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TECHNICAL NOTE

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Development and Validation of a Multiplexed Y-Chromosome STR Genotyping System, Y-PLEX™6, for Forensic Casework*

ABSTRACT: A Y-chromosome multiplex polymerase chain reaction (PCR) amplification kit, known as Y-PLEX™6, has been developed for use in human identification. The Y-PLEX™6 kit enables simultaneous amplification of six polymorphic short tandem repeat (STR) loci located on the non-recombinant region of the human Y-chromosome. These loci are: DYS393, DYS19, DYS389II, DYS390, DYS391, and DYS385. Our studies show that as little as 0.2 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 1:125. Among the six Y-STR loci, the maximum mean stutter percentage was 11.9 for allele at DYS389II locus. Attempts at amplification of DNA from various animal sources revealed that the Y-PLEX™6 primers are human specific. Details of the development of the kit, generation and description of the allelic ladders, and validation of the multiplex PCR are presented. In addition, Y-STR allele and haplotype frequencies in three populations have been investigated. The data indicate that results obtained using the Y-PLEX™6 kit are robust, 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, DYS393, DYS19, DYS389II, DYS390, DYS391, DYS385

Short tandem repeat (STR) loci are distributed ubiquitously throughout the genome and have become useful genetic markers for human identification due to their high power of discrimination and possibility of being amplified in a multiplex fashion (1–3). STR loci are now routinely used in forensic casework and paternity evaluations. In forensic DNA analysis, additional genetic markers, such as mitochondrial DNA (4,5) and Y-chromosome specific STR loci (6–9) are becoming increasingly important in investigating difficult cases.

The ability to identify male specific DNA in a mixed gender DNA sample can be valuable evidentiary information in resolving some cases. The haploid nature of Y-specific genetic markers may aid in genetic characterization of male contributors in a multiple source DNA sample. In assaults where a female victim has struggled with the assailant, her nail scrapings may have minimal amounts of foreign DNA from a male assailant. In some cases, autosomal STR results may contain predominantly female alleles mixed with alleles from the male suspect at minimal levels, making it difficult, if not impossible, to differentiate the suspect's profile from the stutter peaks of the victim's profiles; valuable probative information may be lost. Therefore, polymorphic STR loci residing on the non-recombining region of the Y-chromosome (and with unipaternal mode of inheritance) can play a very useful role in resolving difficult cases where male and female biological material is mixed together.

The importance of Y-chromosome markers in human identification has been evaluated for forensic applications. In fact, there are a number of studies relating to multiplex analysis of Y-chromosome markers (7,9–15). However, a standardized and validated commercial multiplex system with a sequence-verified allelic ladder has not been available for forensic casework. An informative core set of eight Y-STR loci have been described and they are: DYS19, DYS385, DYS389I, DYS389II, DYS390, DYS391, DYS392, and DYS393 (7,16–18). We report here the development and validation of a Y-PLEX™6 PCR kit comprising six Y-STR loci: DYS393, DYS19, DYS389II, DYS390, DYS391, and DYS385. The studies support that this six locus Y multiplex system can be used to obtain reliable results for forensic casework and male lineage studies.

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Materials and Methods

The custom primers, fluorescent labeled and unlabeled, were synthesized and obtained from commercial sources (Life Technologies, Rockville, MD; Operon, Alameda, CA). AmpliTaq Gold®, performance-optimized polymer POP 4, matrix standards (FAM, ROX, TAMRA, and JOE), 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 (100X) 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.

Extraction and Quantitation of DNA

The DNA from anonymous donor samples was obtained from buccal swabs or blood drawn in EDTA vacutainer tubes and was extracted either by phenol-chloroform (19), Chelex® (20) or extraction from FTA paper (21) procedures. The quantity of human DNA was determined by slot blot hybridization using the Quantiblot kit (Applied Biosystems, Foster City, CA) and following the manufacturer's recommended protocols.

Amplification

Amplification reactions were set up as follows: 5.0 μL of 5X Y-PLEX™6 Primer Mix; 0.5 μL of AmpliTaq Gold™ (5 units/μL); 2 to 5 ng of DNA template. The volume was adjusted to 25 μL using sterile water. Final concentration of the buffer in amplification reaction was 1.4X. Amplification reactions were performed in a 9600 or 9700 Thermal Cycler (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; 30 cycles of 94°C, 30 s; 59°C, 1 min and 70°C, 1 min; 60°C, 60 min and 4°C until the samples were removed from the thermal cycler. The positive control (2 to 5 ng of male DNA, ATCC 45514) and the negative control (2 to 5 ng of female DNA, ATCC 45510) were amplified with every batch of amplification reaction.

Analysis of Amplified Product on 310 Genetic Analyzer

Amplified products 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. The samples were denatured at 95°C for 3 min using either a 9600 or 9700 Thermal Cycler. The denatured products were electrophoretically separated on a 310 Genetic Analyzer using performance optimized polymer 4 (POP-4), filter set A, and an injection time of 5 s as described in the "ABI Prism™ Genetic Analyzer, User's Manual" (Applied Biosystems, 1998). 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, JOE, ROX, and TAMRA was generated and used.

Analysis of Amplified Product on 377 DNA Sequencer

Amplified products 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. The samples were denatured at 95°C for 3 min using a 9700 Thermal Cycler. The denatured products were electrophoretically separated on the 377 DNA Sequencer using filter set A as described in the "ABI Prism™ 377 DNA Sequencer User's Manual" (Applied Biosystems, 1998). The run time was approximately 3 to 4 h (time necessary to elute the 450 base pair size standard peak in GS500 ROX). A matrix file using the matrix standards FAM, JOE, ROX, and TAMRA was generated and used.

Sequencing of Alleles

Individual alleles, after amplification, were sequenced by using BigDye™ terminator cycle sequencing using ABI 377 DNA Sequencer (Applied Biosystems, Foster City, CA).

Validation Studies

The methods used in validation studies are described in the text.

Contamination and Environmental Insult Analysis

The contaminant study was grouped into three groups: contaminants—wet; contaminants—dry; and environmental. Bloodstains were prepared using freshly drawn blood without any preservatives or anticoagulants. Air-dried semen stains were prepared from previously pooled ejaculates. The cotton cloth (100%) was used to prepare the stains after laundering, rinsing, and drying. Fifty μL bloodstains and 25 μL semen stains were made for the environmental study. This was done in duplicate and the entire stain for each duplicate was extracted separately. One of the extracted samples was used for various PCR-based validations. The DNA was quantified, and 5 ng of DNA based on slot blot analysis was amplified for each sample. The PCR reaction was run for 28 cycles. The control samples in all studies were stored at −20°C.

Contaminants (Wet)—Cotton cloth pieces were saturated with the contaminants (soil solution, liquid hand soap, unleaded gasoline, 10% bleach, and *Eschecheria coli* bacteria strain HB101) and allowed to air-dry overnight at room temperature. Replicate semen (25 μL) and whole blood (50 μL) stains were made on each cloth and allowed to dry overnight at room temperature. These samples were stored at room temperature in the dark until processed. The blood and semen stains were processed at intervals of 1, 4, 8, 14, and 28 days. An organic extraction method was used to extract and purify the DNA (22). Stains from Day 1 and Day 28 were used for this study except for the bleach/blood mixture, in which Day 14 was used because the stain from Day 28 was not available.

Contaminants (Dried Stains)—Liquid whole blood (50 μL) and semen (25 μL) were applied to the cotton cloth and allowed to air-dry overnight. The cloth pieces were then saturated with either a soil suspension or unleaded gas and allowed to air-dry overnight. These samples were stored at room temperature in the dark until processed. Blood and semen stains were processed at intervals of 1, 4, 8, 14, and 28 days. DNA was organically extracted (22). Stains from Day 1 and Day 28 were used for this study.

Environmental—Liquid whole blood (50 μL) and semen (25 μL) samples were applied to cotton cloth and allowed to air-dry overnight. The stains were placed outdoors in direct sunlight or in the dark and were exposed to daily temperatures that ranged from 10 to 35°C. Stains were processed at intervals of 1, 4, 8, 14, and 28 days. Stains from Day 1 and Day 28 were extracted organically (22).

Statistical Analyses

Allele frequencies for each marker were determined by the gene-count method (23).

Results and Discussion

For construction of the Y-PLEX™6 multiplex system, six loci—DYS393, DYS19, DYS389II, DYS390, DYS391, and DYS385—were selected. Of these loci, the DYS385 locus demonstrates variation at two loci due to gene duplication (24). These six loci contained within the Y-PLEX™6 system are part of the international research haplotype group (7,16). Thus, this multiplex system will be useful for particular forensic analyses. In order for the Y multiplex system to be applicable to forensic analyses, various criteria were addressed including primer design, sensitivity, obtaining allele peak height balance, percentage of stutter peaks, and cross-reactivity with DNA from females. In addition, mixture, environmental insult, and population studies were performed. A minimum value of 75 RFU was used for interpretation of the data.

Development of Multiplex System

The GenBank accession numbers, repeat motifs, and size of PCR products for these six Y-STR loci are summarized in Table 1. The Y-PLEX™6 primer mix was prepared as a mixture of locus specific fluorescent labeled and unlabeled primers in a 1.4 X GeneAmp® PCR Gold buffer (Applied Biosystems, Foster City, CA) containing 8 m*M* dNTPs and salts. Primers for DYS393, DYS19, and DYS389II were labeled with FAM, and the primers for DYS390, DYS391, and DYS385 were labeled with TAMRA.

The performance of the Y-PLEX™6 kit was investigated by first individually amplifying each locus utilizing 15 different male DNA samples. The primer pair titration was tested at 0.5X, 1X, 1.5X, and 2X (1X primer mix provides 0.075 to 0.227 μ*M* concentration of primers in the final reaction mixture). Results showed that at a higher primer concentration (>1X), the loci DYS393 and DYS390 were amplified preferentially, whereas at a lower primer concentration (<1X) the longer fragments from DYS389II and DYS385 were amplified preferentially (data not shown). Although the amount of amplification product varied, as determined by peak area between the loci, no allele dropout at the studied primer pair concentrations was observed. The final concentration (1X) of primers in the Y-PLEX™6 primer mix was selected so that the best locus-to-locus balance could be obtained.

The concentration of $MgCl_2$ in the PCR was varied from 0.5 to 2 m*M*. At 2 m*M* $MgCl_2$, allele dropout at the DYS389II and DYS385 loci was observed (data not shown). At 0.5 m*M* $MgCl_2$, as expected, amplification was poor for all six loci. The 5X primer mix provided with the amplification kit contains optimal concentration of $MgCl_2$ as well as buffer, and further addition of $MgCl_2$ is not required.

The amount of AmpliTaq Gold® was varied, at regular intervals, from 0.1 to 1.0 μL (5 units/μL) in the PCR reaction using a known DNA sample (2 ng). The optimum results were obtained at 0.5 μL of AmpliTaq Gold®. When less than 0.5 μL were used, the RFU of amplified alleles for DYS389II and DYS385 loci were less than 75. Incorporation of more than 0.5 μL of AmpliTaq Gold® in the reaction mixture did not increase the RFU of the amplified product significantly. Hence, 0.5 μL of AmpliTaq Gold® is recommended as the cost effective concentration. However, when highly degraded DNA samples are being amplified, higher amounts of the enzyme up to 1.0 μL were required (data not shown).

TABLE 1—*General criteria of loci in the Y-PLEX™ 6 kit.*

STR Marker	Dye	Color Window	PCR Product Size, bp	Repeat Motif	GenBank Accession No.
DYS 393	FAM	Blue	112–136	AGAT	G09601
DYS 19	FAM	Blue	181–205	TAGA	X77751
DYS 389II	FAM	Blue	286–320	TCTG/TCTA	G09600
DYS 390	TAMRA	Yellow	179–207	TCTA/TCTG	G09611
DYS 391	TAMRA	Yellow	241–257	TCTA	G09613
DYS 385	TAMRA	Yellow	345–394	GAAA	Z93950

Annealing Temperature

The T_m for the primers for six loci amplified using Y-PLEX™6 was calculated to be between 47 and 54°C. Empirically, the annealing temperature for the amplification was determined using a known male DNA sample (2 ng) and performing the PCR for 28 cycles at different annealing temperatures ranging from 55 to 65°C using a PTC200 thermal cycler (MJ Research). The well temperature was recorded. The amplified products were analyzed on a 310 Genetic Analyzer. The best peak height balance among all six loci was achieved at an annealing temperature of 59°C (Fig. 1). At 61°C, allele dropout was observed at the DYS19 locus. At higher temperatures, allele dropout at the loci DYS19, DYS389II, DYS390, and DYS391 was observed.

Number of Cycles

DNA from four different male samples and one female sample was amplified at various cycle numbers to determine the number of amplification cycles necessary to optimize results for the Y-PLEX™6 kit. Each sample (2 ng of template) was amplified using 26, 28, 30, and 32 cycles and an annealing temperature of 59°C. At 26 cycles, allele dropout was observed at one or more of the following loci: DYS385, DYS389II, and DYS19. The amplification of alleles at all loci in the male samples was obtained at 28 cycles or higher. Amplification for 30 cycles was selected since the peak height of alleles was higher than that after 28 cycles (Fig. 2). The quantity of amplification product did not increase when the PCR was run for 32 cycles, as revealed by peak height. The female DNA sample did not exhibit any products when amplified for 26, 28, 30, or 32 cycles.

PCR Volume

Three reaction volumes were tested to determine the range of PCR volume that can be tolerated using the Y-PLEX™6 kit. Four male samples (2 ng of template), which had previously been typed using the Y- PLEX™6 kit, were amplified using 12.5, 25, and 50 μL reaction volumes in a 9700 thermal cycler. The amplified products were analyzed on a 310 Genetic Analyzer. The correct haplotype was obtained for all the samples at all reaction volumes (data not shown). Thus, any of these volumes can be used.

Thermal Cycler

Amplification using the Y-PLEX™6 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 six loci amplified well using any of the three thermal cyclers (data not shown). However, amplification

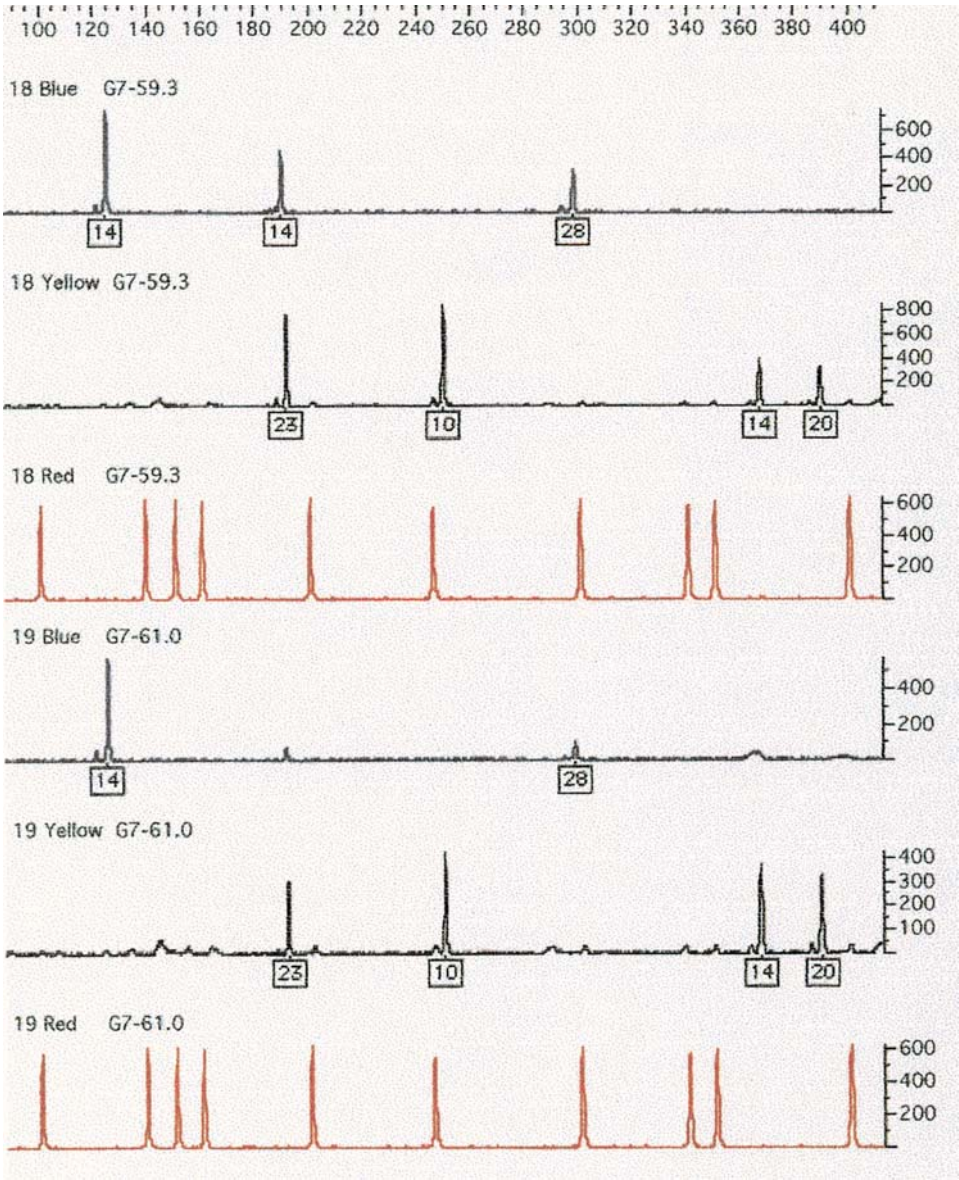

FIG. 1—*Profiles from amplification at 59.3°C* (top) *and 61.0°C* (bottom) *using the Y-PLEX™ 6 kit.*

yield was highest when using the PTC-200, followed by the 9600 and the 9700 using PCR conditions as described in Materials and Methods.

Generation of Allelic Ladder

Anonymous DNA samples from 200 individuals were amplified for each locus, and allele designations were made. Subsequently, DNA samples were chosen and combined so that the desired combination of alleles was obtained for a generation of an allelic ladder. The DNA templates were amplified for each locus individually. Then the locus-specific amplified product was analyzed on a 310 Genetic Analyzer. Based on the results, the amplified products were pooled so that the ladder alleles at all loci provided peaks generally of equal height. The alleles from each locus were sequenced to ensure that the amplified products in the ladder had the correct repeat structures and length. A typical Genotyper® profile of the Y-PLEX™6 ladder is provided in Fig. 3.

Electrophoresis

The electrophoresis conditions using POP-4 polymer and a 310 Genetic Analyzer and that of Long Ranger® gel and a 377 DNA Sequencer are not identical; thus, the estimated size of the alleles will likely differ with each system. Size differences of up to three base pairs have been observed for some alleles for Y-PLEX™6. The sizes of alleles when analyzed on a 310 Genetic Analyzer and a 377 DNA Sequencer are summarized in Table 2. This observation demonstrates the importance of using an allelic ladder for normalizing allele calls.

Precision of Allele Sizing during Repeated Analysis

The Y-PLEX™6 allelic ladder was injected 25 times onto a 310 Genetic Analyzer, and the precision of estimating each allele size in the allelic ladder was calculated. The sizes of the alleles from sequence studies, observed size range, mean size, and standard devi-

ation for each allele in the allelic ladder are summarized in Table 3. The standard deviation values were less than 0.1 except for alleles at the DYS389II and DYS385 loci. Maximum values for standard deviation at the DYS389II and DYS385 loci were 0.255 and 0.160, respectively.

Minimum Sensitivity

The minimum amount of input DNA required to obtain a complete profile was investigated. Serial dilutions were prepared of a known DNA sample. The amount of template DNA in the reactions was: 10.0, 8.0, 5.0, 2.0, 1.0, 0.5, 0.4, 0.3, 0.2, 0.1, and 0.05 ng. Allele dropout at the loci DYS390, DYS391, and DYS385 was observed when 0.05 and 0.1 ng of DNA was used for amplification. When 0.2 ng of template DNA was used, the allele peaks at all six loci were readily detectable; the alleles had peak height between 400 to 600 RFU (Fig. 4). Thus, 0.2 ng of DNA was determined to be the minimum amount of DNA for the Y-PLEX™6 to obtain a complete profile (based solely on the quantities tested). At template levels of 5 ng or greater, off-scale data and excessive stutter peaks were observed.

Stutter Studies

Measurement of stutter for the alleles at all six loci was performed for 50 male samples. The height of the stutter peak was compared with the corresponding allele at each locus (Table 4). The values for standard deviation and upper range stutter percent for the loci DYS393, DYS19, DYS389II, DYS390, DYS391, and DYS385 were comparable to the autosomal loci amplified with AmpFℓSTR® Profiler Plus and AmpFℓSTR® COfiler as reported by Moretti et.al. (25). At Locus DYS19, an N–2 stutter peak was observed.

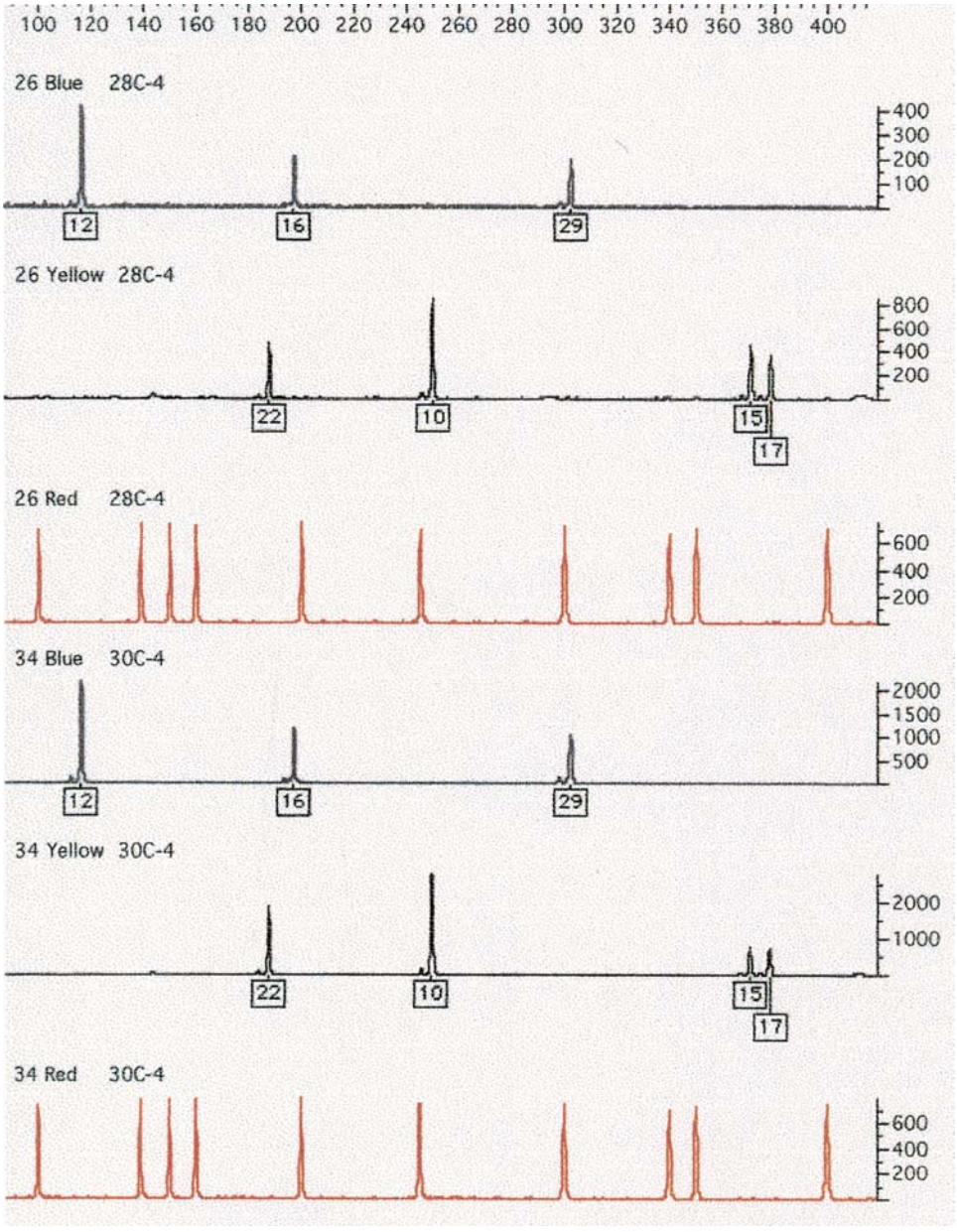

FIG. 2—*Profiles obtained from amplification of 28* (top) *and 30* (bottom) *cycles.*

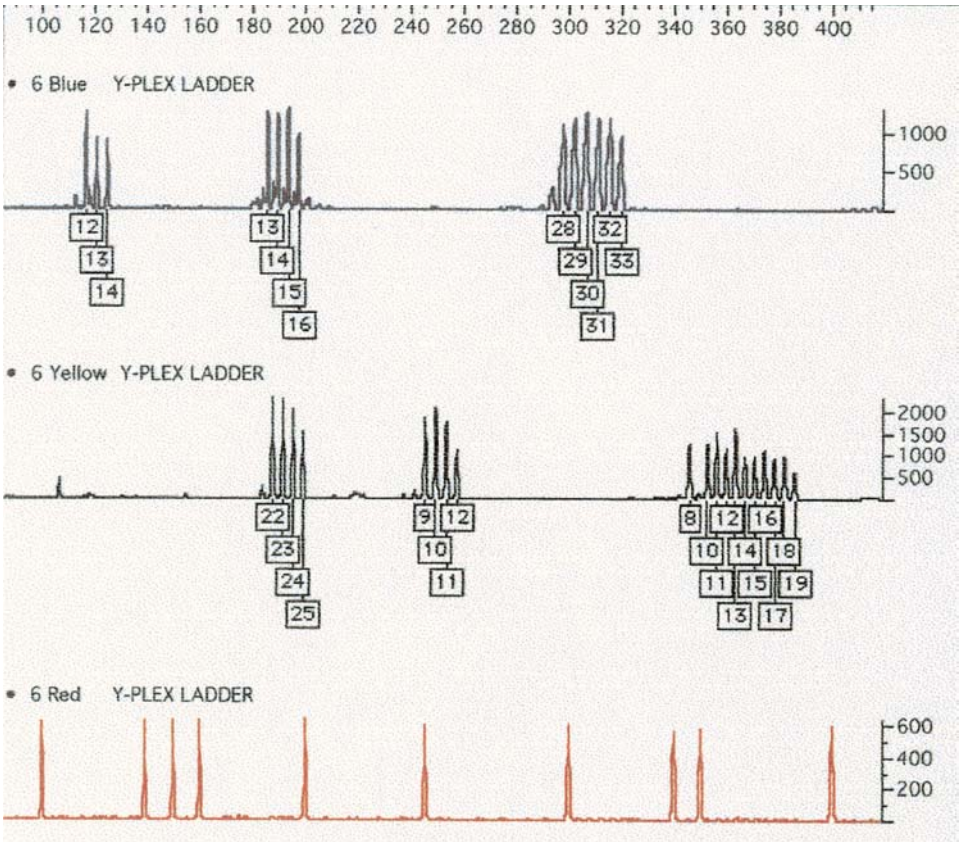

FIG. 3—*Y-PLEX™ 6 Allelic ladder used for the genotyping.*

TABLE 2—*Operational size of alleles in the Y-PLEX™ 6 ladder.*

Color Window	STR Locus	Alleles in Ladder	Size on 310, bp	Size on 377, bp	Other Observed Alleles
Blue	DYS 393	12	116.00	119.00	11, 15,16, 17
		13	120.00	123.00	
		14	124.00	127.00	
	DYS 19	13	185.00	187.50	12, 14.3, 17, 18
		14	189.00	191.50	
		15	193.00	195.50	
		16	197.00	199.50	
	DYS 389II	28	298.00	298.00	25, 26, 27
		29	302.00	302.00	
		30	306.50	306.00	
		31	311.00	310.00	
		32	315.00	314.00	
		33	319.00	318.00	
Yellow	DYS 390	22	187.00	186.00	20, 21, 25.2,
		23	191.00	190.00	26, 27
		24	195.00	194.00	
		25	199.00	198.00	
	DYS 391	9	245.00	247.00	8
		10	249.00	251.00	
		11	253.00	255.00	
		12	257.00	259.00	
	DYS 385	8	345.50	344.00	9, 13.2, 14.2,
		10	352.50	352.00	15.3, 17.1,
		11	356.50	356.00	17.2, 20, 21
		12	360.00	360.00	
		13	363.50	364.00	
		14	367.50	367.50	
		15	371.00	371.00	
		16	375.00	375.00	
		17	378.50	379.00	
		18	382.50	382.50	
		19	386.00	386.00	

TABLE 3—*Precision of migration of alleles in allelic ladder on 310 Genetic Analyzer.*

Loci	Allele	Seq. Size	Observed Range	Mean	S.D.
DYS393	12	116	116.08–116.37	116.23	0.075
	13	120	120.13–120.36	120.24	0.063
	14	124	124.19–124.35	124.28	0.048
DYS19	13	185	185.11–185.42	185.29	0.083
	14	189	189.02–189.31	189.21	0.075
	15	193	192.98–193.21	193.12	0.057
	16	197	196.94–197.13	197.02	0.051
DYS389II	28	298	297.29–297.64	297.44	0.102
	29	302	301.42–301.84	301.64	0.126
	30	306	305.26–306.29	306.02	0.255
	31	310	310.25–310.69	310.47	0.146
	32	314	314.05–315.06	314.8	0.215
	33	318	318.82–319.38	319.08	0.177
DYS390	22	186	186.98–187.33	187.17	0.099
	23	190	190.91–191.22	191.07	0.08
	24	194	194.81–195.11	194.95	0.078
	25	198	198.71–198.87	198.8	0.063
DYS391	9	245	245.21–245.48	245.35	0.064
	10	249	249.27–249.51	249.39	0.054
	11	253	253.33–253.51	253.41	0.049
	12	257	257.31–257.54	257.39	0.064
DYS385	8	345	345.57–345.78	345.71	0.059
	10	352	352.73–352.90	352.79	0.037
	11	356	356.34–356.71	356.45	0.07
	12	359	359.97–360.18	360.08	0.061
	13	363	363.66–363.89	363.75	0.068
	14	367	367.30–367.62	367.45	0.077
	15	370	371.09–371.34	371.23	0.077
	16	374	374.92–375.13	375	0.067
	17	378	378.08–378.96	378.75	0.16
	18	382	382.43–382.75	382.55	0.087
	19	385	386.10–386.52	386.32	0.097

S.D. = Standard Deviation.

Mixture Studies

Two sets of mixtures of DNA samples, male-female and male-male, were prepared. The male-female mixtures were prepared in the proportions 1:0, 1:1, 1:2, 1:5, 1:10, 1:20, 1:30, 1:40, 1:50, 1:125, 1:250, 1:312, and 1:500. The male-male mixtures were prepared in the proportions 1:0, 1:1, 1:2, 1:5, 1:10, 1:20, 1:30, 1:40, 1:50, 0:1, 2:1, 5:1, 10:1, 20:1, 30:1, 40:1, and 50:1. The samples were amplified and analyzed under standard conditions. The quantities of DNA used in preparing mixtures and results of mixture studies are summarized in Table 5 and Table 6.

TABLE 4—*Stutter values for each locus amplified using the Y-PLEX™ 6 kit.*

Locus	*n*	Stutter Range, %	Mean Stutter, %	S.D.	Upper Range Stutter %
DYS393	48	5.4–9.1	7.10	0.80	9.50
DYS19	45	4.8–7.9	6.30	1.00	9.30
DYS389II	46	9.4–14.8	11.90	1.07	15.11
DYS390	47	4.2–10.4	6.40	1.50	10.90
DYS391	45	4.8–7.0	5.54	0.60	7.34
DYS385	49	4.5–11.3	7.00	1.50	11.50

S.D. = Standard Deviation.
Upper Range Stutter % = Mean + 3S.D.

Male-Female Mixtures—A complete male profile was detected in the male-female mixture samples down to the 1:125 ratio, which contained 0.2 ng of male DNA and 25 ng of female DNA (see Table 5). The mixtures generated at proportions of 1:250 and greater exhibited the loss of some of the male alleles. When the template quantity of female DNA was 10 ng or greater, two TAMRA-labeled amplified products of sizes 255 bp and 448 bp were observed, which was the result of nonspecific amplification.

Male-Male Mixtures—Allelic profile of male-1 for Loci DYS393, DYS19, DYS389II, DYS390, DYS391, and DYS385 was 14, 15, 31, 23, 10, and 14.2 and 15, respectively. The allelic profile of male-2 for these loci was 15, 15, 29, 21, 10, and 17, respectively. The interpretation of the results of mixture studies was based on uncommon or distinct alleles at Loci DYS389II, DYS390, and DYS385. The allele calls for male-1 and male-2 at loci DYS19

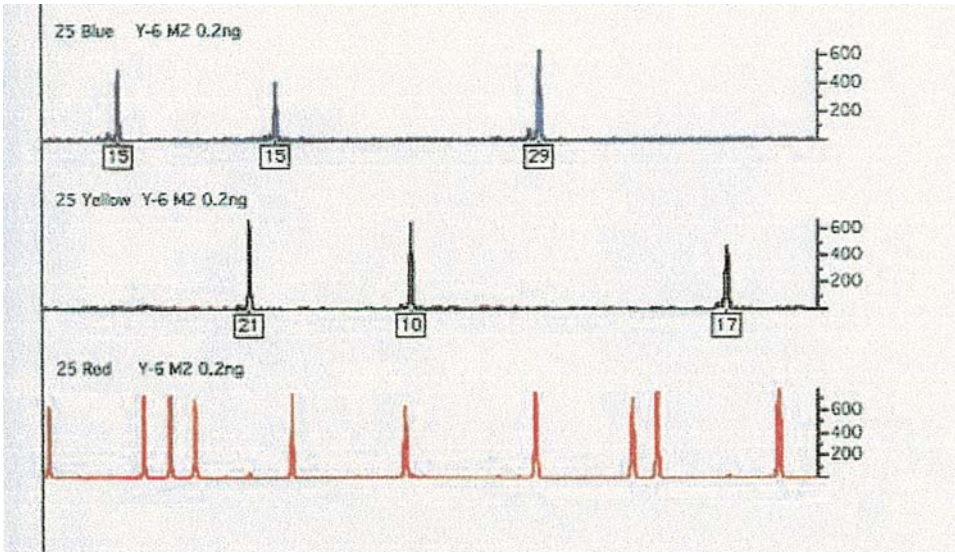

FIG. 4—*Profile of the positive control sample from 0.2 ng of template DNA using the Y-PLEX™ 6 kit.*

TABLE 5—*Results from mixtures of male and female DNA amplified using the Y-PLEX™ 6 kit.*

			Peak Height (rfu) of the Alleles in Male Profile in a Mixture Sample						
Male:Female DNA Ratio	Male DNA, ng	Female DNA, ng	DYS 393 Allele 14	DYS19 Allele 15	DYS 389II Allele 31	DYS 390 Allele 23	DYS 391 Allele 10	DYS 385-1 Allele 14.2	DYS 385-2 Allele 15
1:0	0.5	0	2677	2026	500	1289	1891	287	276
1:1	0.5	0.5	2921	3067	1046	1942	1964	516	455
1:2	0.5	1	3087	2280	969	1716	2173	494	451
1:5	0.5	2.5	2281	2253	893	1726	1949	461	531
1:10	0.5	5	3107	2563	848	1752	1710	399	531
1:20	0.5	10	4294	3584	1024	2053	2199	409	453
1:30	0.5	15	4050	3419	906	2054	2483	403	425
1:40	0.5	20	4216	2811	1185	2072	2279	502	469
1:50	0.5	25	3634	2410	1019	1760	1970	545	431
1:125	0.2	25	593	365	94	1069	970	245	203
1:250	0.1	25	244	181	<50	223	347	<50	<50
1:312	0.08	25	176	95	<50	228	199	80	<50
1:500	0.05	25	326	228	<50	78	158	<50	<50

and DYS391 were identical. Further, the results for the locus DYS393 were not used for interpretation since the allele from male-1 was at the stutter position for the allele from male-2. The results for distinct alleles at DYS389II, DYS390, and DYS385 are summarized in Table 6. The results indicated that the quantities of amplified products are only generally proportional to the DNA present in the mixture. 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 30:1 (male-1:male-2). The loss of some alleles was observed at greater proportions (Table 6).

Nonhuman Studies

Male DNA from the following nonhuman species was amplified using 2 to 4 ng of template DNA and Y-PLEX™6 reagents: Xavier monkey, chimpanzee, mandrill (baboon), gorilla, dog, and cat. No amplification products were observed with any of the nonhuman samples (data not shown). Two ng of DNA from each of these nonhuman species was co-amplified subsequently with 2 ng of the human male positive control DNA sample. The positive control profile was typable in all samples, supporting the contention that DNA extracts from the nonhuman samples did not contain PCR inhibitors affecting the amplification. Thus, the data indicated that the primers present in the Y-PLEX™6 were specific for human DNA (although it may be possible that other primates not tested may yield amplification products).

Contamination and Environmental Insult Analysis

The male DNA samples exposed to the different environmental insults gave the same haplotype as the known blood of the donor except for the blood and semen samples treated with soil suspension (both wet and dry) (data not shown). These samples were treated with Chelex® for additional cleanup. An aliquot of extract containing about 10 ng of DNA was mixed with 200 μL of 5% Chelex®, incubated at 56°C for 2 h, heated at 100°C for 8 min, and subjected to centrifugation. The supernatant was washed using a 100-μm filter (Am-

TABLE 6—*Results from mixtures of two male DNA samples amplified using the Y-PLEX™ 6 kit.*

			Peak Height (rfu) of the Distinct Alleles							
			DYS 389II		DYS 390		DYS 385 -1		DYS 385 -2	
Male-1:Male-2 DNA Ratio	Male-1 DNA, ng	Male-2 DNA, ng	Male-1	Male-2	Male-1	Male-2	Male-1	Male-2	Male-1	Male-2
1:0	0.5	0	1129	0	2329	0	520	0	469	NA*
1:1	0.5	0.5	807	1175	1650	2289	422	1419	287	NA
1:2	0.5	1	491	2100	1221	3749	392	2599	396	NA
1:5	0.5	2.5	460	3930	905	6802	274	4999	318	NA
1:10	0.5	5	304	5855	<50	6041	180	5780	195	NA
1:20	0.5	10	193	5097	<50	6644	85	4922	121	NA
1:30	0.5	15	89	4810	<50	6848	84	6067	112	NA
1:40	0.5	20	82	4695	<50	6278	<50	6781	88	NA
1:50	0.5	25	75	5346	<50	6887	<50	7110	109	NA
0:1	0	0.5	0	1222	0	2396	0	1359	0	NA
2:1	1	0.5	1079	1109	2263	1815	705	1312	621	NA
5:1	2.5	0.5	2910	1167	5854	1963	1402	1111	1537	NA
10:1	5	0.5	3889	875	5954	1608	1778	753	1799	NA
20:1	10	0.5	4494	435	5467	1014	2578	472	2641	NA
30:1	15	0.5	4498	226	6271	691	3438	366	3344	NA
40:1	20	0.5	3681	<50	6608	380	3402	234	3314	NA
50:1	25	0.5	6310	<50	5339	335	3605	181	3486	NA

*NA = No Allele; Male-2 sample exhibited only one allele at DYS385.

TABLE 7—*Analysis of the non-probative samples using the Y-PLEX™ 6 kit.*

Sample Information	Sample Description	DYS393	DYS19	DYS389II	DYS390	DYS391	DYS385	
CASE 1-Q1	Vaginal swab—female fraction	13, (14*)	14	27	24	10	12*	13*
CASE 1-Q2	Vaginal swab—male fraction	13, (14)	14	27, (29)	24	10	12*	13*
CASE 1-K1	Suspect	13	14	27	24	10	12	13
CASE 2-Q1	Semen on sock—female fraction	13	0	0	0	0	0	0
CASE 2-K1	Suspect	13	14	29	24	11	11	14
CASE 3-Q1	Saliva on perineal swab	0	0	0	0	0	0	0
CASE 3-K1	Suspect	12	14	29	24	10	11	14
CASE 4-Q1	Semen on underwear—female fraction	0	0	0	0	0	0	0
CASE 4-K1	Suspect	0	0	0	0	0	0	0
CASE 5-Q1	Semen on perineal swab—male fraction	15	15	31	23	10	15*	16*
CASE 5-K1	Suspect	15	15	31	23	10	15	16
CASE 6-Q1	Semen on sheet—female fraction	0	0	0	0	0	0	0
CASE 6-Q2	Semen on sheet—female fraction	12	0	0	0	0	0	0
CASE 6-K1	Suspect	12	14	29	24	11	11	14

(Allele) = Weaker Allele.
Allele* = Allele less than 75 RFU.

icon). In addition, eight μg of bovine serum albumin (BSA, stabilizing agent) and an additional 0.5 μL of TaqGold™ polymerase were added to the PCR mix to overcome PCR inhibition. All the samples treated with soil suspension could be amplified correctly after the cleanup procedure except for the blood and semen samples stored for 28 days after the wet treatment with soil suspension.

TABLE 8—*Allele frequencies among the African American, Caucasian, and Native American population groups.*

Locus	Allele	Frequency		
		African American (n = 543)	Caucasian (n = 581)	Native American (n = 49)
DYS393	8	0.002	N.D.	N.D.
	9	0.024	0.024	0.245
	10	0.707	0.492	0.367
	11	0.252	0.454	0.388
	12	0.015	0.029	N.D.
DYS19	12	0.002	0.002	N.D.
	13	0.031	0.060	0.184
	14	0.241	0.651	0.510
	14.3	N.D.	0.002	N.D.
	15	0.403	0.198	0.224
	16	0.182	0.072	0.082
	17	0.138	0.012	N.D.
	18	0.002	0.003	N.D.
DYS389II	25	0.002	N.D.	N.D.
	26	0.002	N.D.	N.D.
	27	0.009	0.015	N.D.
	28	0.125	0.177	0.163
	29	0.203	0.477	0.469
	30	0.344	0.251	0.306
	31	0.250	0.062	0.061
	32	0.055	0.012	N.D.
	33	0.009	0.005	N.D.
DYS390	20	0.017	0.002	N.D.
	21	0.517	0.014	0.020
	22	0.116	0.124	N.D.
	23	0.103	0.255	0.571
	24	0.162	0.444	0.286
	25	0.077	0.150	0.122
	25.2	N.D.	0.002	N.D.
	26	0.007	0.009	N.D.
	27	N.D.	0.002	N.D.
DYS391	8	0.002	N.D.	N.D.
	9	0.024	0.024	0.245
	10	0.707	0.492	0.367
	11	0.252	0.454	0.388
	12	0.015	0.029	N.D.
DYS385	8	0.001	N.D.	N.D.
	9	0.001	N.D.	N.D.
	10	0.007	0.013	N.D.
	11	0.079	0.309	0.224
	12	0.020	0.050	0.020
	13	0.047	0.105	0.051
	13.2	0.001	N.D.	N.D.
	14	0.153	0.316	0.173
	14.2	N.D.	0.001	N.D.
	15	0.133	0.115	0.306
	15.3	N.D.	0.002	N.D.
	16	0.177	0.049	0.082
	17	0.194	0.020	0.082
	17.1	0.001	N.D.	N.D.
	17.2	N.D.	0.001	N.D.
	18	0.124	0.014	0.041
	19	0.050	0.005	0.020
	20	0.011	0.001	N.D.
	21	0.001	N.D.	N.D.

N.D. = Not Detected.

Nonprobative Samples

Six nonprobative cases containing various DNA sources, which were previously analyzed with the AmpFℓSTR® Profiler Plus and AmpFℓSTR® COfiler kits (Applied Biosystems), were reanalyzed using the Y-PLEX™6 kit. The samples included female and male DNA fractions of semen identified on vaginal swabs. The interpretation of the results obtained from Y-PLEX™6, AmpFℓSTR® Profiler Plus, and AmpFℓSTR® COfiler kits were consistent. The results obtained for Y-PLEX™6 are summarized in Table 7. The weak secondary contributor in samples Case 1-Q1 and Case 1-Q2 indicates a minor contribution from a second male. These results are consistent with the results obtained from AmpFℓSTR® Profiler Plus and AmpFℓSTR® COfiler. In addition, the consensual sex partner of the victim in this case refused to give a known sample for elimination purpose.

Interlaboratory Comparison

Four known and five unknown male DNA samples kindly provided by Dr. Lutz Roewer (Institute for Rechtsmedizin, Berlin, Germany) were analyzed using the Y-PLEX™6 kit. The results for all samples at all six loci were typed correctly and in accordance with the published nomenclature and the ISFG guidelines for STR analysis (data not shown) (26). Since the allele designation was in 100% concordance, the European database can be used for comparison of the results typed by the Y-PLEX™6 kit.

Population Studies

Three population groups, African American (n = 543), Caucasian (n = 581) and Native American Indian (n = 49), were profiled for the six Y-STR loci using the Y-PLEX™6 kit. About 95% of the samples were buccal swabs and the other 5% were whole blood. The reinjection rate was about 10%. Table 8 shows the allele frequency distributions for each Y-chromosome marker in Caucasian and African-American sample populations, respectively. Polymorphic diversity was maximal for Locus DYS385 followed by DYS390, DYS389II, DYS19, DYS393. and DYS391. In general, these allele frequencies were consistent with data published for the same loci in populations of similar anthropological affinity (17,27,28). A few alleles were observed that did not exist in the allelic ladder. These alleles are listed in Table 2. Microvariant alleles were confirmed by two independent amplifications (and thus two typings). The genetic diversity and random match probability for the three population groups studied is summarized in Table 9. Results showed a lower genetic diversity for Native Americans, possibly due to a smaller sample size. The random match probability for the Y-PLEX™6 system was 0.0096 and 0.0039 for Caucasian and African American population groups, respectively. Though the Y-PLEX™6 system is not as discriminative as autosomal STR systems (AmpFℓSTR® Profiler Plus, AmpFℓSTR® COfiler, AmpFℓSTR® Identifiler, or PowerPlex® 16), it offers certain advantages when

TABLE 9—*Genetic diversity and random match probability for Y-PLEX™ 6 kit.*

Population	Genetic Diversity, h	Random Match Probability
Caucasian	0.9921	0.0096
African American	0.9979	0.0039
Native American	0.9847	0.0354

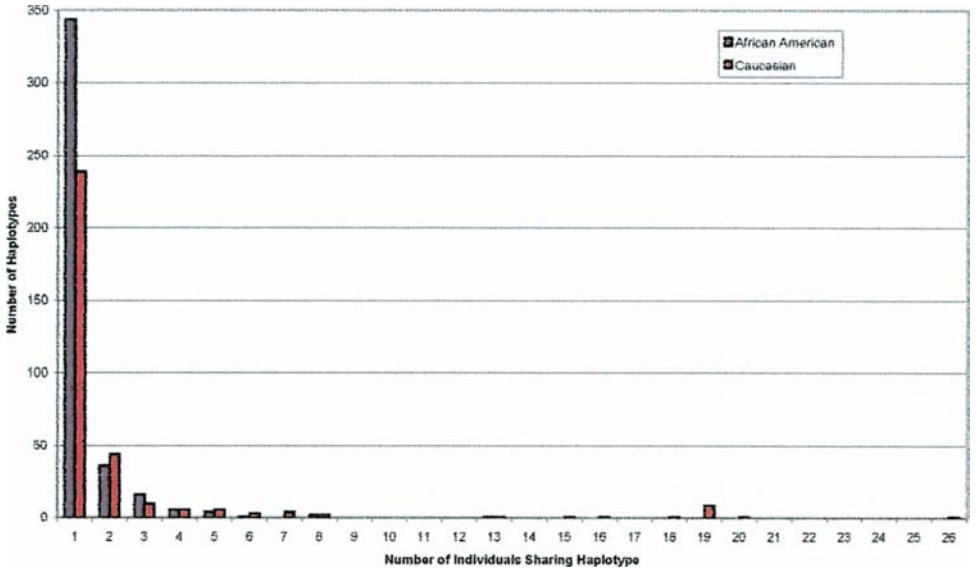

FIG. 5—*Haplotype frequency distribution in the Caucasian Population(red) (n = 581) and the African American population (blue) (n = 543).*

TABLE 10—*Observed frequency of distribution of most frequent haplotypes analyzed by using Y-PLEX™ 6 kit.*

African American Population (n = 543)		Caucasian Population (n = 581)	
Y Haplotype (DYS393, DYS19, DYS389II, DYS390, DYS391, DYS385)	*n*	Y Haplotype (DYS393, DYS19, DYS389II, DYS390, DYS391, DYS385)	*n*
13-15-31-21-10-16,17	13	13-14-29-24-11-11,14	26
13-14-29-24-11-11,14	8	13-14-30-24-11-11,14	20
13-15-31-21-10-16,16	8	13-14-29-24-10-11,14	18
13-14-28-25-10-14,14	6	13-14-29-23-11-11,14	16
13-15-30-21-10-15,16	5	13-14-29-24-11-11,15	15
13-15-32-21-10-16,17	5	13-14-30-24-10-11,14	13
13-17-30-21-10-17,18	5	13-14-29-25-11-11,14	8
14-16-30-21-10-17,18	5	13-14-29-24-10-11,15	8
13-14-29-23-11-11,13	4	13-14-29-23-10-11,14	7
13-14-30-24-11-11,14	4	13-15-29-24-11-11,14	7
13-15-30-21-10-16,17	4	13-14-30-23-11-11,14	7
13-17-30-21-10-18,18	4	13-14-29-25-11-11,13	7
14-15-30-21-10-15,18	4	13-14-28-24-11-11,14	6
15-16-30-21-10-17,18	4	13-14-29-25-10-11,14	6
13-14-28-24-11-11,14	3	13-14-29-25-11-11,15	6
13-14-29-24-10-11,14	3	13-14-28-22-10-13,14	5
13-14-30-24-11-11,15	3	13-14-28-22-10-13,13	5
13-15-30-21-10-17,17	3	13-14-28-22-10-14,14	5
13-15-31-21-10-15,18	3	13-14-30-23-10-11,14	5
13-15-31-21-10-18,19	3	13-14-30-25-11-11,14	5
13-15-31-21-11-16,17	3	14-14-29-24-11-11,14	5
13-15-31-22-11-15,17	3	13-14-28-23-10-13,14	4
14-15-30-21-10-15,16	3	13-14-28-23-10-14,14	4
14-15-30-21-10-16,17	3	13-14-28-23-11-11,14	4
14-15-31-21-10-17,18	3	13-14-31-24-10-11,14	4
14-17-30-21-10-17,18	3	13-15-29-24-10-11,14	4
15-16-30-20-10-17,18	3	13-14-29-24-11-11,13	4
15-16-30-21-10-16,17	3	13-14-29-25-11-11,12	3
15-16-30-21-10-17,19	3	12-14-29-24-11-11,14	3
		13-13-30-24-10-16,18	3
		13-14-28-23-10-11,14	3
		13-14-29-24-10-12,14	3
		13-14-30-24-10-11,15	3
		13-15-28-22-10-13,14	3
		13-15-29-25-11-11,14	3
		14-14-29-23-10-11,14	3
		14-14-30-24-11-11,14	3

dealing with mixtures of male and female DNA; only the male profile is typed and a single peak at each marker (except for DYS385) enables determining the number of male contributors, e.g., multiple assault cases. Thus, the Y-PLEX™6 system is very useful in DNA analysis of certain forensic casework samples such as fingernail scrapings, P30 positive but sperm negative samples as well as sample mixtures of male and female. Prinz et. al. (29) have achieved a higher success rate in detecting the semen donor's alleles for the Y-STR as compared to autosomal loci; the study involved analysis of 56 nonprobative semen stains and swabs by using multiplexes for Y-STR and autosomal loci.

The haplotype frequencies for Caucasians and African Americans provide some interesting information (Fig. 5 and Table 10). Strikingly, 239 of the 581 Caucasians and 344 of the 543 African Americans profiled were observed only once in each database (Fig. 5). The 37 and 29 most frequent haplotypes and their frequency distribution in Caucasian and African American population, respectively, are presented in Table 10. Roewer et al. (17) 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. Nevertheless, analysis for additional loci should increase the power of discrimination afforded by using the Y-PLEX™6 kit.

Conclusions

The Y-PLEX™6 multiplex system enables analysis of the six Y-STR Loci DYS393, DYS19, DYS389II, DYS390, DYS391, and DYS385. The primers present in this system were specific for human DNA. The Y-PLEX™6 system was robust, valid, and reliable for forensic casework. Population data are available that can be used for human identity testing cases. In addition to the six loci multiplexed in the Y-PLEX™6 kit, the Loci DYS389I and DYS392 are part of a larger haplotype group in which substantial population data are available (7,17). A second Y-STR multiplex system that incorporates these additional Y STR loci is being developed.

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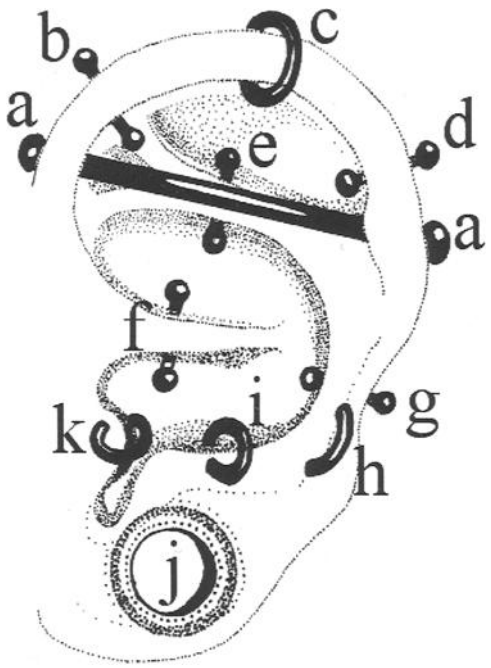
ERRATA

Erratum/Correction of Swift B and Rutty GN. The Human Ear: Its Role in Forensic Practice. J Forensic Sci 2003 Jan.;48(1):153–160.

It has come to the attention of the Journal the fact that the hand-drawn image (Fig. 3) of the ear with piercings isn't labeled with regards to the figure legend. Below is the new/correct Fig. 3.

The Journal regrets this error. Note: Any and all future citations of the above-referenced paper should read Swift B and Rutty GN. The Human Ear: Its Role in Forensic Practice. [published erratum appears in J Forensic Sci 2003 May;48(3)] J Forensic Sci 2003 Jan;48(1): 153–160.



Erratum/Correction of Sinha SK, Budowle B, et al. Development and Validation of a Multiplexed Y-Chromosome STR Genotyping System, Y-PLEX™6 for Forensic Casework. J Forensic Sci. 2003 Jan.;48(1):93–103.

On page 99, the legend reads as:

FIG. 4—Profile of the positive control sample from 0.2 ng of template DNA using the Y-PLEX™6 kit.

should read:

FIG. 4—Profile from 0.2 ng of template DNA using the Y-PLEX™6 kit.

The Journal regrets this error. Note: Any and all future citations of the above-referenced paper should read: Sinha SK, Budowle B, et al. Development and Validation of a Multiplexed Y-Chromosome STR Genotyping System, Y-PLEX™6, for Forensic Casework. [published erratum appears in J Forensic Sci 2003 May;48(3)] J Forensic Sci 2003, 48(1):93–103.