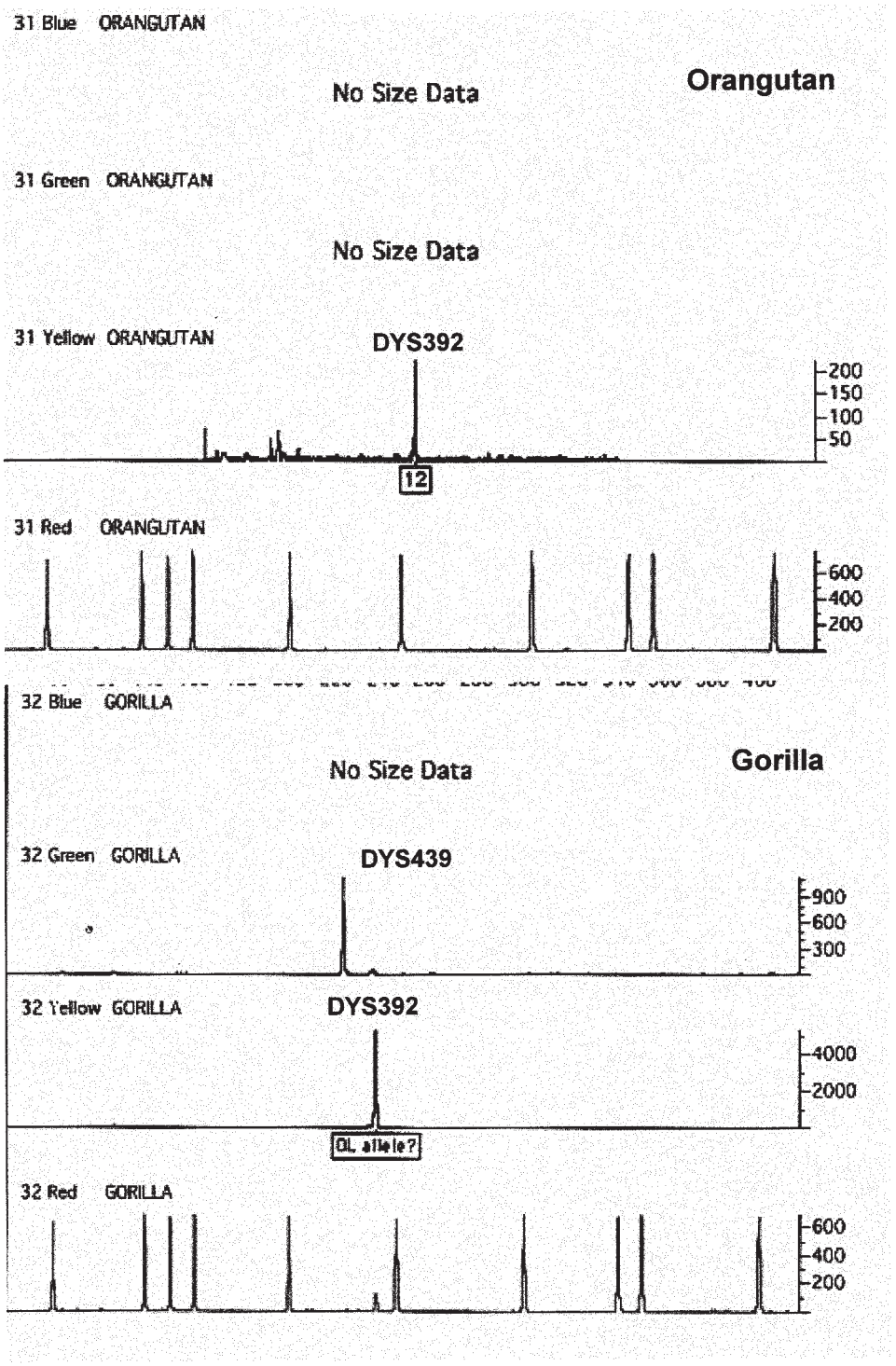


FIG. 7—*Profile of the DNA (2.0 ng) from orangutan (Top) and gorilla (Bottom) using the Y-PLEX™5 system.*

TABLE 6—*Analysis of the nonprobative casework samples using the Y-PLEX™5 system.*

| Sample Info | Sample Description | DYS389I | DYS389II | DYS439 | DYS438 | DYS393 |
|---|---|---|---|---|---|---|
| Case #1-Q1 | Vaginal swab–female fraction | 11 | NR* | 12 | 12 | NR |
| Case #1-Q2 | Vaginal swab–male fraction | 11, (13)** | 27, (29) | 12 | (10), 12 | 13 |
| Case #1-K1 | Suspect | 11 | 27 | 12 | 12 | 13 |
| Case #2-Q1 | Semen on sock–female fraction | NR | NR | NR | NR | NR |
| Case #2-K1 | Suspect | 13 | 29 | 12 | 12 | 13 |
| Case #3-Q1 | Saliva on perineal swab | 13 | 29 | 13 | 12 | 13 |
| Case #3-K1 | Suspect | 13 | 29 | 13 | 12 | 13 |
| Case #5-Q1 | Semen on perineal swab–male fraction | NR | NR | NR | NR | NR |
| Case #5-Q2 | Semen on vaginal swab–male fraction | 14 | 31 | 11 | 10 | 13 |
| Case #5-K1 | Suspect | 14 | 31 | 11 | 10 | 13 |
| Case #6-Q2 | Semen on sheet–female fraction | 13 | 29 | 13 | 12 | NR |
| Case #6-K1 | Suspect | 13 | 29 | 13 | 12 | 13 |

* NR = No results.
** (Allele) = Weaker allele.

fected the most. It should be emphasized that DNA extracted from soil-contaminated substrates, whether the soil contamination occurred before or after the biological fluid was applied, was never mistyped, just not typeable.

*Nonprobative Samples*

Five nonprobative cases containing various DNA sources, which were previously analyzed with the AmpF$\ell$STR® Profiler Plus and AmpF$\ell$STR® COfiler kits (Applied Biosystems), were re-analyzed using the Y-PLEX™5 kit. The samples included female and male DNA fractions of semen identified on vaginal swabs, perineal swabs, clothing, and bedsheets and saliva indicated on perineal swabs. The results obtained for Y-PLEX™5 are summarized in Table 6. The haplotype frequencies for the samples Case#1Q1, Case#1Q2, Case#3Q1, Case#5Q2, Case#6Q2, as calculated from the database currently available was 1,1, 49, 1, and 56 in 1298 (22). Thus, the haplotypes for the samples Case#1Q1, Case#1Q2 and Case#5Q2 were unique in the current database. The interpretation of the results obtained from the Y-PLEX™5, AmpF$\ell$STR® Profiler Plus and AmpF$\ell$STR® COfiler kits were consistent (data not shown). The results were also consistent with the previously reported studies for these cases using the Y-PLEX™6 (9). Results were concordant for the DYS389II locus between the Y-PLEX™5 and Y-PLEX™6 kits for those samples yielding results for both kits. The weak secondary contributor in samples Case#1-Q2 indicates a minor contribution from a second male. The conclusions derived from the results obtained from Y-PLEX™5 and the AmpF$\ell$STR® Profiler Plus, AmpF$\ell$STR® COfiler and Y-PLEX™6 genotyping systems are consistent.

*Population Studies*

Caucasian ($n$ = 103) and African American ($n$ = 378) population groups were profiled using the Y-PLEX™5 genotyping system. All DNA samples were buccal swabs. The re-injection rate was about 15%, which is higher than may be routinely expected. These samples were stored Chelex® extracts that have been frozen and thawed several times. The allele frequency distributions for each Y-STR locus in Caucasian and African-American sample populations are presented in Table 7. The haplotype random match probability for the Y-PLEX™5 system was 0.0749 and 0.0433 for Caucasian and African American population groups, respectively (Table 8). In Caucasians, 31 of the 103 and in African Americans 73 of the 378 individuals profiled were observed only once (Fig. 8).

TABLE 7—*Allele frequencies among the Caucasian and African American population groups typed using the Y-PLEX™5 system.*

| | | Caucasian ($n$ = 103) | | African American ($n$ = 378) | |
|---|---|---|---|---|---|
| Locus | Allele | Number | % | Number | % |
| DYS389I | 11 | … | … | 2 | 0.529 |
| | 12 | 16 | 15.534 | 59 | 15.608 |
| | 13 | 72 | 69.903 | 253 | 66.931 |
| | 14 | 14 | 13.592 | 63 | 16.667 |
| | 15 | 1 | 0.971 | 1 | 0.265 |
| DYS389II | 27 | 2 | 1.942 | 5 | 1.323 |
| | 28 | 12 | 11.650 | 33 | 8.730 |
| | 29 | 53 | 54.456 | 87 | 23.016 |
| | 30 | 26 | 25.243 | 137 | 36.243 |
| | 31 | 9 | 8.738 | 87 | 23.016 |
| | 32 | 1 | 0.971 | 27 | 7.143 |
| | 33 | … | … | 2 | 0.529 |
| DYS439 | 9 | … | … | 1 | 0.265 |
| | 10 | 6 | 5.825 | 5 | 1.323 |
| | 11 | 25 | 24.272 | 104 | 27.513 |
| | 12 | 52 | 50.485 | 203 | 53.704 |
| | 13 | 20 | 19.417 | 57 | 15.079 |
| | 14 | … | … | 8 | 2.116 |
| DYS438 | 8 | 1 | 0.971 | 10 | 2.646 |
| | 9 | 4 | 3.883 | 1 | 0.265 |
| | 10 | 19 | 18.447 | 38 | 10.053 |
| | 11 | 12 | 11.650 | 239 | 63.228 |
| | 12 | 64 | 62.136 | 88 | 23.280 |
| | 13 | 3 | 2.913 | 2 | 0.529 |
| DYS392 | 10 | … | … | 3 | 0.794 |
| | 11 | 26 | 25.243 | 277 | 73.280 |
| | 12 | 6 | 5.825 | 14 | 3.704 |
| | 13 | 65 | 63.107 | 71 | 18.783 |
| | 14 | 6 | 5.825 | 12 | 3.175 |
| | 15 | … | … | 1 | 0.265 |

TABLE 8—*Genetic diversity and haplotype random match probability for the Y-PLEX™5 system.*

| Population | Genetic Diversity (h) | Haplotype Random Match Probability |
|---|---|---|
| Caucasian ($n$ = 103) | 0.9342 | 0.0749 |
| African American ($n$ = 378) | 0.9592 | 0.0433 |

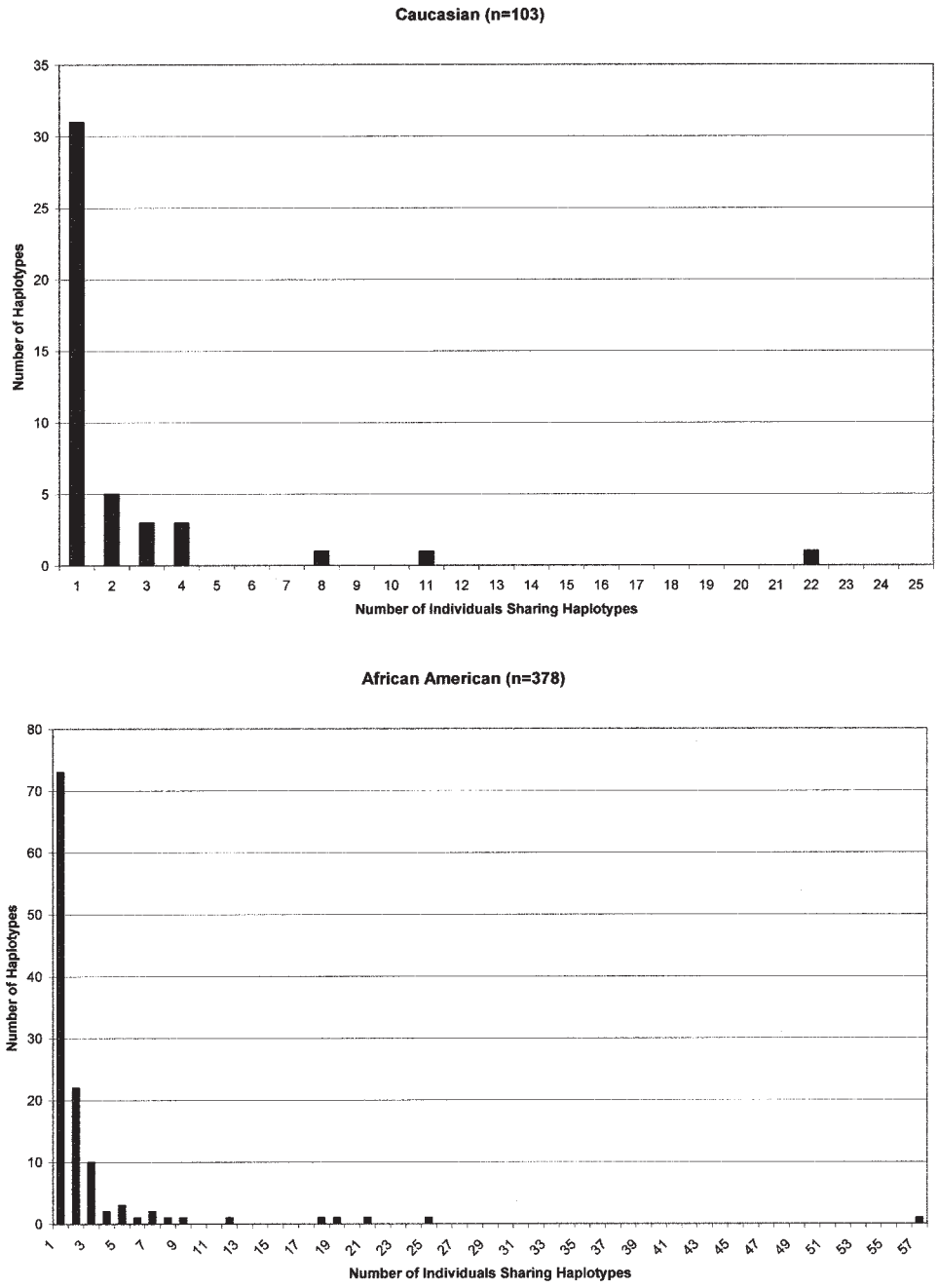


FIG. 8—*Haplotype count distribution in the Caucasian population (Top) (n = 103) and the African American population (Bottom) (n = 378).*

The most frequent haplotypes observed in the Caucasian and African American population datasets are presented in Table 9.

## Discussion

The Y-PLEX™6 multiplex system enables amplification of seven of the nine loci, which provides minimal haplotypes, identified by the International Y-STR user group (5,9). In order to achieve the analysis for all nine loci, we have developed the Y-PLEX™5 system. The five loci namely DYS389I, DYS389II, DYS439, DYS438 and DYS392 were selected for generating the Y-PLEX™5 system. Of these, the DYS389I, DYS389II and DYS392 loci are members of the nine loci generating minimal haplotype. The other two loci DYS438 and DYS439 exhibit relatively high gene diversity values compared with other reported Y-STR loci (11). The locus DYS389II resides in both the Y-PLEX™5 and Y-PLEX™6 systems. Validation studies for the Y-PLEX™5 were performed according to the FBI Director's Quality Assurance Standards and included the following experiments: annealing temperature, primer ratio, primer concentration, salt concentration, different DNA polymerases, concentration of dNTPs, different thermal cyclers, denaturation time, annealing time, cycle extension time, final extension time, number of PCR cycles, reaction volume, female DNA, sensitivity, non-human studies, reproducibility, precision, additives, inter-laboratory studies, female-male mixtures, male-male mixtures, stutter, DNase degradation, environmental insult, and nonprobative casework.

TABLE 9—*Observed number of the most frequent haplotypes analyzed using the Y-PLEX™5 system.*

| Caucasian ($n$ = 103) | | African American ($n$ = 378) | |
|---|---|---|---|
| Y Haplotype* | *n* | Y Haplotype* | *n* |
| 13-29-12-12-13 | 22 | 13-30-12-11-11 | 57 |
| 13-29-13-12-13 | 11 | 13-29-12-12-13 | 25 |
| 14-30-12-12-13 | 8 | 13-31-12-11-11 | 21 |
| 12-28-11-10-11 | 4 | 13-30-11-11-11 | 19 |
| 13-29-11-12-13 | 4 | 14-31-12-11-11 | 19 |
| 13-30-10-11-11 | 4 | 13-30-13-11-11 | 18 |
| 13-29-12-12-14 | 3 | 13-31-11-11-11 | 12 |
| 13-30-12-10-12 | 3 | 13-29-11-12-13 | 9 |
| 13-30-12-12-13 | 3 | 13-30-12-12-13 | 8 |
| | | 12-28-11-11-11 | 7 |
| | | 13-31-13-11-11 | 7 |
| | | 14-32-12-11-11 | 6 |
| | | 12-29-12-11-11 | 5 |
| | | 13-29-13-12-13 | 5 |
| | | 13-32-12-11-11 | 5 |
| | | 13-29-12-11-11 | 4 |
| | | 13-29-12-12-14 | 4 |
| | | 12-28-11-10-11 | 3 |
| | | 12-28-11-12-13 | 3 |
| | | 12-29-11-8-11 | 3 |
| | | 12-29-13-11-11 | 3 |
| | | 12-30-11-11-11 | 3 |
| | | 13-28-11-12-13 | 3 |
| | | 13-29-11-11-11 | 3 |
| | | 13-30-10-11-11 | 3 |
| | | 14-30-11-11-11 | 3 |
| | | 14-30-12-12-13 | 3 |

* The haplotype is DYS389I, DYS389II, DYS439, DYS438, DYS392.

The Y-PLEX™5 allelic ladder provides profiles for most frequently observed alleles in the general population. The nucleotide sequences of the alleles were consistent with the number of repeat units and devoid of any microvarients. The allele nomenclature for the alleles at DYS389I, DYS389II and DYS392 were made according to the ISFG guidelines (5,7). Ayub et al. (11) have suggested to count the variable GATA and TTTTC repeat units at the DYS439 and DYS438 loci, respectively, for allele designation. The DYS439 locus contains stretches of constant repeat unit GATA preceding the variable repeat unit GATA:$[GATA)_2N_4(GATA)_3N_{14}(GATA)N_3(GATA)N_7(GATA)_n]$. In a recent study, Gusmao et al. (21) have compared the sequences from human and chimpanzees and concluded that no variation is expected in the constant repeat unit. Thus, in the present study only variable repeat unit $(GATA)_n$ was used for the nomenclature of alleles at this locus, which is in accord with the published literature (11,21). At the DYS438 locus, Gusmao et al. (21) have reported occurrence of one TTTTA unit within the variable TTTTC repeat units in some humans: $[(TTTTC)_1(TTTTA)_{0,1}(TTTTC)_n]$. Occurrence of such TTTTA unit in sequences of all alleles in the allelic ladder for the Y-PLEX™5 system was not observed. For the nomenclature of alleles at DYS438 locus, all TTTTC repeat units were counted, which includes complete stretch of variable and constant repeat unit observed by the Gusmao et al. (21). The allele designation for alleles at all five loci amplified with the Y-PLEX™5 system were confirmed by sample exchange with the IPATMUP, NIST, and Institute of Legal Medicine, Humboldt-University, Berlin, Germany. Further, nomenclature for the alleles at DYS439 and DYS438 loci was discussed at the III International Y-User Workshop and the group accepted the nomenclature followed in the present investigation (26).

The minimum sensitivity of 0.1 ng of male DNA for the Y-PLEX™5 kit compares favorably with the sensitivity for the Y-PLEX™6 system of 0.2 ng of male DNA (9). The profiles of male-male mixture samples tested provided clear indication of contribution by two male components. It should be noted that the mixture generated from two or more male donors sharing the same paternal lineage would provide identical profiles, which may be misinterpreted as a single source sample. This is true for all Y-STR systems. It was possible to obtain the profile of male DNA in a mixture sample containing female DNA as high as 300 ng. The results demonstrate that the Y-PLEX ™5 system is highly specific in amplification of male DNA.

The phenomenon of difference in the size of alleles up to 4 bases on the 310 Genetic Analyzer and 377 DNA Sequencer is attributed to different electrophoretic conditions. Though the electrophoresis was performed under denaturing conditions on 310 Genetic Analyzer and 377 DNA Sequencer, the electrophoresis conditions on former instrument for individual sample (injection) were 60°C, 7–8 μA and 15 kV and on the latter instrument for a gel plate for 96 samples were 51°C, 25–30 mA and 3 kV. Further, the electrophoresis medium on the 310 Genetic Analyzer was poly (N,N-dimethylacrylamide), which is a liquid porous medium and on the 377 DNA Sequencer was polyacrylamide (solid porous medium). Thus, it is important to use an allelic ladder for genotyping. The standard deviation and upper stutter percentage for the loci DYS389I, DYS389II, DYS439, DYS438, and DYS392 were comparable to the Y-STR loci amplified with Y-PLEX™6 (9) and autosomal loci amplified with AmpFℓSTR® Profiler Plus and AmpFℓSTR® COfiler (23).

The allele frequencies obtained for the Caucasian and African American population groups were consistent with data published for populations of similar anthropological affinity (5,9,11,24,25). The haplotype random match probability for the haplotype for Caucasian and African American population groups by obtained using Y-PLEX™5 was 0.0749 and 0.0433 and Y-PLEX™6 was 0.0096 and 0.0039, respectively (9). The discriminating power - using the Y-PLEX™5 is not as great as the Y-PLEX™6. However, the discriminating power of the haplotype for 11 Y-STR loci generated using the Y-PLEX™5 and Y-PLEX™6 genotyping systems is higher than for the minimal haplotype generated for nine loci (manuscript in preparation). Thirty-one of the 103 Caucasians and 73 of the 378 African Americans exhibited unique haplotype when profiled with the Y-PLEX™5. The database studies using Y-PLEX™6 revealed that 239 of the 581 Caucasians and 344 of the 543 African Americans profiled were observed only once (9). Roewer et al. (5) profiled 4688 individuals, which is the largest European database for the minimal Y-STR haplotypes. In this database, 139 individuals share one profile and 14 or more individuals share the 30 most frequent haplotypes.

## Conclusions

The Y-PLEX™5 multiplex system enabled analysis of the five Y-STR loci namely DYS389I, DYS389II, DYS439, DYS438, and DYS392. The system's sensitivity was sufficient to obtain a complete profile from 0.1 ng of male DNA. The data support that the Y-PLEX™5 system is valid and reliable for forensic casework. The conclusions for nonprobative casework obtained with the Y-PLEX™5 system were concordant with the conclusions obtained from autosomal STR loci.

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