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Performance Characteristics of Commercial Y-STR Multiplex Systems*

ABSTRACT: In this work, a number of performance checks were carried out to evaluate the efficacy of commercial Y-short tandem repeats (Y-STR) kits for casework applications. The study evaluated the sensitivity, specificity and stability of the Y-STR markers used and the ability to obtain a male profile from postcoital samples taken at various time points after intercourse. All systems performed well with 1–3 ng of male DNA as recommended by the manufacturers. All systems gave full profiles at 100 pg of input DNA, which is within the realm of low copy number DNA analysis. Moreover all, except Y-Plex™ 12, gave full profiles with 30–50 pg of male DNA. No increased performance was obtained with any of the systems by increasing the cycle number beyond that recommended by the various manufacturers. When up to 1 μg of female DNA was used (in the absence of male DNA) no female DNA cross reactivity was observed with the Y-Plex™ 12 and Y-Filer™ systems. PowerPlex® Y produced female DNA derived products near the DYS438 and within the DYS392 loci at a rare allele position with high input DNA levels (300 ng and 1 μg, respectively). Male/female DNA admixture experiments indicated the particularly high specificity of the Y-Filer™ and PowerPlex® Y systems under conditions of several thousand fold female DNA excess. All systems were able to detect the minor alleles in male/male DNA admixtures at a 1:5 dilution with the PowerPlex® Y and Y-Filer™ being able to detect some minor alleles at 1:20. Species testing indicated some limited, minor cross reactivity of the commercial systems with some domestic male mammals although it is easily recognizable and would not pose any problems in casework analysis. As expected a significant number of cross-reacting products were obtained with nonhuman primate species. All Y-STR multiplex systems tested were able to produce complete Y-STR profiles from bloodstains and semen stains exposed up to 6 weeks when the samples were protected against precipitation and sunlight. However, exposure of the samples to precipitation either in the presence or absence of sunlight resulted in Y-STR profile loss over time, with total profile loss occurring with all systems after 3 weeks or more. Complete Y-STR profiles of the male donors up to 72 h postcoitus were obtained with all of the multiplex systems tested, except for Y-Plex™ 12, which gave partial profiles.

KEYWORDS: forensic science, DNA analysis, Y-short tandem repeats, multiplex systems, Applied Biosystems AmpFℓSTR® Yfiler™ PCR Amplification Kit, Promega PowerPlex® – Y System, Reliagene Y-PLEX™ 12, postcoital interval, MPI, MPB

Y-chromosome specific DNA typing systems can prove invaluable for the identification of the genetic profile of the male component in mixed male/female specimens in those instances in which the female DNA portion is present in overwhelming quantities relative to the male (1,2). This could be due to the deposition of semen by azoospermic or oligospermic males, cases of oral sodomy where only trace amounts of male buccal epithelial cells may be present or due to the normal postcoital degradative and sample loss processes that occur with the passage of time. Additionally, Y-chromosome systems can be used to determine the presence of the number of semen donors in cases of multiple perpetrator rape. A third reason for employing Y-chromosome polymorphisms would be for criminal paternity analysis or disaster victim identification. The Y-chromosome haplotype of a missing individual may be determined by typing a male relative such as a son, brother, father, nephew or uncle. Fourthly, the ability to specifically detect a male profile could obviate the need for the time-consuming and oft-times inefficient differential extraction procedure for the separation of sperm and nonsperm fractions. Finally, male specific systems may aid the investigation of cases involving mixtures or close biological relatives by providing additional statistical discriminatory power.

A set of nine Y-short tandem repeats (Y-STR) loci, commonly referred to as the ''minimal haplotype loci'' set (MHL), were recommended for forensic use by the European Y-chromosome typing community (3). These loci comprise DYS19, DYS 385a and b, DYS389I and II, DYS 390, DYS 391, DYS 392 and DYS 393. The MHL loci have proved to be a particularly robust set of genetic markers and have been successfully employed in casework analysis (4–8). Despite their utility, however, additional Y-STR loci have been evaluated to improve the discriminatory capacity of the MHL. In 2003, the Scientific Working Group on DNA Analysis Methods (SWGDAM) group recommended for US laboratories a core set of 11 loci that comprised the MHL loci together with DYS 438 and DYS 439 (9). A variety of commercial Y-STR kits are currently available for forensic casework use and three incorporate 11 or more Y-STR markers, which include the SWGDAM core loci. The Reliagene Corporation (New Orleans, LA) manufactures the Y-PLEX™ 12 kit that comprises the 11 SWGDAM core loci in addition to the AMEL gender determining locus (hereafter referred to as Y-Plex™ 12) (10). The Promega Corporation's (Madison, WI) PowerPlex®–Y System possesses the SWGDAM loci plus DYS 437 (designated PowerPlex® Y hereafter)(11), whereas Applied Biosystem's (Foster City, CA) AmpFℓSTR® Yfiler™ PCR Amplification Kit (hereafter described as Yfiler™) includes these same markers plus five additional loci (DYS 448, DYS456, DYS 458, Y-GATA C4, and Y-GATA H4)(12).

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Previously, we independently tested and evaluated all of the Y-STR loci present in these commercial kits, and confirmed their forensic utility, using a variety of multiplex assay formats (1,13–15). Specifically the commercial kit loci are contained within three of our in-house multiplexes, designated MPI, MPII and MPIV, which comprise a total of 40 Y-STR loci (13–15). These in-house multiplex systems were developed, tested, and evaluated prior to the release of the commercial kits. In order to provide higher specificity and sensitivity with samples containing low copy number male templates in a background of overwhelming female templates, ultra high sensitivity (UHS) multiplex systems, designated MPA and MPB, were developed (16). MPA and MPB were fabricated from subsets of the MPI and MPII loci which, when co-amplified with increased cycle number, demonstrated the least amount of cross reactivity of the MPI/II loci with X-derived sequences. These UHS systems, in contrast to the MPI/II multiplexes, were able to dissect out the male profile in postcoital samples recovered 4 days after intercourse (16).

Forensic laboratories that seek to add Y-STR testing to their analytical armamentarium need to choose an appropriate commercial kit and, subsequently, test and evaluate it prior to use. If comprehensive developmental validation studies have already been performed on the commercial kit and have been reported in the forensic literature, then the laboratory need only conduct a more limited internal in-house validation exercise. The function of the latter is to demonstrate satisfactory performance of the kit with the particular types of samples, personnel, equipment, and facilities employed by the laboratory. While for practical reasons such developmental validation studies of the commercial kits are often sponsored and managed by the kit manufacturers themselves, it is important that the results be verified by other studies conducted by vendor-independent groups.

In order to facilitate Y-STR technology transfer to the crime laboratory community, a vendor-independent comprehensive comparative testing and evaluation of the most commonly used commercial kits was carried out. A particular emphasis of this study, using well characterized in-house Y-STR systems as performance yardsticks, was the extent to which different commercial kits were able to obtain male profiles from a range of challenging samples that could be encountered in casework.

Materials and Methods

DNA Isolation and Purification

All body fluid samples were collected in accordance with procedures approved by the University's Institutional Review Board. Blood was collected from human subjects by venepuncture. The blood stains obtained originated from four human male individuals (Y2, Y3, Y4, Y5) and one human female individual (X2). Fifty-microliter drops of blood were aliquoted onto a cotton swatch and dried overnight. Semen was collected from two human males (Y2, Y3) who deposited semen in sterile plastic containers. Fifty-microliter drops of semen were aliquoted onto a cotton cloth and dried overnight. Buccal swabs were collected by a swabbing a subject's inside cheek with sterile swabs and allowing them to dry overnight. The buccal swabs obtained originated from six human male individuals (Y1, Y2, Y3, Y4, Y5, and Y6) and three human female individuals (X1, X2, and X3). Body hairs (3–6 hairs from various body regions) were collected from six human male individuals (Y1, Y2, Y3, Y4, Y5, and Y6) and one human female individual (X1)

DNA was extracted from the samples using either a standard organic extraction protocol (17) or a standard differential lysis protocol, with minor modifications, which separates sperm and non-sperm cells (18). Swab tips or hairs were placed in a Spin-Ease tube (Gibco-BRL, Grand Island, NY) and incubated overnight at 56°C in 400 μL DNA extraction buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 0.5% SDS, 0.1 mg/mL Proteinase K and, for samples containing semen, 39 mM DTT). The swab tip was removed from the tube and placed into a Spin-Ease basket and placed back into its original tube and centrifuged at 16,100 g for 5 min to ensure all absorbed fluid was removed from the swab tip. The extract was purified by adding 400 μL of 25:24:1 phenol/chloroform/isoamyl alcohol (Fisher Scientific, Norcross, GA) to the Spin-Ease tube. The upper aqueous layer was removed and subjected to spin filtration using a Microcon 100 concentrator (Millipore, Bedford, MA) according to the manufacture's instructions. Samples were stored in TE^{-4} (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5) and kept at 4°C until analysis.

DNA Quantitation—Yield Gel

Extracted DNA from nonhuman sources was electrophoresed in a 1% agarose gel and stained using 1% ethidium bromide (Fisher Scientific). Samples were visualized using an Ultra-lum Omega 10 (Ultra-lum Inc., Claremont, CA). Quantitation was accomplished by comparing the intensities of the unknown bands to a set of known standards run concurrently with the samples.

DNA Quantitation—Real Time PCR Assay

All human DNA extracts were quantitated using the Quantifiler™ Human DNA Quantification Kit and/or the Quantifiler™ Human Male DNA Quantification Kit (Applied Biosystems, Foster City, CA) (19) in accordance with the manufacturer's instructions.

Standard PCR Conditions

PCR reaction components were as follows: *MPI/II and MPB*: 25 μL total volume reaction containing 250 μM dNTP's, 10× PCR Buffer II (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 3.25 mM $MgCl_2$, 10 μg nonacetylated BSA (Sigma-Aldrich, St. Louis, MO), 2.5 units AmpliTaq Gold™ DNA Polymerase (Applied Biosystems). *MP1/11* 3 ng template DNA, 0.69–1.25 μM primers (1), *MPB*: 25 μL total volume reaction containing 1 ng template DNA, 0.0276–0.075 μM primers (see below).

Commercial Kits— Amplification proceeded in accordance with the manufacturers' recommended conditions.

Primers— Either the forward or reverse primers of MP1 and MPB were labeled with a fluorescent phosphoramidite dye (Invitrogen, Grand Island, NY) (see Tables 1 and 2). The forward and reverse primer concentrations for the two in-house multiplexes were as follows: *MP1*: DYS393—0.055 μM, DYS392—1.0 μM, DYS391—0.065 μM, DYS389—0.15 μM, DYS438—0.15 μM, Y-GATA-A7.2—0.10 μM, and DYS385—1.0 μM. *MPB*: DYS393—0.0276 μM, DYS389—0.075 μM, Y-GATA-H4—0.05 μM, Y-GATA-A7.2—0.045 μM. The commercial Y-STR kit primer concentrations are proprietary and are provided by the manufacturers pre-mixed.

Cycling Conditions— The cycling conditions were: *MP1*: 95°C 11 min hot start; two cycles: 96°C 30 sec, 62°C 1 min, 72°C 1 min; two cycles: 96°C 30 sec, 60°C 1 min, 72°C 1 min; 31 cycles: 96°C 30 sec, 58°C 1 min, 72°C 1 min; final extension 72°C

TABLE 1—*Characteristics of the Y-STR multiplex systems surveyed. Information provided includes the loci included in each system, the fluorescent dyes used, optimum cycle number, manufacturer's recommended amount of DNA amplified, and total PCR volume.*

	Number of Loci	Labels	Optimum Number of PCR Cycles	Manufacturer's Recommended Amount of DNA (ng) Amplified	PCR Reaction (μL) Volume
Commercial Y-STR multiplexes					
Applied Biosystems AmpFℓSTR® Yfiler™ PCR Amplification Kit	(16) *DYS456, DYS389I, DYS390, DYS389II, DYS458, DYS19, DYS385 a/b, DYS393, DYS391, DYS439, DYS635, DYS392, Y-GATA H4, DYS437, DYS438, DYS448*	6-FAM VIC NED PET	30	1	25
Promega Promega PowerPlex® – Y System	(11) *DYS391, DYS389I, DYS439, DYS389II, DYS438, DYS437, DYS19, DYS392, DYS 393, DYS390, DYS385a/b*	FL TMR JOE	32	2	25
Reliagene Y-PLEX™ 12	(11) *DYS392, DYS390, DYS385a/b, DYS393, DYS389I, DYS391, DYS389II, AMEL, DYS19, DYS439, DYS438*	6-FAM JOE NED	30	1–2	25
UCF multiplexes					
Multiplex I (MPI)	(8) *DYS391, DYS392, DYS393, DYS389I, DYS389II, Y-GATA A7.2, DYS438, DYS385a/b*	6-FAM TET HEX	35	1	25
Multiplex B (MPB)	(5) *DYS393, DYS389I, DYS389II, Y-GATA A7.2, Y-GATA H4*	6-FAM TET HEX	45	3	25

45 min. *MPB*: 95°C 11 min hot start; two cycles: 96°C 30 sec, 62°C 1.5 min, 72°C 1 min; two cycles 96°C 30 sec, 60°C 1.5 min, 72°C 1 min; 41 cycles: 96°C 30 sec, 58°C 1.5 min, 72°C 1 min; final extension 72°C 45 min. *Yfiler™* (12)*:* 95°C 11 min hot start; 30 cycles: 94°C 1 min, 61°C 1 min, 72°C 1 min; final extension 60°C 80 min. *PowerPlex® – Y* (11): 95°C 11 min hot start; 96°C 1 min, 10 cycles: ramp 100% 94°C 30 sec, ramp 29% 60°C 30 sec, ramp 23% 70°C 45 sec; 18–22 cycles: ramp 100% 90°C 30 sec, ramp 29% 58°C 30 sec, ramp 23% 70°C 45 sec; final extension 60°C 30 min. *Y-PLEX™ 12* (10): 95°C 10 min hot start; 30 cycles: 94°C 1 min, 58°C 1 min, 70°C 1 min; final extension 60°C 60 min.

Sample Electrophoresis and Data Analysis

Amplified product was separated and detected using a Macintosh-based ABI Prism 310 capillary electrophoresis system (Applied Biosystems). *MP1 and MPB*: 1 μL of PCR product was added to 24 μL Hi-Di formamide (Applied Biosystems) and 1 μL of the GeneScan 500 TAMRA internal lane standard (Applied Biosystems). *Yfiler™*: 1.5 μL of PCR product was added to 24.5 μL Hi-Di formamide and 0.5 μL of the GeneScan 500 LIZ internal lane standard (Applied Biosystems). *PowerPlex® – Y System*: 1 μL of PCR product was added to 24 μL Hi-Di formamide and 1 μL of the ILS 600 internal lane standard (Promega). *Y-PLEX™ 12*: 1.5 μL of PCR product was added to 24.5 μL Hi-Di formamide and 0.5 μL of the GeneScan 500 ROX internal lane standard (Applied Biosystems). Samples analyzed with MP1 and MPB were injected using Module C (5 sec injection, 15 kV, 60°C, Filter set C). Samples analyzed with *Yfiler™* were injected using Module G5v2 (5 sec injection, 15 kV, 60°C, Filter set G5v2). Samples analyzed with *PowerPlex® – Y* were injected using Module A (5 sec injection, 15 kV, 60°C, Filter set A). Samples analyzed with *Y-PLEX™ 12* were injected using Module F (5 sec injection, 15 kV, 60°C, Filter set F). All samples were heated at 95°C for 3 min and snap-cooled on ice for no less than 3 min. Data were analyzed using GeneScan Analysis 3.1.2 software (Applied Biosystems), using a 100 RFU threshold.

TABLE 2—*Sensitivity of Y-STR multiplexes. The quantity of DNA (in ng) required to produce a full or partial profile and for the profile to be totally lost is listed. The reported limits are provided for comparison.*

Y-STR Multiplex	Full Profile	Partial Profile	Lost Profile	Reported Limits
Applied Biosystems AmpFℓSTR® Yfiler™ PCR Amplification Kit	0.045	0.040	0.010	0.050–0.100
Promega PowerPlex® – Y System	0.030	0.015	0.005	<0.250
Reliagene Y-PLEX™ 12	0.100	0.050	0.005	0.100
Multiplex I (MPI)	0.050	0.040	0.010	0.200
Multiplex B (MPB)	0.030	0.010	0.004	Not applicable

PCR Cycle Modifications

The number of cycles was increased for each multiplex system (except MPB) in order to potentially improve kit performance. Cycle numbers tested were 34, 35, and 40, since the preliminary determined standard cycling conditions were 30 (Yfiler™), 35 (Multiplex 1), 32 (PowerPlex® – Y), and 30 (Y-PLEX™ 12).

Sensitivity

Buccal DNA from three males (Y1, Y2, and Y3) and three females (X1, X2, and X3) were used. The quantities of male DNA used were: 1–5 pg, 10 pg, 20 pg, 30 pg, 45 pg, 50 pg, 60 pg, 100 pg, 200 pg, 500 pg, 1 ng, 3 ng, 30 ng, and 300 ng. The quantities of female DNA tested were 3 ng, 30 ng, 300 ng, and 1 μg.

Mixture Studies

Male/Female Admixtures— In the first set of male/female mixture experiments, the male component was held constant at 1 ng (Buccal DNA) and the female (Buccal DNA) component was added in varying quantities, and the entire sample amplified. The admixed male/female samples comprised two mixtures with different male and female contributors: *Mixture 1*: (Y3 & X2); *Mixture 2*: (Y2 & X3). Male/female ratios were tested maintaining 1 ng male DNA throughout each ratio: 1:1, 1:10, 1:100, 1:500, 1:600, 1:800, 1:1000, 1:2000, 1:3000, 1:4000, 1:5000 and 1:6000.

In the second set of male/female mixture experiments, the total input buccal cell DNA was held constant at 300 ng, and the entire sample amplified. In this case, the admixed male/female samples comprised three mixtures with different males and females; *Mixture 1*: (Y1 & X1); *Mixture 2*: (Y2 & X3), *Mixture 3*: (Y3 & X2). Male/female ratios tested were 1:1, 1:10, 1:100, 1:500, 1:600, 1:800, 1:1000, 1:2000, 1:3000, 1:4000, 1:5000, 1:6000, 1:8000, 1:10,000, 1:15,000, 1:20,000, 1:21,000, 1:22,000, 1:25,000, 1:28,000, 1:30,000, and 1:32,000.

Male/Male Admixtures— Buccal cell DNA from six males was combined to make three male/male mixtures; *Mixture 1*: (Y3 & Y2), *Mixture 2*: (Y4 & Y6), and *Mixture 3*: (Y5 & Y1). One nanogram of DNA from the minor component was used in the amplification reaction together with increasing quantities of DNA from the major component. The ratios tested were 1:1 (1 ng/1 ng), 1:5 (1 ng/5 ng), 1:10 (1 ng/10 ng), and 1:20 (1 ng/20 ng).

Female DNA Cross-Reactivity

To test for female DNA cross-reactivity, multiplex systems were tested using different input quantities of female Buccal DNA (3 ng, 30 ng, 300 ng, and 1 μg).

Species Specificity

Thirteen different species were tested. Nonprimate blood was obtained from a number of sources: Tuscawilla Oaks Animal Hospital, Oviedo, FL (male cat, male dog); HemoStat Laboratories, Dixen, CA (male cow, male horse, and male sheep); Charles R. Daniels, DeLand, FL (male deer); West End Animal Hospital, Gainesville, FL (male ferret). Nonhuman primate blood was acquired from Coriell Cell Repository, Camden, NJ (male and female gorilla, male and female orangutan, male and female chimpanzee, male macaque, female pygmy chimpanzee, male spider monkey). The DNA was extracted using a standard organic extraction method as described above. For amplification 1–3 ng of male DNA or 300 ng female DNA was added to the PCR reaction.

Stability Studies

Consistency of Y-STR Haplotypes in Different Tissues from the Same Individual— The consistency of Y-STR typing within different tissues of the same individual was assessed by amplifying DNA extracted from blood, saliva, hair, and semen (or, if female, vaginal secretions) collected from the same individual. Samples were obtained from four male individuals and one female individual.

Environmental Effects on Stability

Samples were prepared by pipetting 50 μL of liquid blood or semen onto a sterile cotton swatch, and allowed to dry overnight. The samples were exposed to the environment (i.e. heat, light, humidity, and precipitation) beginning on July 11, 2005, in a small, fenced in, metropolitan area in Orlando, Florida. Samples were exposed for 1 day, 2 days, 1 week, 3 weeks, and 6 weeks then collected and stored at −20°C until analysis. Samples were extracted using a standard organic extraction as described above. The quantity of template added to each reaction varied (1.0–1.3 ng) depending on the condition of the sample.

Postcoital Samples

Postcoital cervico-vaginal swabs were obtained in pairs simultaneously by two female volunteers who, after separate acts of intercourse, recovered the samples postcoitus at various time points. The time points collected were: 0, 12, 24, 48, and 72 h. To insure that the postcoital swabs were devoid of any male DNA from previous acts of sexual intercourse, volunteers refrained from sexual intercourse for a minimum of 5 days. A pair of precoital cervicovaginal swabs was obtained simultaneously before coitus commenced and only those samples from time points that had an associated negative precoital sample were used in the study. A negative precoital sample result is defined as one in which replicate analysis by qPCR using the Quantifiler™ Human Male DNA Quantification Kit failed to detect the presence of male DNA.

DNA was isolated using both a standard nondifferential extraction procedure, resulting in an extract containing both male- and female-derived DNA, and using a differential extraction procedure, the latter resulting in separate sperm and nonsperm extracts; both methods described previously. Three hundred nanograms of DNA (from the nondifferential extract) or 1 ng of sperm fraction DNA (from the differential extraction) was amplified.

Results and Discussion

A comparison of the characteristics and experimental parameters of the multiplex Y-STR systems evaluated in this study including the identity of the incorporated loci, dye labels, recommended number of PCR cycles, input quantity of DNA, and volume of the PCR reaction is provided in Table 1. After an initial check of the optimal PCR cycling conditions the Y-STR systems were tested for their sensitivity, specificity, stability, and mimicking casework-type samples, the ability to discern a male profile in postcoital cervicovaginal samples taken at various times after intercourse.

PCR Cycling Parameters

In order to ascertain whether improved performance was possible by adjusting the PCR cycling conditions, the number of amplification cycles was increased for the three commercial kits (34, 35, and 40 cycles) and the in-house MPI system (to 40 cycles). The MPB system was not tested in this exercise as it already employed an increased cycle number protocol (45 cycles). Three human male DNA samples at concentrations determined by this study to produce full and partial profiles with standard cycling conditions (see Table 2) were used for this performance evaluation. No improvement in performance was obtained with increasing the cycle number for any of the systems tested. Contrariwise, a decrease in performance was observed in that either full profiles became partial profiles or, in the case of 40 cycles, produced a loss of the profile altogether due to detector saturation and raised stutter peak artifacts. In light of these results the cycle number was kept at the recommended optimal cycle number as determined by the manufacturer (Table 1).

Sensitivity

The sensitivity of each of the Y-STR systems was tested using three different male DNA samples (input 1 pg–300 ng) and, as a negative control, one female DNA sample (input 3–300 ng). Identical results were obtained for the three male samples and no profiles from any of the systems were obtained with the female DNA. While the manufacturer's recommended input DNA for each

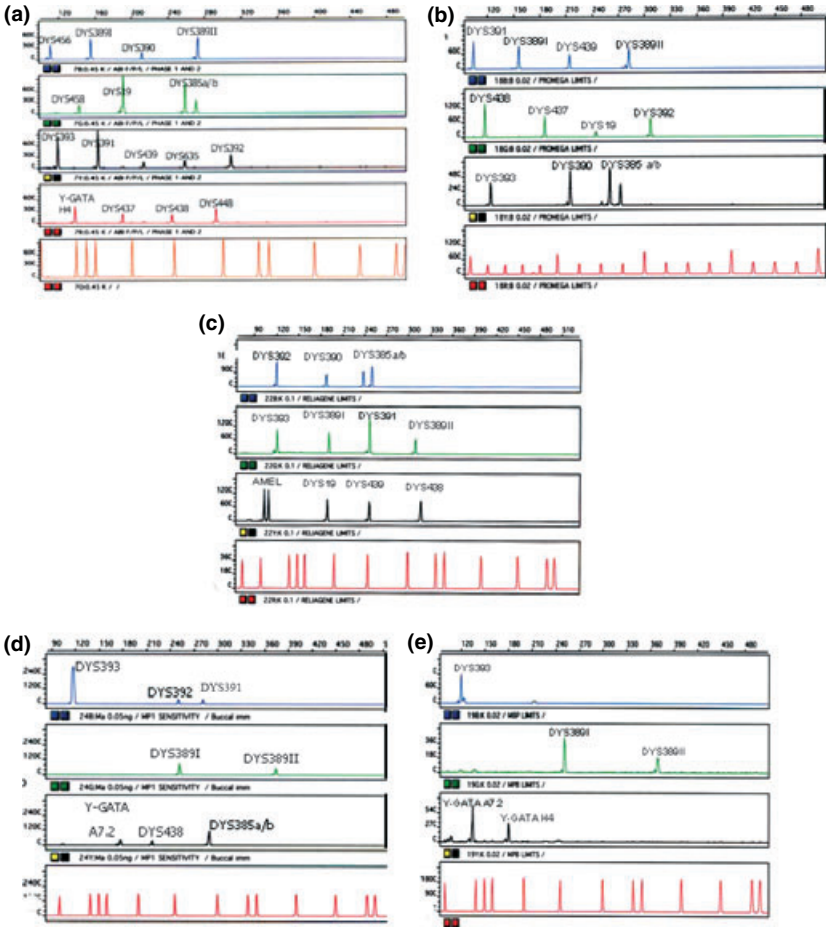

FIG. 1—*Sensitivity of Y-STR multiplex systems. Electropherograms showing the quality of the complete profiles at each system's sensitivity limit. (a) Applied Biosystems AmpFℓSTR® Yfiler™ PCR Amplification Kit (0.045 ng male DNA); (b) Promega PowerPlex® – Y System (0.02 ng male DNA); (c) Reliagene Y-PLEX™ 12 (0.10 ng male DNA); (d) Multiplex I (0.05 ng male DNA); (e) Mulitplex B (0.02 ng male DNA).*

system ranged from 1 to 3 ng, all Y-STR systems proved effective with a significantly reduced amount of DNA input (Table 2). Examples of the resulting electropherograms depicting the full profiles obtained at their sensitivity limits are depicted for each Y-STR multiplex system in Fig. 1. The full profiles for each Y-STR multiplex system at their detection limit in Fig. 1 show unbalanced inter locus peak heights, which is characteristic of low copy number samples. Each multiplex displayed relatively balanced inter and intra locus peak heights when the optimum amount of template DNA (1–3 ng) was used for amplification (data not shown).

The minimum amount of template DNA necessary for a full seventeen-locus Y-Filer™ DNA profile was 0.045 ng (Fig. 1*a*). A thirteen-, ten-, and nine-locus partial profile was obtained with 0.040, 0.035, and 0.020 ng of template DNA respectively, but was completely lost with 0.010 ng of DNA.

The minimum template DNA necessary for a full twelve-locus PowerPlex® – Y profile was 0.030 ng (Fig. 1*b*). A partial eight locus profile was obtained using 0.015 ng of template DNA but the profile was completely lost with 0.005 ng of template DNA.

The minimum limit of template DNA necessary for a full twelve-locus Y-PLEX™ 12 profile, was 0.10 ng (Fig. 1*c*). A partial profile (11 loci) was obtained using 0.05 ng of template DNA and was completely lost when 0.005 ng of template DNA was used.

The minimum amount of template DNA necessary for a full nine-locus MPI profile was 0.050 ng (Fig. 1*d*). A six-, five-, and two-locus partial MPI profile was obtained using 0.040, 0.030, and 0.020 ng of template DNA respectively. The profile was completely lost when 0.010 ng of template DNA was amplified.

The lower limit of template DNA necessary for a full five-locus MPB profile was 0.030 ng (Fig. 1e). A three-locus MPB partial profile was obtained using 0.010 ng of template and was completely lost when 0.004 ng of template DNA was amplified.

In summary, some of the commercial Y-STR systems proved to be extremely sensitive with Y-Filer and PowerPlex® Y producing full profiles with 5–8 cell equivalents. Y-Plex™ 12 proved to be somewhat less sensitive, requiring ~ 17 cell equivalents of DNA, although even this latter level of sensitivity is perfectly adequate for forensic use.

Specificity

Female DNA Cross-Reactivity— Due to its evolutionary history, the Y-chromosome still retains a considerable degree of sequence homology to the X-chromosome (20). Although primers for Y-STR kits are designed to recognize specific Y-STR loci they may still possess homology to sequences on the X-chromosome. The degree of homology will determine the extent of X-chromosome artifacts detected when using isolated DNA from admixed male and female DNA samples. In this study, the multiplex systems were amplified using varying input quantities of female DNA (3 ng, 30 ng, 300 ng, and 1 μg). The results are summarized in Table 3.

TABLE 3—*Female DNA artifacts. The locus yielding female DNA artifacts are listed along with the quantity of female input DNA required to produce them.*

Y-STR Multiplex	Locus	ng Female DNA Added
Promega PowerPlex® – Y System	DYS391	300
	DYS392	1000
Multiplex I (MPI)	DYS393	3–1000
	DYS385a	30
Multiplex B (MPB)	DYS393	3–1000

The Y-PLEX™ 12 and Y-Filer™ systems proved to be highly specific for the Y-chromosome because no significant female DNA products were observed with any of the input quantities of female DNA tested. Two female monomorphic products were observed using the PowerPlex® – Y with high levels of input female DNA: one of the products was observed just outside the DYS391 locus at 308 bp with 300 ng of female DNA, whereas the second product was observed in the DYS438 locus at 104 bp (indistinguishable from the rare 9.2 allele) when 1 μg of female DNA was amplified.

The MPI and MPB in-house systems both displayed female DNA-derived products in the DYS393 locus with as little as 3 ng of female DNA. These female DNA artifacts were ameliorated by using HPLC purified primers (data not shown) indicating the likelihood that they were due to a "dye blob" effect. More troublesomely, MPI possessed a female DNA artifact in the DYS385a locus at a position indistinguishable from the 15 allele when ≥ 30 ng of female DNA was amplified.

Male/Female Mixtures— The ability of the multiplex Y-STR systems to discern the male donor profile in male/female admixtures was evaluated. The intent was to replicate the types of mixture scenarios found in casework. Two different ways of assessing the Y-STR profiling efficacy of male/female mixtures were used (1,15). In the first study a series of admixed samples were prepared in which the total amount of DNA amplified was 300 ng, with variable proportions of male/female DNA (from 1:1 to 1:32,000). The second male/female mixture study involved the amplification of 1 ng of male DNA (determined by quantitation of male specific DNA) from each sample with the concomitant amplification of a variable quantity of female DNA (male: female ratios ranging from 1:1 to 1:6000). The lowest male: female admixture ratios at which the Y-STR systems were able to produce full or partial profiles or when the systems failed to produce profiles were determined and the results are summarized in Table 4.

The results from the male/female admixtures in which 1 ng of male DNA was amplified indicate the high sensitivity and specificity of the PowerPlex® Y and Y-Filer™ systems. For the Y Filer™ system, a full profile was obtained with the 1:3000 male:female DNA ratio (Fig. 2(i)) and was totally lost at 1:8000. For the PowerPlex® Y, a full profile was obtained with the 1:1000 male: female DNA ratio (Fig. 2(iii)) and was totally lost at 1:5000. A full profile was obtained with the MPB and MPI systems with a 1:1000 male:female DNA ratio (MPB, Fig. 2(ii)) and was totally lost at 1:8000 (MPB) or 1:5000 (MPI). Y-Plex™12 proved to be significantly less sensitive with the aforementioned admixtures than the other multiplex systems.

The results from the male/female admixtures in which 300 ng of total DNA was amplified confirm the particularly high sensitivity and specificity of the PowerPlex® Y and Y-Filer™ systems. For the Y Filer system, a full profile was obtained with the 1:4000 male: female DNA ratio and was totally lost at 1:15,000 (data not shown). For the PowerPlex® Y system, a full profile was obtained with the 1:10,000 male:female DNA ratio and was totally lost at 1:22,000 (data not shown). As expected, the in-house ultra-high sensitivity system, MPB, was extremely sensitive and a full profile was obtained with the 1:16,000 male:female DNA ratio and was totally lost at 1:30,000 (data not shown). Y-Plex-12 and MPI proved to be significantly less sensitive with these admixtures than the other multiplex systems.

TABLE 4—*Specificity of Y-STR multiplexes with male/female DNA mixtures. The most dilute male DNA sample still able to produce a full or partial male donor profile, and for the profile to be totally lost, is listed for both 1 ng total male DNA input and 300 ng of total DNA. The manufacturers' reported limits are provided for comparison.*

Y-STR Multiplex	Full Profile	Partial Profile	Lost Profile	Reported Limits
AmpFℓSTR® Yfiler™				
1 ng male	1:3000	1:4000	1:8000	Not reported
300 ng total DNA	1:4000	1:5000	1:15,000	1:2000
Promega PowerPlex® – Y System				
1 ng male	1:1000	1:2000	1:5000	Not reported
300 ng total DNA	1:10,000	1:15,000	1:22,000	Not reported
Reliagene Y-PLEX™ 12				
1 ng male	1:100	1:300	1:1000	Not reported
300 ng total DNA	1:100	1:300	1:2000	1:600–1:800*
Multiplex I (MPI)				
1 ng male	1:1000	1:2000	1:5000	Not reported
300 ng total DNA	1:100	1:500	1:5000	1:2000
Multiplex B (MPB)				
1 ng male	1:1000	1:2000	1:8000	Not reported
300 ng total DNA	1:16,000	1:20,000	1:30,000	Not reported

*Reported limit not given in terms of full profiles

Male/Male Mixtures— In theory, due to the hemizygous state of the Y-chromosome, it should be straightforward to detect the presence of DNA in admixtures resulting from the presence of two male contributors. Specifically, the presence of multiple alleles at several mono-local Y-STR loci (as opposed to bi-local markers such as DYS385 a/b) indicates the presence of a mixture and the number of contributors is determined by the maximal number of alleles found at one or more of the mono-local loci. However, as mixtures encountered in casework will not consist exclusively of 1:1 admixture ratios, it is important to determine the extent to which minor admixture contributors are detected. DNA from six male individuals were admixed into three bi-component mixtures in various ratios (1/1, 1/5, 1/10, 1/20) and the results are summarized in Table 5. The number of minor contributor alleles detected is greater with the Y-Filer kit than with the other systems since that kit contains more loci than the others. The presence of two male individuals was determined by the presence of two allelic signals at each locus (except the bi-local locus, DYS385). The mixtures were chosen because each individual male component differed from the other at two or more loci.

All discernible minor alleles were detected with all multiplex Y-STR systems when both DNA contributors were present in equal amounts (1:1 ratio). Although there was some loss of minor alleles, every system was able to discern a number of minor contributor alleles in the 1:5 mixtures. More extensive dilution of the minor contributor beyond 1:5 resulted in progressive loss of minor component alleles. The PowerPlex® – Y, Y-Filer™, and MPB systems were able to discern some minor component alleles at 1/10 and 1/20 ratios. Although the other two systems (Y-Plex™ 12 and MPI) detected putative minor component allelic signals at the 1/10 and 1/20 dilutions, it was not possible to differentiate these from stutter products.

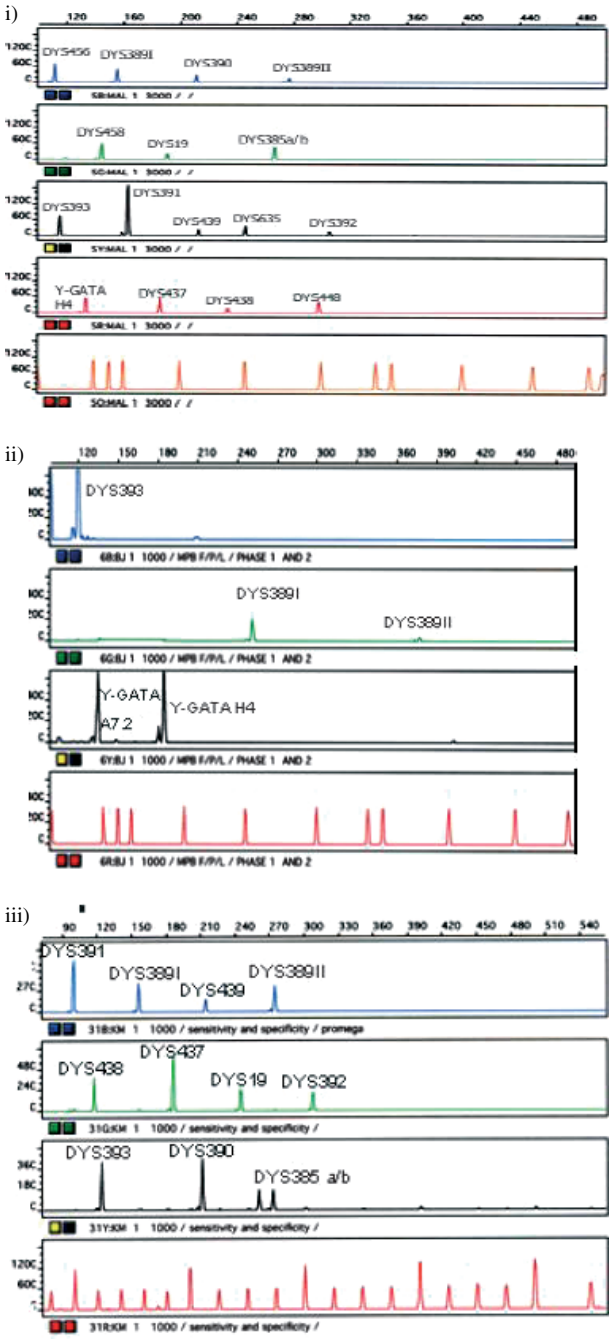

FIG. 2—*Specificity limits of Y-STR systems of male/female DNA mixtures using 1 ng Male DNA input. (i) Applied Biosystems AmpFℓSTR® Yfiler™ PCR Amplification Kit 1:3000; (ii) Multiplex B 1:1000; (iii) Promega PowerPlex® – Y System 1:1000.*

Stability Studies

Consistency of Y-STR Profiles in Different Tissues from the Same Individual— All Y-STR systems were typed using 1 ng of DNA isolated from blood, semen, saliva, and hair from four male individuals and 300 ng of DNA from blood, vaginal secretions, saliva, and hair from one female (data not shown). All tissue types from the males produced complete (and different) Y-STR profiles with each of the multiplex systems tested. None of the female tissue samples produced a full Y-STR profile with any of the Y-STR systems. However, as expected, Y-PLEX™ 12 produced an amelogenin X allele with all female samples due to the incorporation into that multiplex of the amelogenin gender determining system.

TABLE 5—*Y-STR resolution of male/male mixtures. Results indicate for each dilution and multiplex system the number of unshared minor contributor alleles detected.*

Y-STR Multiplex	1:1	1:5	1:10	1:20
AmpFℓSTR® Yfiler™				
Mixture 1	10/10	7/10	4/10	4/10
Mixture 2	13/13	11/13	6/13	6/13
Mixture 3	12/12	7/12	5/12	3/12
Promega PowerPlex® – Y System				
Mixture 1	6/6	4/6	5/6	3/6
Mixture 2	8/8	8/8	4/8	2/8
Mixture 3	9/9	7/9	5/9	4/9
Reliagene Y-PLEX™ 12				
Mixture 1	5/5	5/5	4/5	3/5
Mixture 2	6/6	5/6	3/6	1/6
Mixture 3	9/9	6/9	4/9	3/9
Multiplex I (MPI)				
Mixture 1	6/6	6/6	4/6	2/6
Mixture 2	4/4	3/4	1/4	1/4
Mixture 3	6/6	6/6	2/6	1/6
Multiplex B (MPB)				
Mixture 1	4/4	4/4	3/4	1/4
Mixture 2	2/2	2/2	2/2	2/2
Mixture 3	3/3	2/3	2/3	2/3

Environmental Effects

The ability to obtain a Y-STR profile from environmentally damaged DNA was evaluated using three different conditions: heat/humidity (H), heat/humidity/precipitation (P), and heat/humidity/precipitation/sunlight (S). Fifty microliters semen and blood stains from two male individuals and a bloodstain from a female were placed in an outdoor, metropolitan area in Central Florida and subjected to the natural elements. Protection against precipitation and sunlight was achieved by appropriate engineering controls which included a covered and UV screened patio. Exposure of the samples continued from July to October of 2005, with temperatures ranging from 79–95°F (90°F average high; 88°F average low). The relative humidity averaged 57% and 10.5 inches (4.2 cm) of rain fell during this period. Samples were exposed for 1 day, 2 days, 1 week, 3 weeks and 6 weeks and subsequently subjected to Y-STR analysis, the results of which are summarized in Table 6.

All Y-STR multiplex systems tested were able to produce complete Y-STR profiles from bloodstains and semen stains exposed up to 6 weeks when the samples were protected against precipitation and sunlight (Table 6, columns labeled H). However, exposure of the samples to precipitation either in the presence (S) or absence (P) of sunlight resulted in Y-STR profile loss over time, with total profile loss occurring with all systems after 3 weeks or more.

After 1 day exposure to heat/humidity/precipitation (R), all Y-STR multiplex systems tested were able to produce complete Y-STR profiles from bloodstains and semen stains. Two days or more exposure of the bloodstains to precipitation resulted in the loss of the Y-STR profiles from all multiplexes tested, with the exception of the PowerPlex® Y system, which permitted the detection of a partial profile comprising two loci after 1 week. Precipitation exposed semen stains were more amenable to Y-STR profiling than bloodstains, with partial profiles being obtained for the Y-Filer™ (13 loci), Y-Plex™ 12 (12 loci) and MPB (4 loci) systems and complete profiles being obtained from the PowerPlex® Y and MPI

TABLE 6—*Y-STR typing of environmentally challenged blood and semen stains. A full profile is designated '+' and total loss of the profile is designated '−'. A partial profile is designated by '(+)' and the number of typeable loci indicated. The environmental conditions used were: Heat and Humidity (H); Heat, Humidity, and Precipitation (P); Heat, Humidity, Sunlight, and Precipitation (S).*

	1 day			2 days			1 week			3 weeks			6 weeks		
	H	P	S	H	P	S	H	P	S	H	P	S	H	P	S
AmpFℓSTR® Yfiler™	+	+	+	+	−	−	+	−	−	+	−	−	+	−	−
Blood semen	+	+	+	+	(+)13	(+)13	+	(+)4	(+)4	+	−	−	+	−	−
Multiplex I	+	+	+	+	−	−	+	−	−	+	−	−	+	−	−
Blood semen	+	+	+	+	+	+	+	−	−	+	−	−	+	−	−
Multiplex B	+	+	+	+	−	−	+	−	−	+	−	−	+	−	−
Blood semen	+	+	+	+	(+)4	(+)4	+	(+)4	(+)3	+	−	−	+	−	−
Promega PowerPlex® – Y System	+	+	+	+	(+)2	−	+	(+)2	(+)2	+	−	−	+	−	−
Blood semen	+	+	+	+	+	+	+	(+)8	(+)6	+	−	−	+	−	−
Reliagene Y-PLEX™ 12	+	+	+	+	−	−	+	−	−	+	−	−	+	−	−
Blood semen	+	+	+	+	(+)12	(+)12	+	−	(+)3	+	−	−	+	−	−

systems after 2 days exposure. After 1 week exposure to precipitation the semen stains provided partial Y-Filer™ (four loci), PowerPlex Y (eight loci) and MPB (four loci) profiles, with complete profile loss being obtained for the Y-Plex™ 12 and MPI systems.

After 1 day exposure to heat/humidity/precipitation, all Y-STR multiplex systems tested were able to produce complete Y-STR profiles from bloodstains and semen stains. Two days or more exposure of the bloodstains to precipitation plus sunlight resulted in the loss of the Y-STR profiles from all multiplexes tested, with the exception of the PowerPlex® Y system, which permitted the detection of a partial profile comprising two loci after 1 week. Precipitation and sunlight exposed semen stains were more amenable to Y-STR profiling than bloodstains, with partial profiles being obtained for the Y-Filer™ (13 loci), Y-Plex™ 12 (12 loci), and MPB (4 loci) systems and complete profiles being obtained from the PowerPlex® Y and MPI systems after 2 days exposure. After 1 week exposure to precipitation and sunlight the semen stains provided partial Y-Filer (four loci), PowerPlex® Y (six loci), Y-Plex™ 12 (three loci), and MPB (three loci) profiles, with complete profile loss being obtained for the MPI system.

Species Specificity

Species specificity was assessed by testing the Y-STR systems using DNA from a number of different common domestic male mammals (male sheep, cow, cat, dog, horse, deer, and ferret) and male and female nonhuman primates. Some of the mammals tested produced PCR products that co-migrated with Y-STR loci or demonstrated significant fluorescence signals: horse (DYS391), cow (DYS391, DYS437, DYS393), sheep (DYS439), and deer (DYS389II) with the PowerPlex® – Y System; a 101 bp product in the yellow channel in cow, cat, dog, sheep, and deer with Y-PLEX™ 12; DYS456 (ferret) and Y-GATA 4 (ferret), DYS393 (dog) with the Y-Filer™ system; DYS393 (cow, MPB) (data not shown).

A much higher level of sequence homology exists between humans and nonhuman primates than with domestic mammals. We tested 1 ng of DNA from five male nonhuman primates (gorilla, chimpanzee, orangutan, spider monkey, and macaque) and 300 ng of DNA from four female nonhuman primates (gorilla, chimpanzee, pygmy chimpanzee, and orangutan). As expected the number of cross reacting products was found to be significantly larger than that found with domestic mammals (data not shown).

The PowerPlex® – Y System demonstrated a number of nonhuman primate products: female orangutan (DYS389I, DYS391, DYS437), female chimpanzee (DYS389I, DYS390, DYS391, DYS439), male macaque (DYS19, DYS389I, DYS393, DYS438, DYS439), female pygmy chimpanzee (DYS389I, DYS390, DYS439), and male chimpanzee (DYS437). The Y-PLEX™ 12 system detected: Amel in all nonhuman primate species tested; male gorilla (DYS385, DYS389II), male chimpanzee (DYS389I, DYS392, DYS393, DYS439), female chimpanzee (DYS393, DYS439), male macaque (DYS391), and female pygmy chimpanzee (DYS391). The Y-Filer™ detected: male chimpanzee (DYS389I), female orangutan (DYS393), female chimpanzee (DYS393 and DYS439), and male macaque (DYS448). MP1 exhibited a large number of nonhuman primate products: female chimpanzee (DYS393), female pygmy chimpanzee (DYS393), male macaque (DYS385a, DYS392, DYS438), and male chimpanzee (DYS389I). MPB exhibited no PCR products due to nonhuman primate homology (data not shown).

Postcoital Cervico-vaginal Studies

A postcoital cervico-vaginal swab was recovered independently by two females at specified intervals after coitus (0, 12, 24, 48,

TABLE 7—*Postcoital samples. The success of each Y-STR multiplex is indicated for cervicovaginal samples taken 0, 12, 24, 48, and 72 h after intercourse. +, Full profile; −, Lost profile; (+), Partial profile.*

Pair 1	0 h	12 h	24 h	48 h	72 h
Nondifferential extraction 300 ng					
AmpFℓSTR® Yfiler™	+	+	+	+	+
Multiplex I	+	+	+	(+)	−
Multiplex B	+	+	+	+	+
Promega PowerPlex® – Y System	+	+	+	+	+
Reliagene Y-PLEX™ 12	+	+	(+)	(+)	(+)
Differential extraction 1 ng male					
AmpFℓSTR® Yfiler™	+	+	+	+	+
Multiplex I	+	+	+	+	+
Multiplex B	+	+	+	+	+
Promega PowerPlex® – Y System	+	+	+	+	+
Reliagene Y-PLEX™ 12	+	+	+	+	(+)
Pair 2					
Nondifferential extraction 300 ng					
AmpFℓSTR® Yfiler™	+	+	+	+	+
Multiplex I	+	+	+	(+)	(+)
Multiplex B	+	+	+	+	+
Promega PowerPlex® – Y System	+	+	+	+	+
Reliagene Y-PLEX™ 12	+	+	(+)	(+)	(+)
Differential extraction 1 ng male					
AmpFℓSTR® Yfiler™	+	+	+	+	+
Multiplex I	+	+	+	+	+
Multiplex B	+	+	+	+	+
Promega PowerPlex® – Y System	+	+	+	+	+
Reliagene Y-PLEX™ 12	+	+	+	(+)	(+)

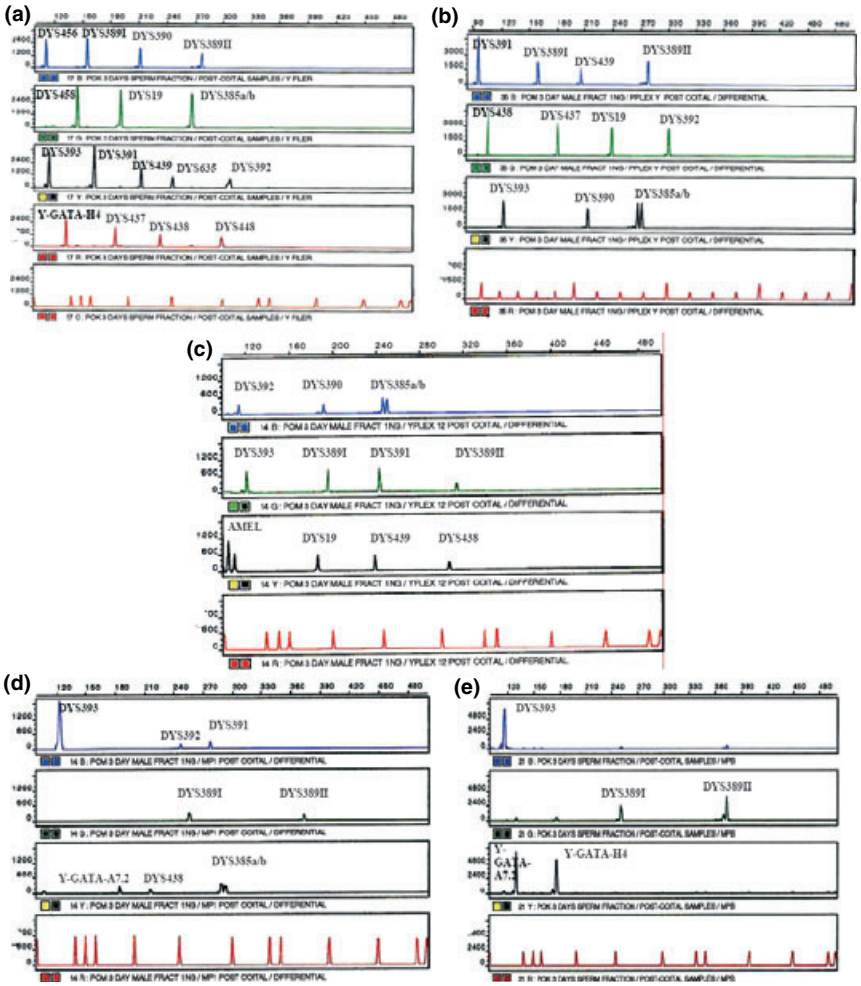

FIG. 3—*Y-STR profiles obtained from 72 h postcoital cervicovaginal samples subjected to a differential extraction. Three hundred nanograms of total DNA added to each multiplex system. (a) Applied Biosystems AmpFℓSTR® Yfiler™ PCR Amplification Kit; (b) Promega PowerPlex® – Y System; (c) Reliagene Y-PLEX™ 12; (d) Multiplex I; (e) Multiplex B.*

and 72 h). Each time point sample was collected after a separate act of sexual intercourse and was preceded by a 5-day abstention period. As a negative control, a precoital swab was also recovered prior to coitus for each sampling and only data from postcoital samples that demonstrated a lack of male DNA in the associated precoital sample was used. Two different DNA extraction procedures were evaluated (17,18). Firstly, a standard differential lysis procedure was performed in which 1 ng of the sperm fraction was used for Y-STR analysis. Secondly, a nondifferential extraction method was used that does not attempt to separate the DNA from the sperm and nonsperm cells. Instead, a standard organic extraction protocol was used to lyse (in the presence of DTT) the admixed sperm/nonsperm cell population. This resulted in co-extracted sperm and nonsperm DNA, after which a substantially larger than normal amount of DNA (300 ng) was used for Y-STR amplification. The results are summarized in Table 7.

A standard differential extraction procedure gave complete Y-STR profiles of the male donors up to 72 h postcoitus with all of the multiplex systems tested, except for Y-Plex™ 12, which gave partial profiles (Table 7 and Fig. 3).

The use of 300 ng of nondifferentially extracted DNA produced complete Y-STR profiles up to 72 h postcoitus with the Y-Filer™, PowerPlex® Y, and MPB systems. In contrast, the Y-Plex™ 12 system exhibited partial profiles with the 24 h (one donor pair), 48 h, and 72 h samples. Similarly the MPI system produced partial profiles after 48 h and, in one of the donor pairs, complete profile loss after 72 h (Table 7).

Conclusions

A small, but increasing, number of laboratories in the United States perform Y-STR analysis in casework. In order to facilitate the transfer of Y-STR technology to the crime laboratory community, a sensitivity and specificity performance comparison between commercial products from three of the main vendors (Applied Biosystems AmpFℓSTR® Yfiler™ PCR Amplification Kit, Promega PowerPlex®-Y System, Reliagene Y-PLEX™ 12) and two in-house Y-STR multiplexes (MPI and MPB) was carried out. One of the main goals of this vendor-independent study was to ascertain, using our well-characterized in-house Y-STR systems as a yardstick, the extent to which different commercial Y-STR kits were able to obtain male profiles from challenging samples such as mixtures, postcoital specimens, and environmentally impacted stains. The results confirm the efficacy of the commercial products for casework applications, particularly the PowerPlex-Y and Y-Filer systems.

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