TECHNICAL NOTE

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Development and Validation of the AmpFℓSTR® Yfiler™ PCR Amplification Kit: A Male Specific, Single Amplification 17 Y-STR Multiplex System*

ABSTRACT: In the past 5 years, there has been a substantial increase in the use of Y-short tandem repeat loci (Y-STRs) in forensic laboratories, especially in cases where typing autosomal STRs has met with limited success. The AmpFℓSTR® Yfiler™ PCR amplification kit simultaneously amplifies 17 Y-STR loci including the loci in the "European minimal haplotype" (DYS19, DYS385a/b, DYS389I, DYS389II, DYS390, DYS391, DYS392, and DYS393), the Scientific Working Group on DNA Analysis Methods (SWGDAM) recommended Y-STR loci (DYS438 and DYS439), and the highly polymorphic loci DYS437, DYS448, DYS456, DYS458, Y GATA H4, and DYS635 (formerly known as Y GATA C4). The Yfiler™ kit was validated according to the FBI/National Standards and SWGDAM guidelines. Our results showed that full profiles are attainable with low levels of male DNA (below 125 pg) and that under optimized conditions, no detectable cross-reactive products were obtained on human female DNA, bacteria, and commonly encountered animal species. Additionally, we demonstrated the ability to detect male specific profiles in admixed male and female blood samples at a ratio of 1:1000.

KEYWORDS: forensic science, DNA typing, DYS19, DYS385, DYS389, DYS390, DYS391, DYS392, DYS393, DYS438, DYS439, DYS437, DYS448, DYS456, DYS458, DYS635, Y GATA C4, Y GATA H4

When autosomal short tandem repeats (STRs) are employed in sexual assault cases the amplification and eventual detection of male DNA in a mixture sample may be masked by the presence of high levels of female DNA. The application of Y-STRs simplifies the analysis of these mixtures by removing the female contribution from the amplification profile. Typically in the U.S., the Y-STR loci used consists of the European minimal haplotype markers (DYS19, DYS385a/b, DYS389I, DYS389II, DYS390, DYS391, DYS392, and DYS393) and two additional loci (DYS438 and DYS439) recommended by the Scientific Working Group on DNA Analysis Methods (SWGDAM). The widespread use of these 11 loci has been facilitated by commercially available PCR amplification kits and corresponding databases used to estimate haplotype frequencies (1,2).

The application of Y-STR markers to male/female mixture analysis bypasses the need for performing differential extraction of sperm and epithelial cells (3). Differential extraction procedures are of limited value in sample mixtures derived from vasectomized or azoospermic males or other body-fluid mixtures not containing sperm. Other specialized applications for Y-STRs include paternal lineage studies (4) and deficiency paternity cases where the father of a male child is missing but a paternal male relative's profile is used as a reference to support/exclude relatedness (5).

An intrinsic limitation of Y-STRs compared with autosomal STRs is a reduced power of discrimination due to a lack of recombination throughout most of the Y-chromosome (6). Thus, in an effort to increase the power of discrimination of current single amplification Y-STR multiplexed systems, we have developed a 17 plex Y-STR system that includes the European minimal haplotype and SWGDAM markers and six additional highly polymorphic Y-STR markers (DYS437, DYS448, DYS456, DYS458, DYS635, and Y GATA H4). This article describes the developmental validation performed according to guidelines issued by the Director of the FBI (7), and the revised guidelines issued by SWGDAM (8). We also describe the haplotype diversity, and discriminatory capacity calculations for the three major U.S. population groups (Caucasians, Hispanics, and African Americans).

Materials and Methods

DNA Samples

Anonymous DNA samples were purchased from Seracare Life Sciences (Oceanside, CA), and nonhuman DNA samples were purchased from Pel-Freez (Rogers, AR). The female DNA 9947A

chased from Marligen Biosciences (Ijamsville, MD). The male DNA 9948 and Centre d'Etude du Polymorphisme Humain (CEPH) family DNA samples were purchased from Coriell Cell Repositories (Camden, NJ). The population study encompassed DNA samples from Seracare Life Sciences as well as samples analyzed by six population test sites. DNA samples were quantitated prior to amplification using Quantifiler™ Y Human Male DNA and Quantifiler™ human DNA quantification kits (Applied Biosystems, Foster City, CA) on the ABI Prism® 7000 SDS according to the manufacturer's specifications.

Primer Set Optimization

Each of the Y-STR loci amplified by the Yfiler™ kit has been characterized previously by other groups, and their nomenclature is consistent with that used in the certification of the National Institute of Standards and Technology (NIST) Standard Reference Material® 2395. The Yfiler™ kit primers were designed and optimized to obtain amplified products with robust signal intensity and balanced peak heights from only male samples and from male components in mixtures of male and female DNA. Initially, we tested singleplex reactions using our designed primer sets for each locus to ensure locus-specific amplification even in the presence of excess female DNA. We then carried out multiplex PCR studies through primer concentration adjustment and empirical performance testing in an effort to generate sensitive, balanced, and specific signals for 17 Y-STR loci in a single PCR reaction. The Yfiler™ kit STR loci span a range between 103 and 327 bp. In order to accommodate the co-amplification of 17 Y-STR markers within this narrow size range, we employed a five-dye fragment analysis system developed at Applied Biosystems and utilized in the AmpFℓSTR® SEfiler™ (9) and Identifiler™ kits (10). The five dyes (6-FAM™, NED™, PET®, VIC®, and LIZ®) expand the detection range to 660 nm thereby enabling more loci to be multiplexed into a single PCR. The spacing between different loci was optimized by introducing monomeric nonnucleotide linkers between the DNA oligonucleotide sequence and the fluorescent tag during the primer synthesis process (11,12) for the loci DYS456 and DYS438. The resulting PCR product has a slower mobility, which correlated with the number of linkers used. This approach was successfully employed in the Identifiler kit (10). The corresponding allelic ladders also have mobility modifiers. The NIST Standard Reference Material® 2395 kit was used to verify that the allele calls as a result of Yfiler™ kit analysis were concordant with the information in the Certificate of Analysis published by NIST (data not shown).

The Yfiler™ kit primer concentration ranged from 0.12 – 1.5 μM. Samples were amplified in triplicate at the standard primer concentration and at 10% intervals up to ± 30% levels to evaluate primer performance (data not shown).

PCR Reaction Components

The buffer components tested were: MgCl₂, KCl, AmpliTaq Gold® DNA polymerase, bovine serum albumin (BSA), sodium azide, and dNTPs. Each of these components was tested individually as a series of titrations around the standard condition used in the AmpFℓSTR® Yfiler™ PCR reaction mix. The components were tested in increments of ± 10% up to ± 30% from the standard concentration to test for reliability and robustness of the amplification components. Each concentration was tested with three samples (including the AmpFℓSTR® kit male control DNA 007) and amplified in triplicate using 1 ng of template DNA. The

components were tested at the following concentrations (standard concentration is shown in bold):

- MgCl₂: 1.12, 1.28, 1.44, **1.60**, 1.76, 1.92, and 2.08 mM.
- KCl: 35, 40, 45, **50**, 55, 60, and 65 mM.
- AmpliTaq Gold® DNA polymerase: 2.8, 3.2, 3.6, **4**, 4.4, 4.8, and 5.2 U per reaction.
- BSA: 112, 128, 144, **160**, 176, 192, and 208 μg/mL.
- Sodium azide: 0.014%, 0.016%, 0.018%, **0.020%**, 0.022%, 0.024%, and 0.026%.
- dNTP mixture (equimolar dATP, dCTP, dGTP, and dTTP): 560, 640, 720, **800**, 880, 960, and 1040 μM.

Thermal Cycling Parameters

Thermal cycling parameters were evaluated to establish the optimal performance window of amplification for the Yfiler™ kit. Two or more cycling parameters around the standard set of conditions were tested. For each study, 1 ng of DNA from three samples was prepared in triplicate and stored at 4°C while awaiting amplification in the same thermal cycler for each of the parameters tested.

The following thermal cycler parameters were examined (the recommended parameters are shown in bold):

- Cycle number: 28, 29, **30**, 31, and 32 cycles.
- Denaturation temperature: 92.5°C, **94.0°C**, and 95.5°C.
- Annealing temperature: 59°C, 60°C, **61°C**, 62°C, and 63°C.
- Final extension time: 50, 65, **80**, 95, and 110 min.

Precision and Stutter Studies

Sizing precision allows for determining accurate and reliable genotypes. Seventy-eight DNA samples from four populations (Caucasian, Hispanic, African American, and Native American) were purchased from Seracare Life Sciences (Oceanside, CA) and used for sizing precision and stutter experiments. The DNA samples (1 ng input) were amplified with the Yfiler™ kit using the standard conditions and electrophoresed on the ABI Prism® 3100 genetic analyzer. The deviation of each sample allele size from the corresponding allelic ladder allele size was calculated. All sample alleles tested were within ± 0.5 bp of a corresponding allele in the allelic ladder. The data from this experiment were also used to calculate stutter percentages. Percent stutter was calculated on 1264 alleles by dividing the height of the stutter peak by the height of the main allele peak. Allelic ladder sizing precision was calculated from nine injections of ladder run on the ABI Prism® 310 genetic analyzer. The standard deviation of the mean was calculated and shown to be within 0.15 bp or less (13).

Species Specificity

DNA samples extracted from male primates (1 ng each from gorilla, chimpanzee, orangutan, and macaque), male nonprimates (10 ng each from mouse, dog, pig, cat, horse, chicken, and cow) and microorganisms (approximately 100,000 copies each from *Candida albicans, Staphylococcus aureus, Excherichia coli, Neisseria gonorrhoeae, Bacillus subtilis,* and *Lactobacillus rhamnosus*) were subjected to PCR amplification using the Yfiler™ kit primers. The microbial DNAs were pooled together prior to amplification. Results were analyzed using the ABI Prism® 3100 genetic analyzer with GeneScan® 3.7 analysis software.

Stability Studies

Stability studies were simulated to assess performance of the kit with inhibited and degraded samples. Degraded DNA was produced by DNase I digestion in a time course manner using the DNA-free[TM] DNase treatment and removal kit (Ambion, Austin, TX) according to the manufacturer's instructions. The resulting DNA was examined by agarose gel analysis to assess the level of DNA degradation at each time point. A hematin inhibition study was performed with hematin (Sigma, St. Louis, MO) diluted to 1 mM in 0.1 N NaOH and added to the PCR to obtain final concentrations ranging from 0 to 24 μM.

Mixture Studies

Male/female mixture studies were performed on three different sets of male and female DNAs at 1:1000, 1:2000, 1:4000, and 1:8000. The amount of female DNA was kept constant at 500 ng, and the amount of male control DNA was varied from 500 to 62 pg.

Mixtures of two male DNA samples were examined at various ratios (1:1, 1:3, 1:10, and 1:15) while holding the total amount of input DNA to 1 ng. A 1:1 mixture contains 0.5 ng of each individual. Mixture ratios of 1:3, 1:10, and 1:15 contain 0.25, 0.09, and 0.0625 ng of the minor component, respectively.

A simulated casework sample series was created by combining 1 μL of male blood with a larger amount of female blood (100, 500, and 1000 μL). A 50 μL aliquot of the blood mixture was then spotted onto a cotton cloth. The typical size of the stain used for extraction was 4 mm². The DNA was extracted using the phenol/chloroform method and the quantity of male DNA was determined using the Quantifiler[TM] Y human male DNA quantification kit prior to amplification with the Yfiler[TM] kit. The Quantifer Y results were 0.1 ng/μL for the 1:100 ratio and 0.02 ng/μL for the 1:500 and 1:1000 ratios. Male DNA at 0.7 ng was amplified by the PCR.

PCR Amplification

Unless noted otherwise, the protocols in the AmpFℓSTR® Yfiler[TM] kit user's manual were followed. The PCR amplification was performed in a reaction volume of 25 μL containing 0.8 μL (4 units) of AmpliTaq Gold® DNA polymerase (Applied Biosystems), 9.2 μL of AmpFℓSTR® Yfiler[TM] kit PCR reaction mix, 5 μL of AmpFℓSTR® Yfiler[TM] kit primer set, and a maximum volume of 10 μL of target DNA. Samples were amplified in MicroAmp® reaction tubes (Applied Biosystems) in the GeneAmp® PCR system 9700 with a gold-plated silver or silver block (Applied Biosystems). The standard thermal cycling conditions in the 9600 emulation mode consisted of enzyme activation at 95°C for 11 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 1 min, and extension at 72°C for 1 min. A final extension was performed at 60°C for 80 min with a 4°C temperature hold if the PCR product was to remain in the thermal cycler.

Sample Electrophoresis and Data Analysis

The AmpFℓSTR® Yfiler[TM] kit employs a five-dye set system consisting of 6-FAM[TM], VIC®, NED[TM], PET®, and LIZ®. Amplification products were separated and detected on the ABI PRISM® 3100, 3100—Avant, and the 310 genetic analyzer using the specified G5 variable binning module as described in the user's manual [13]. Sample preparations and electrophoresis on the ABI PRISM® 3100 analyzer occurred as follows: 1 μL of the amplified product or allelic ladder and 0.3 μL of GeneScan[TM]-500 LIZ® size standard were added to 8.7 μL of deionized Hi-Di[TM] formamide (Applied Biosystems), denatured at 95°C for 3 min, and then chilled on ice for 3 min. Samples were injected for 10 s at 3 kV and electrophoresed at 15 kV for 1500 s in Performance Optimized Polymer (POP-4[TM] polymer) with a run temperature of 60°C as indicated in the GeneScan36vb_POP4DyeSetG5Module. The data were collected using the ABI PRISM® 3100 data collection software application v1.1 or 2.0. Electrophoresis results were analyzed using ABI PRISM® GeneScan® analysis software v3.7.1 and Genotyper® software v3.7 with AmpFℓSTR® Yfiler[TM] kit template or GeneMapper® ID software v3.2, as appropriate. Allele peaks were interpreted when greater than or equal to 50 relative fluorescence units (RFUs). Data on the mode of inheritance of DNA markers, stutter, sizing, and precision on the various ABI PRISM® instruments were documented in the AmpFℓSTR® Yfiler[TM] kit user's manual [13].

Statistical Analysis

Intracolor peak balance was calculated by dividing the lowest peak height by the highest peak height within a color. For the loci DYS385a/b and DYS389 I-II, the two allele peak height values were averaged prior to the calculation of intracolor peak balance.

Allele frequencies were calculated by dividing the number of occurrences for each allele by the sample population size. The allele frequency of the multicopy locus DYS385a/b was analyzed as a combination of the two alleles. Allele frequencies and haplotype diversity were calculated using the Arlequin software [14]. Gene diversity, D, of each locus was computed using the formula $D = (n/n - 1)(1 - \Sigma p_i^2)$, where n represents the sample size and p_i is the allele frequency [15]. Haplotype diversity (HD) was calculated with the same equation using haplotype frequencies rather than allele frequencies. Discriminatory capacity was determined by dividing the number of different haplotypes (not unique haplotypes) seen in a given population by the total number of samples [15,16].

Inheritance Study

Three CEPH/Utah families (#1333, #1340, and #1345) were analyzed using the AmpFℓSTR® Identifiler[TM] and AmpFℓSTR® Yfiler[TM] kits. All pedigrees consisted of two sets of grandparents, mother and father, and multiple siblings. Pedigrees #1333 had seven sons, pedigree #1340 had five sons, and pedigree #1345 had five sons.

One nanogram of DNA was amplified with either kit. Samples with discordant alleles (i.e., an allele at a locus that differed between father and son) were reamplified and reinjected to confirm allele calls.

Results and Discussion

Optimization of PCR Reagents

A representative Y-STR profile for 1 ng of the AmpFℓSTR® kit control DNA 007 using recommended conditions is shown in Fig. 1.

The optimization study consisted of testing individual components of the PCR while keeping the remaining components constant. Primers were designed to perform efficiently in a multiplex format. Full profiles were generated at all primer concentrations tested without any spurious amplification products. However,
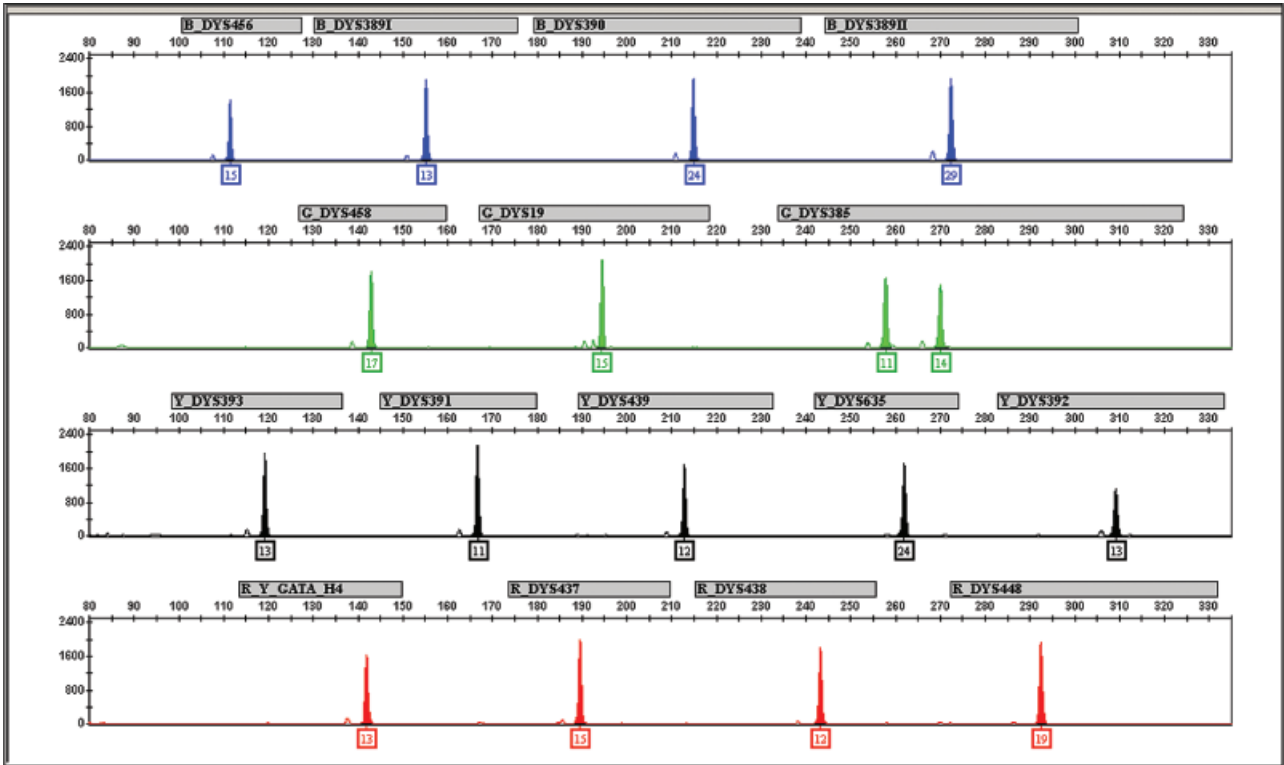
FIG. 1—*Representative electropherogram from GeneMapper® ID software v3.2 showing the profile of 1 ng of AmpFℓSTR® control DNA 007 amplified with the Yfiler™ kit. The four panels correspond to 6-FAM™, VIC®, NED™, and PET® dye-labeled peaks. The haplotype is shown with the allele number displayed underneath each peak.*

optimal intracolor peak balance was obtained within a window of $\pm 10\%$ from the standard primer mix concentration (data not shown). Magnesium plays a critical role in amplification efficiency and specificity. In order to identify the optimal $MgCl_2$ concentration, a series of formulations were prepared from 1.12 to 2.08 mM in 0.16 mM increments. At the lowest concentration of $MgCl_2$ tested (1.12 mM), the amplification profile lacked balance and occasionally resulted in partial profiles (Fig. 2). The loci Y GATA H4 and DYS19 were the most affected by the reduction in $MgCl_2$ concentration with four of the nine profiles demonstrating allele dropout at the 1.12 mM $MgCl_2$ concentration. Intracolor peak balance of 50% or greater was obtained between the concentrations of 1.44 and 1.92 mM (range: 57%–82%, $N = 35$ profiles). No allele dropouts were observed between the
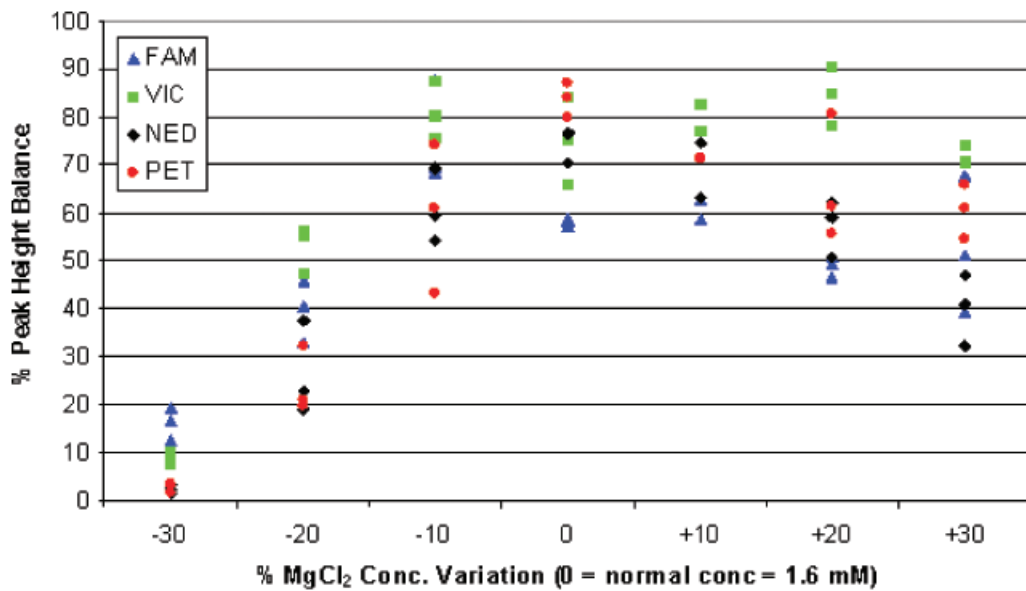

FIG. 2—*Effect of $MgCl_2$ titration on the intracolor peak balance of 1 ng male control DNA 007. Samples were amplified with PCR reaction mixes at $\pm 10\%$, 20%, and 30% of the optimal 0% (1.60 mM) and resolved on an ABI PRISM® 3100 genetic analyzer. The $MgCl_2$ concentrations tested were: 1.12 mM (-30), 1.28 mM (-20), 1.44 mM (-10), 1.60 mM (0), 1.76 mM ($+10$), 1.92 mM ($+20$), and 2.08 mM ($+30$). The peak height balance from loci in the same color were averaged and shown as data points ($n = 3$).*

concentrations of 1.28–2.08 mM ($N = 884$ alleles). The optimal concentration of $MgCl_2$ was set at 1.60 mM. This concentration is higher than what is used in other AmpFℓSTR® PCR kits (1.25 mM) (10).

Varied KCl concentrations ranging from 35 to 65 mM were tested and shown to affect both peak height and intracolor peak balance (data not shown). At 35 mM, the lowest intracolor peak balance for FAM® dye- (41%), NED® dye- (31%), and PET® dye- (46%) were observed but VIC® dye-labeled loci (70%) were not affected. KCl concentrations of 45–60 mM produced optimal intracolor peak balance (47–76%). The optimal KCl concentration was set at 50 mM.

The amount of Amplitaq Gold® DNA polymerase (range: 2.8–5.2 U/rxn) did not have a significant effect on intracolor peak balance of 1 ng of male template DNA with values greater than 49% for all the concentrations tested (range: 49–82%). However, 4 U/rxn were needed for amplification of small amounts of male DNA (i.e., 125 pg) in male/female mixtures (data not shown).

No meaningful differences were observed in peak height or intracolor peak balance when varying bovine serum albumin, sodium azide, and dNTP concentrations.

Thermal Cycling Parameters

The optimal thermal cycling parameters were determined to be in the middle of a window that balances optimal specificity and sensitivity. The results show that PCR cycle numbers did not have a significant effect on peak height balance in the range studied (28–32 cycles). Each increase in cycle number led to an approximately corresponding two-fold increase in peak height. The average peak height at 28 cycles was 509 RFUs. The average peak height at the standard cycle number of 30 was 1964 RFUs. Several peaks were offscale at 32 cycles (average peak height 5841 RFUs) while no off-scale peaks were detected at lower cycle numbers (data not shown).

The optimal annealing temperature was based on a balance among specific amplification of male DNA, sensitivity, and reproducible intracolor peak balance. No locus dropout was observed with annealing temperatures between 59°C and 62°C. A significant decrease in peak heights as well as occasional allele dropouts were noted at 63°C (Fig. 3). The loci DYS456, DYS390, DYS389 II, DYS19, DYS439, and Y GATA H4 were affected the most at 63°C. In contrast, the loci DYS393, DYS391, DYS635, DYS437, and DYS448 were not affected by the increase in annealing temperature. We also observed an inverse relationship between an increase in the annealing temperature and cross-reactivity with 500 ng of female DNA (data not shown). These experiments indicated that a 1°C window around the set point of 61°C yields male specific PCR products with the desired sensitivity for the DNA samples tested. At the standard annealing temperature (61°C), intracolor peak balance greater than 53% was observed.

Although the primers used in the Yfiler™ kit were designed to promote nonspecific terminal nucleotide addition by AmpliTaq Gold DNA polymerase (17), a final extension step was added to the protocol. The final extension step ensures the completion of +A addition to the 3' end of all double-stranded PCR products. This is especially important in mixtures containing a high background of female DNA. The optimal final extension step was determined to be 60°C for 80 min (data not shown).

Stutter products are a result of strand slippage (18) during PCR amplification. The most common stutter is one unit in length
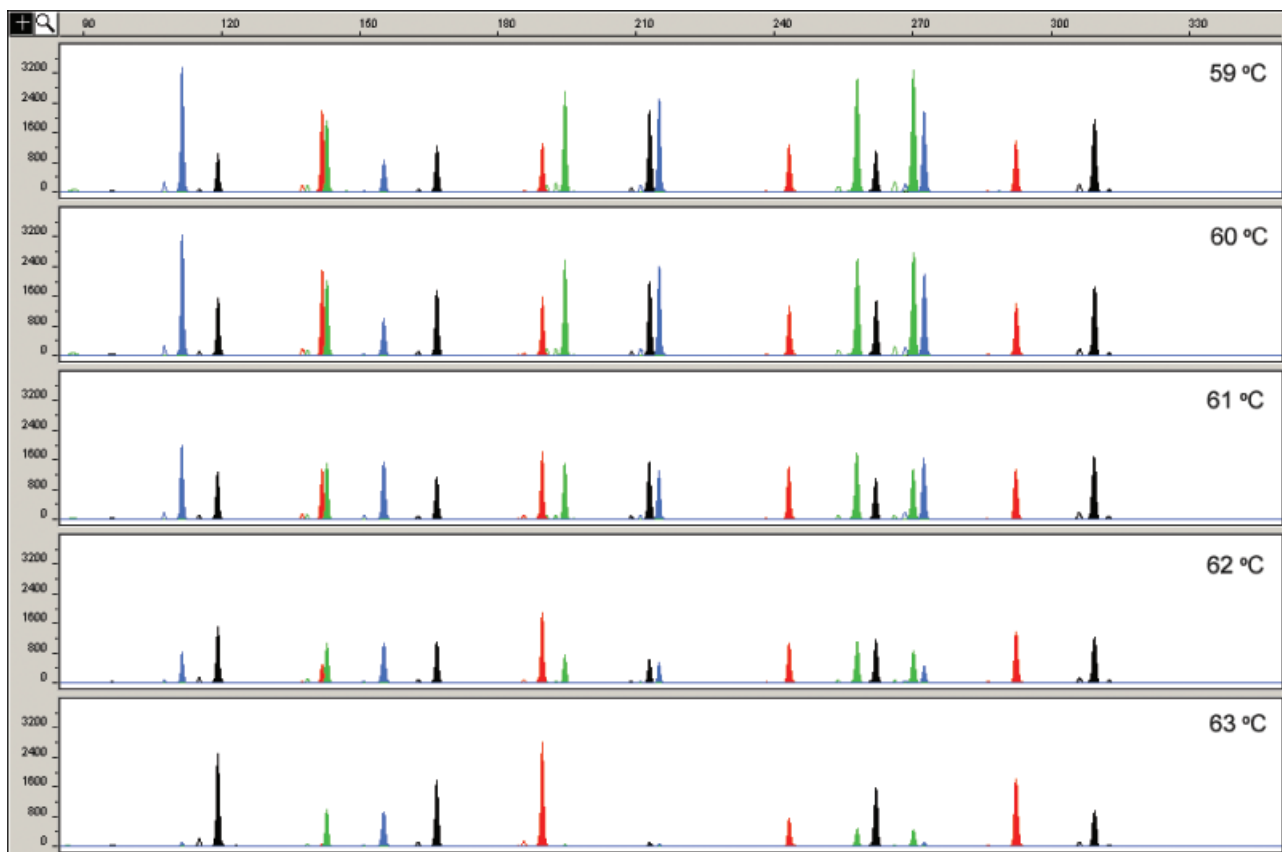

FIG. 3—*Representative electropherograms from an annealing temperature study of the male 007 DNA. The DNA was amplified with Yfiler™ kit primers at the indicated temperatures and analyzed on the ABI P*RISM® *3100 genetic analyzer. The peak height was measured in relative fluorescent units (RFUs).*

TABLE 1—*Stutter range for Yfiler™ kit loci.*

Locus	Stutter Type (bp)	Stutter Range (%)	Standard Deviation
DYS456	$N-4$	7.07–13.21	1.19
DYS389I	$N-4$	4.01–11.79	1.07
DYS390	$N-4$	4.82–10.40	1.48
DYS389II	$N-4$	9.39–13.85	1.12
DYS458	$N-4$	5.82–12.20	1.26
DYS19	$N-4$	4.65–11.04	1.37
DYS19	$N-2$*	7.24–10.21	0.68
DYS385	$N-4$	6.71–13.90	2.08
DYS393	$N-4$	6.95–12.58	1.09
DYS391	$N-4$	2.85–11.62	1.35
DYS439	$N-4$	4.19–11.18	1.14
DYS635	$N-4$	2.57–10.75	1.64
DYS392	$N-3$	6.91–16.22	2.19
DYS392	$N+3$*	2.88–7.90	1.36
Y GATA H4	$N-4$	3.68–11.08	1.13
DYS437	$N-4$	0.71–8.59	1.11
DYS438	$N-5$	1.67–4.28	0.55
DYS448	$N-6$	2.07–4.96	0.57

*In addition to the stutter product, one repeat unit smaller than the true allele (N) stutter was observed at $N-2$ for DYS19 and $N+3$ for DYS392.

smaller than the true allele resulting in a product that could be, for example, three bases smaller for the trinucleotide repeat marker DYS392, five bases smaller for the pentanucleotide repeat marker DYS438, six bases smaller for the hexanucleotide repeat marker DYS448, and four bases smaller for the remaining loci (all tetranucleotide repeat markers) in the Yfiler™ kit. Other locus specific

stutters observed were two bases smaller for DYS19 ($N-2$) and three bases larger for DYS392 ($N+3$). The percent stutter range for the Yfiler™ kit markers is shown in Table 1. As seen by others, we observed that the level of stutter product formation was correlated with repeat core size (2,19). Trinucleotide and tetranucleotide repeat markers yielded higher stutter percentages than the penta- and hexanucleotide repeat markers DYS438 and DYS448. All loci showed the trend of increasing stutter percentages with larger alleles. On average, the stutter product formation for Yfiler™ is slightly higher than that for the Identifiler kit (10). This could be due to the higher number of amplification cycles for the Yfiler™ kit (30 vs. 28 cycles) increasing the chance of slippage events. The Yfiler™ kit also has a higher amount of $MgCl_2$ in the amplification reaction (1.6 mM vs. 1.25 mM) potentially leading to higher slippage events by the polymerase (20).

Species Specificity

A variety of animal and microbial species were tested to assess the human specificity of the assay. Of the primate DNA samples, chimpanzee (Fig. 4) and to a lesser extent gorilla (data not shown) yielded partial profiles but with a migration pattern different from that of known human alleles. Chimpanzee Y-STRs homologous to human Y-STRs have been previously characterized (21). The amplification of genomic DNA from nonprimate species and pooled DNA from a number of microorganisms did not yield reproducible detectable products. A representative electropherogram containing selected species is shown in Fig. 4.
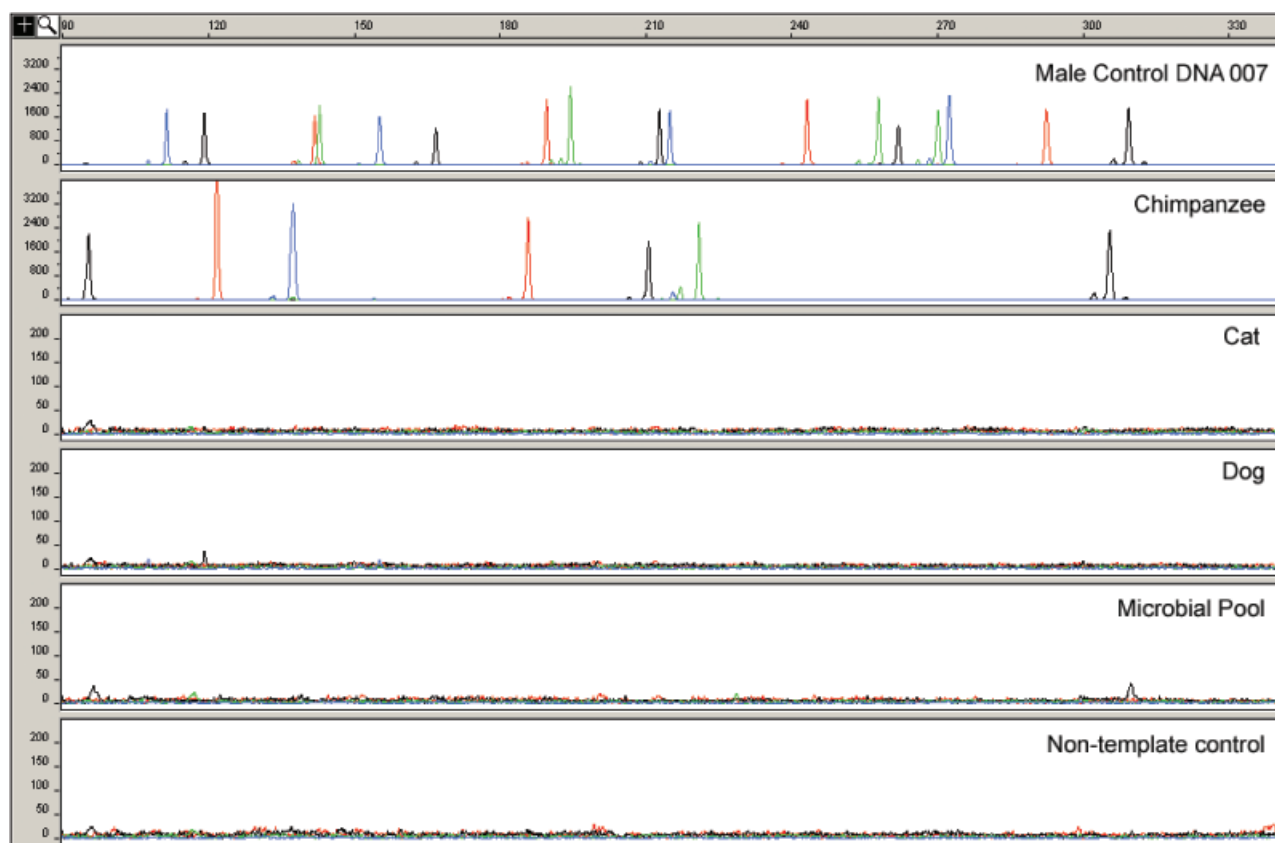

FIG. 4—*Representative electropherograms from a species specificity study. From top to bottom: 1 ng of the AmpFℓSTR® control DNA 007 (human), 1 ng of chimpanzee DNA, 10 ng of cat DNA, 10 ng of dog DNA, microbial pool (approx. 100,000 copies each of* Candida albicans, Escherichia coli *and* Lactobacillus rhamnosus *amplified together) and a nontemplate control (NTC).*

Sensitivity

Sensitivity studies were performed using serial dilutions of three male DNA samples including the male control DNA 007 contained within the kit (Fig. 5). The optimal quantity of template DNA for the Yfiler™ kit PCR ranged from 0.5 to 1.0 ng. Full profiles were obtained with amounts as low as 0.125 ng. Low input DNA amounts (< 100 pg) yielded more variable results occasionally resulting in partial profiles. Some minor dye artifacts near the calling-region may appear in the electropherogram (VIC® 88 bp, NED® 80 and 95 bp, and PET® 80 bp) (data not shown). These artifacts can be distinguished from actual allele peaks by using appropriate negative controls and should therefore not compromise the accurate typing of samples. Excess template input could result in a fluorescent signal that exceeds the dynamic range of detection (> 8191 RFUs) by the instrument (data not shown).

Degraded DNA

Environmental exposures of forensic samples may result in DNA degradation or damage at random chromosomal locations. As with any multilocus system, the possibility exists that not every locus will amplify if the DNA sample has been severely degraded. The ability of the Yfiler™ kit primers to amplify degraded DNA was investigated by amplifying high molecular weight genomic DNA incubated with DNase I for several time periods ("Materials and Methods"). Two nanograms of degraded DNA (and 1 ng undegraded DNA) were amplified using the AmpFℓSTR® Yfiler™ kit. As expected, with increasing DNase I digestion, there was a reduction in PCR product yield at all loci. After 12 min incubation with DNase I, the largest amplicon loci became undetectable (Fig. 6).

PCR Inhibition

DNA samples from crime scenes can contain inhibitors that can affect amplification of DNA samples. Heme compounds have been identified as PCR inhibitors in DNA samples extracted from bloodstains (22). The effect of hematin on the amplification efficiency was examined by varying concentrations of hematin (0–24 μM) in the PCR. Overall inhibition of the PCR reaction was observed as the concentration of hematin increased over 16 μM. Addition of hematin at 24 μM resulted in an almost complete inhibition of amplification (data not shown) (13).

Mixture Studies

Evidence samples that contain body fluids and/or tissues originating from more than one individual are commonly encountered in forensic casework. In the Yfiler™ kit reactions, the female DNA component is not amplified by the Y-chromosome-specific primers and therefore, it does not contribute to the profile (Fig. 7). Full profiles at 1:2000 (250 pg male DNA: 500 ng female DNA) were routinely obtained in our experiments, and mixtures at 1:4000 and 1:8000 occasionally resulted in partial profiles. Low-level cross-reactivity with female DNA (> 500 ng) was occasionally observed in the NED® (136 bp) and PET® (291 bp)
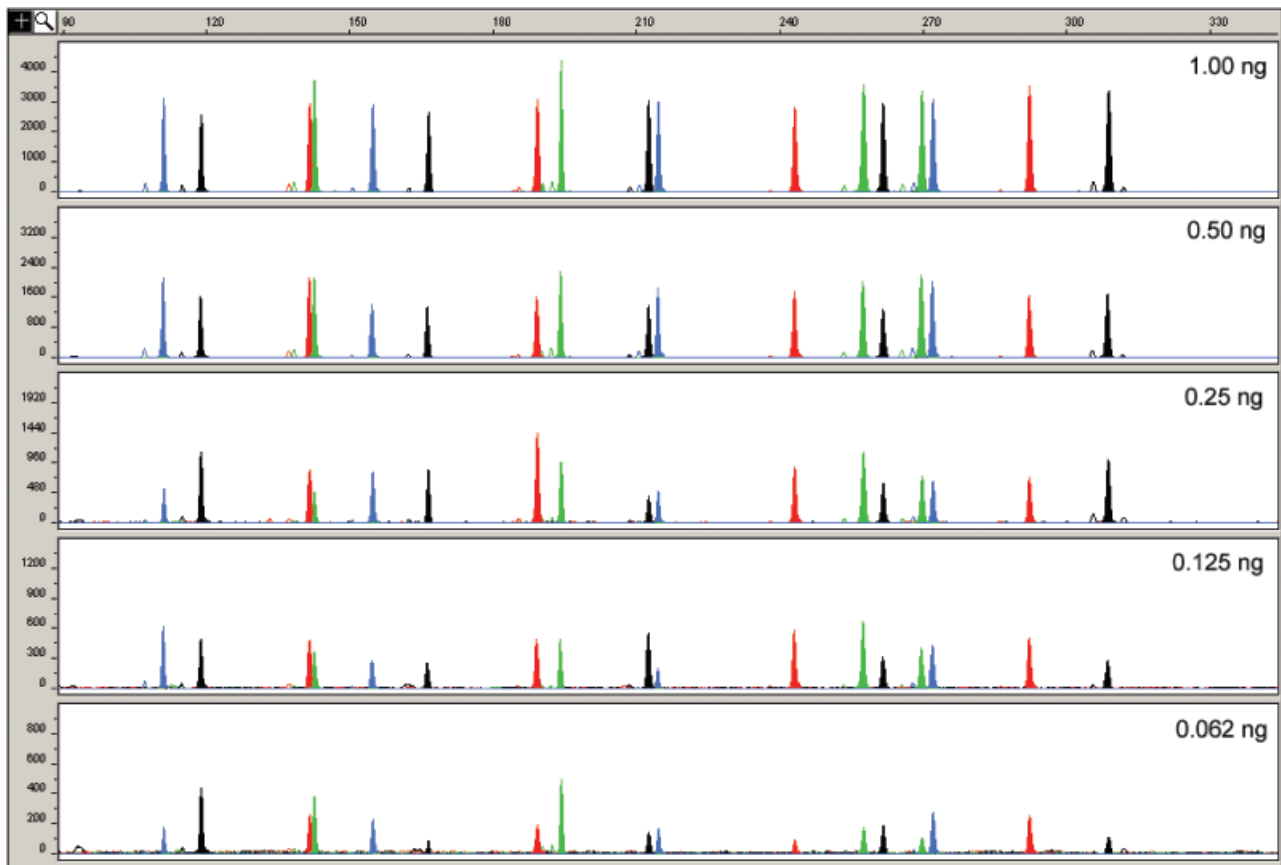

FIG. 5—*Effect of varying inputs of template DNA on intracolor peak height. The results depicted are representative from the amplification of the male control DNA 007 at the indicated amounts (n = 3). The data were analyzed with a peak amplitude threshold of 50 RFUs. One instance of allele dropout at Y GATA H4 was observed in one of the 62 pg profiles using an ABI PRISM® 3100 genetic analyzer.*
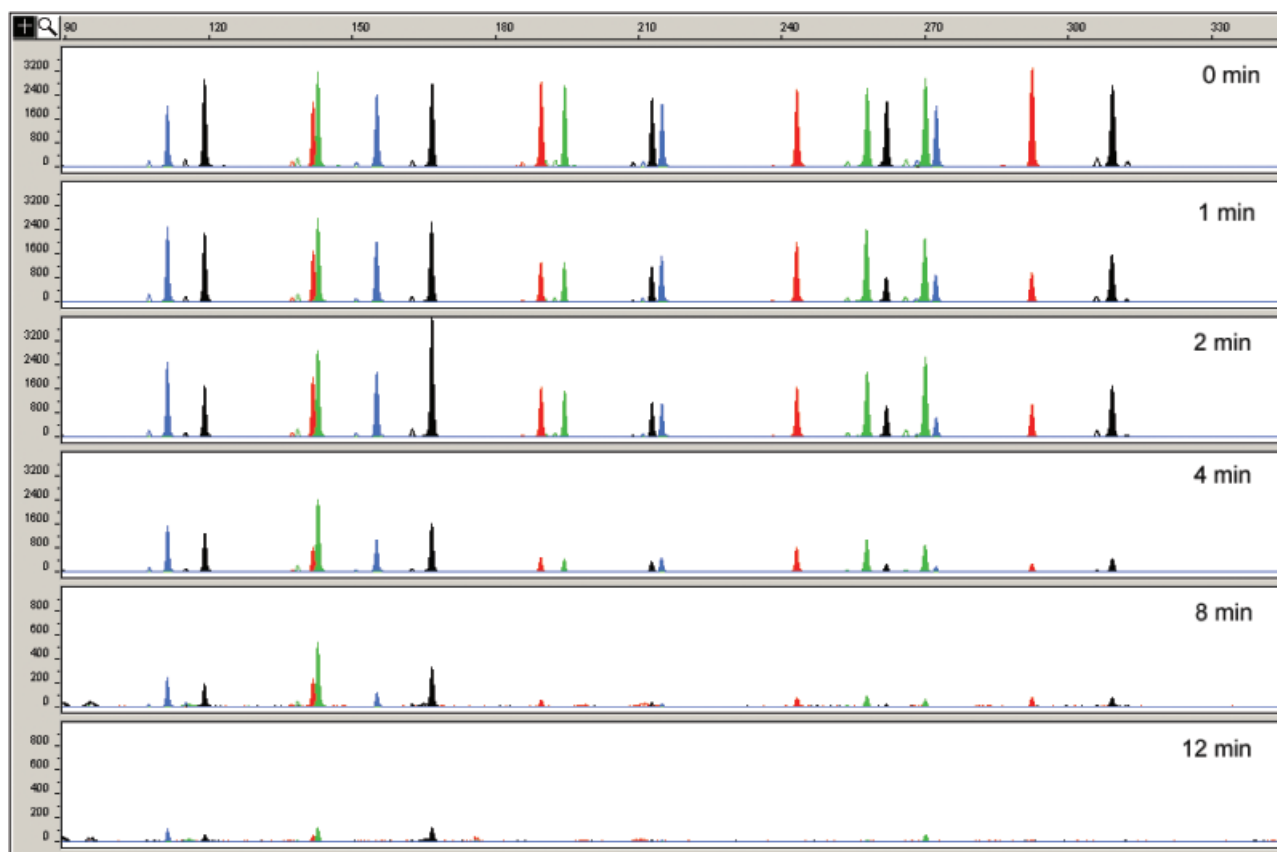
FIG. 6—*Profiles of AmpFℓSTR® control DNA in the absence of DNaseI (panel 1), incubated with DNAse I for 1, 2, 4, 8, and 12 min (panels 2–6). The Y-scale has been expanded for 8 and 12 min.*

dyes. In general, these peaks will not affect interpretation owing to their low peak heights (<50 RFUs) (13).

Forensic samples may contain body fluids or tissues originating from more than one male. Table 2 depicts the haplotype of the minor component of two individuals mixed at several mixture ratios. The alleles at each locus for the two individuals are different and thus do not overlap; however, some alleles of the minor contributors do reside at stutter positions of alleles from the major contributor. The minor component of the 1:3 mixture ratios was readily typeable. At a 1:10 ratio, all three profiles yielded complete haplotypes; however, alleles DYS390 and DYS385a for one of the profiles were filtered out by the Yfiler™ kit Kazam macro because they were below the stutter filter threshold. Ratios greater than 1:10 generally resulted in partial profiles for the minor male component.

In order to assess the performance of the Yfiler™ kit with specimens for which differential extraction is not an option, we created mixtures of male and female blood. As shown in Fig. 8, a complete Y-STR profile was obtained that matched the profile of the male donor at the concentrations tested.

Population and Inheritance Studies

The significance of a match between genetically typed samples depends on the frequency at which a haplotype occurs in a population. If the haplotype of the relevant evidence sample is different from the haplotype of the suspect's reference sample, then the suspect is "excluded" as the donor of the biological evidence. An exclusion is independent of the frequency of the two haplotypes in the population.

If the suspect and evidence samples have the same haplotype, then the suspect (and paternal relatives) cannot be excluded as a possible source of the evidence sample. The probability that another, unrelated, individual would also match the evidence sample is estimated by the frequency of the haplotype in the relevant population group. Barring mutation, the Y-chromosome is inherited from father to son unchanged as a haplotype of physically linked markers. Owing to the fact that the product rule cannot be applied to Y-STR markers, a counting method is used to empirically determine how many times a particular haplotype is observed in a population database (23).

Samples from U.S. Caucasians ($N = 778$), U.S. Hispanics ($N = 381$) and African Americans ($N = 786$) were analyzed using the Yfiler™ kit. As shown in Table 3, overall gene diversity ranged from 0.381 (DYS393 in U.S. Caucasians) to 0.950 (DYS385a/b in African Americans). The markers were ranked within each group according to their gene diversity values. DYS385a/b and DYS458 ranked the first and second, respectively, in gene diversity values across the three population groups. However, the lowest gene diversity values in each of the population groups were attributed to different markers (DYS393 in U.S. Caucasians and U.S. Hispanics and DYS391 in African Americans). The different gene diversity ranking order among the three population groups indicates that while some markers could be very informative in some population groups they could also be of limited value in the analysis of other population groups. The gene diversity values and relative rankings (Table 3) were also consistent with very few exceptions with the data previously published for the same population groups (24).
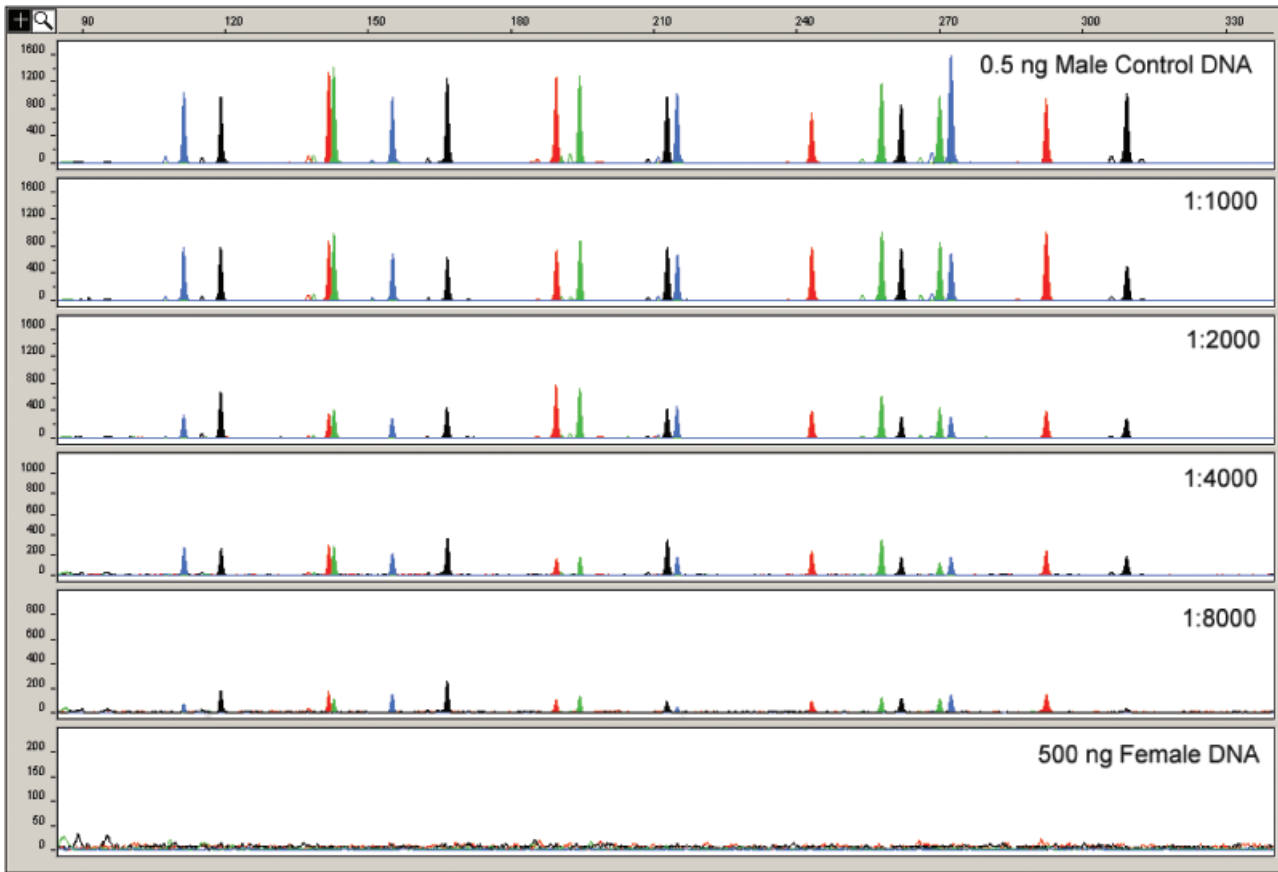
FIG. 7—*Mixture studies. Amplification of male control DNA 007 in the presence of female DNA 9947A. Profiles shown in the panels from top to bottom: 500 pg of male DNA, 500 pg male DNA with 500 ng female DNA (1:1000), 250 pg male DNA with 500 ng female DNA (1:2000), 125 pg male DNA with 500 ng female DNA (1:4000), 63 pg male DNA with 500 ng female DNA (1:8000), and 500 ng female DNA.*

As part of the analysis, we also calculated haplotype diversity (HD) for different marker combinations in order to gain insight to the added value of utilizing 17 Y-STR markers (Table 4). The marker combinations analyzed consisted of frequently utilized sets including the "European minimal haplotype," the "U.S. haplotype," the "U.S. Haplotype+DYS437," and the "Yfiler™ kit 17-marker set." Our results indicated that the HD value increased in the population groups studied as the number of markers in-creased. The Yfiler™ kit, which contains the most markers, scored the highest HD values across the populations studied. These results are proportionally similar to previous haplotype diversity analyses performed on the same population groups when comparing the U.S. haplotype marker set to a 20 Y-STR multiplex (24).

The discriminatory capacity (DC) and the number of unique haplotypes (UH) for various marker set combinations were also

TABLE 2—*Male–male mixture study.*

Mixture Ratio	Haplotype	Locus With No Overlapping Alleles									Locus With Minor Component Allele Overlapping With Major Component Stutter Peak				
		DYS456	DYS389II	DYS458	DYS19	DYS393	DYS439	DYS392	DYS437	DYS448	DYS389I	DYS390	DYS385a/b	DYS635	DYS438
0:1	Major HT	17	31	18	14	13	12	13	15	19	13	24	11,14	23	11
1:0	Minor HT	15	28	16	15	14	13	11	16	21	12	23	13,15	22	10
1:3	Minor HT	15	28	16	15	14	13	11	16	21	12	23	13,15	22	10
1:3	Minor HT	15	28	16	15	14	13	11	16	21	12	23	13,15	22	10
1:3	Minor HT	15	28	16	15	14	13	11	16	21	12	23	13,15	22	10
1:10	Minor HT	15	28	16	15	14	13	11	16	21	12	23	13,15	22	10
1:10	Minor HT	15	28	16	15	14	13	11	16	21	12	23	13,0	22	10
1:10	Minor HT	15	28	16	15	14	13	11	16	21	12	*	*, 15	22	10
1:15	Minor HT	15	28	16	15	0	13	11	16	0	*	23	13,15	*	10
1:15	Minor HT	15	0	16	15	0	13	11	16	21	*	23	*, 15	22	10
1:15	Minor HT	15	28	16	15	14	0	11	16	21	12	23	13, 15	*	10

Minor component allele calls at nonoverlapping Y-STR loci from replicate mixture amplifications. Detected haplotype of minor component using a peak amplitude threshold of 50 RFU and the Yfiler™ kit Kazam macro.
*Stutter peak present, which may mask allele.
STR, short tandem repeat; Major HT, major component haplotype; Minor HT, minor component haplotype; 0, no allele called.
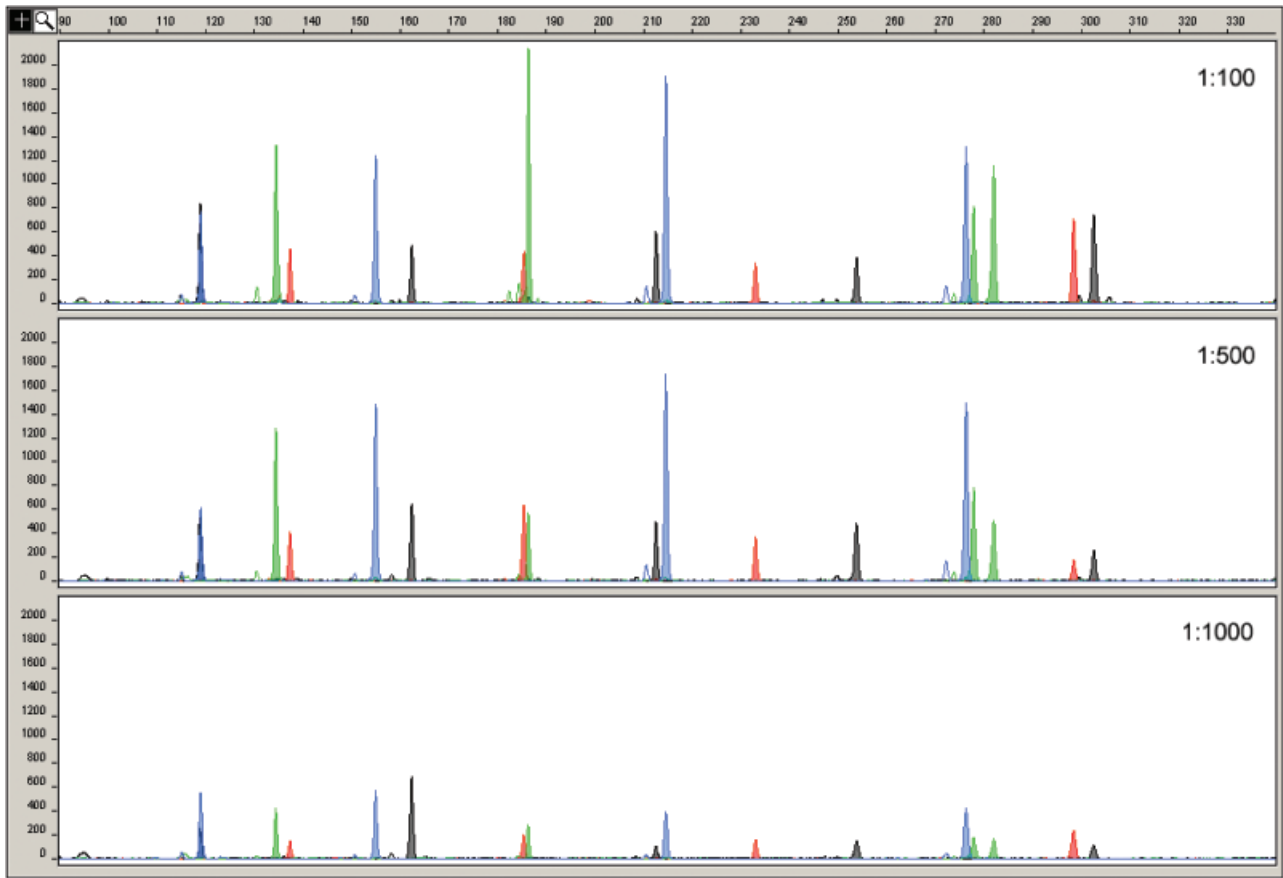
FIG. 8—*Mixture studies of male and female blood samples. Profiles shown in the panels from top to bottom correspond to the samples extracted from the cotton cloth containing 1 μL of male blood combined with 100, 500, and 1000 μL of female blood, respectively.*

determined (Table 5). As expected, the DC and UH correlated with the number of markers employed in the analysis. For example, in the Caucasian population, an increase of 21 percentage points was observed from the "U.S. haplotype" 11 Y-STR set to the Yfiler[TM] kit 17 Y-STR set. The most common haplotype group in Caucasians occurred 48 times when using the "European

minimal haplotype" markers, 21 times when using the "U.S. haplotype" set, and five times when employing the Yfiler[TM] kit markers.

The mode of inheritance of the Yfiler[TM] kit STR loci was examined using CEPH family DNA sets. Three CEPH family sets were examined (#1333, 1340, and 1345), representing 23 meiotic divisions. The haplotype results confirmed that the loci were inherited patrilineally. Two cases where the DYS458 allele was not in agreement with the other paternal lineage family members were identified: in sibling 1345–7356, a discordant DYS458-18 allele was identified instead of the DYS458-17 allele and in sibling 1340–7342, a discordant DYS458-16 allele was identified instead of the DYS458-17 allele. Paternity was confirmed by genotyping using the Identifiler kit. The mutations at the locus DYS458 are

TABLE 3—*AmpFℓSTR® Yfiler[TM] kit gene diversity across three U.S. populations.*

Y-STR	African Americans	Caucasians	Hispanics
DYS385a/b	0.950 (1)	0.842 (1)	0.935 (1)
DYS458	0.753 (2)	0.777 (2)	0.782 (2)
DYS19	0.747 (3)	0.500 (15)	0.666 (8)
DYS389II	0.747 (4)	0.676 (5)	0.730 (3)
DYS635	0.715 (5)	0.643 (7)	0.724 (4)
DYS448	0.693 (6)	0.596 (10)	0.716 (5)
DYS390	0.644 (7)	0.708 (4)	0.680 (7)
DYS439	0.629 (8)	0.648 (6)	0.665 (9)
Y GATA H4	0.610 (9)	0.599 (9)	0.572 (13)
DYS393	0.607 (10)	0.381 (16)	0.504 (16)
DYS456	0.603 (11)	0.722 (3)	0.663 (11)
DYS438	0.544 (12)	0.590 (11)	0.708 (6)
DYS389I	0.527 (13)	0.520 (14)	0.559 (14)
DYS437	0.498 (14)	0.576 (12)	0.573 (12)
DYS392	0.411 (15)	0.604 (8)	0.664 (10)
DYS391	0.404 (16)	0.546 (13)	0.541 (15)

The numbers in parentheses correspond to the rank order by gene diversity values.

STR, short tandem repeat.

TABLE 4—*Haplotype diversity (HD) for various Y-STR marker combinations.*

	HD		
Y-STR Marker Combinations	African Americans (N = 786)	U.S. Caucasians (N = 778)	U.S. Hispanics (N = 381)
"Minimal" haplotype	0.9985	0.9930	0.9967
"U.S. haplotype"	0.9994	0.9972	0.9985
"U.S. haplotype"+DYS437	0.9995	0.9978	0.9990
Yfiler[TM] kit	0.9999	0.9999	0.9998

The "Minimal" haplotype is the European minimal haplotype. The "U.S. haplotype" includes the minimal haplotype loci plus DYS438 and DYS439.

STR, short tandem repeat.

TABLE 5—*Discriminatory capacity (DC) and number of unique haplotypes (UH) for the three U.S. populations.*

Y-STR Marker Combinations	African Americans (N = 786)		Caucasians (N = 778)		Hispanics (N = 381)	
	DC (%)	UH	DC (%)	UH	DC (%)	UH
"Minimal" haplotype	75.8	496	61.7	382	79.8	266
"U.S. haplotype"	86.8	618	74.3	503	85.6	295
"U.S. haplotype" +DYS437	87.7	628	76.7	524	88.2	306
Yfiler™ kit	97.6	749	95.5	714	95.8	350

STR, short tandem repeat.

consistent with one-step changes observed for most Y-STR loci (25–29). The mutation rate for DYS458 was not calculated due to the small sample size.

Conclusions

The Yfiler™ PCR amplification kit simultaneously amplifies 17 loci, making it a highly discriminating Y-STR detection system for human identification. The combination of a five-dye chemistry (6-FAM™, NED™, PET®, VIC®, and LIZ®) and the inclusion of non-nucleotide linkers made it technologically possible to incorporate additional highly polymorphic markers (DYS448, DYS456, DYS458, DYS635, and Y GATA H4) not previously seen in other single amplification systems. These markers have been extensively characterized by other groups and have been independently shown to provide additional power of discrimination in combination with the "European minimal haplotype" markers (16,24,30–42). We have demonstrated that the Yfiler™ kit markers in population studies provided higher haplotype diversity (0.9999 in U.S. Caucasians ([N = 778]), 0.9999 in African Americans ([N = 786]) and 0.9998 in U.S. Hispanics ([N = 381])), and discriminatory capacity (95.5% in U.S. Caucasians, 97.6% in African Americans, and 95.8% in U.S. Hispanics) than the "U.S. haplotype" (Tables 4 and 5). A new haplotype database for the 17 Y-STR markers in the Yfiler™ kit is available at www.applied-biosystems.com\yfilerdatabase for use by the forensic community.

The validation of the Yfiler™ kit encompassed the verification of the best reaction conditions and reagent concentrations for the amplification of male DNA as well as the specific amplification of male DNA in the presence of a high background of female DNA. The performance criteria included overall peak heights, intracolor peak balance, and lack of cross-reactive peaks in the presence of female DNA. The thermal cycling parameters of the Yfiler™ kit differ from other AmpFℓSTR® kits in three ways: (1) the number of amplification cycles (30 cycles), (2) the annealing temperature (61°C), and (3) the final extension time (80 min). These changes significantly enhanced sensitivity for detection of small amounts of male DNA mixed with large amounts of female DNA. The sensitivity studies demonstrated that an input amount of 1 ng of male DNA does not produce off-scale peaks while allowing enough signal strength for reproducible detection of 125 pg or less of template DNA (Fig. 5). The male/female mixture studies (Figs. 7 and 8) demonstrate that the Yfiler™ kit is capable of producing robust, male-specific profiles in the presence of excess female DNA (500 ng). The studies on nonoptimal amplification conditions such as DNA inputs in the presence of confounding factors (e.g., nonhuman DNA, PCR inhibitors, partially degraded DNA, etc.) also showed that the Yfiler™ kit is capable of producing reliable profiles under a wide range of reaction conditions.

The reagent manufacturer performed the validation studies described in this paper. It is recommended that each laboratory conduct its own internal validation according to FBI/National Standards and SWGDAM guidelines (7,8).

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