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Research paper

Developmental validation of the Yfiler® Plus PCR Amplification Kit: An enhanced Y-STR multiplex for casework and database applications

Siddhita Gopinath*, Chang Zhong, Vivian Nguyen, Jianye Ge, Robert E. Lagacé, Marc L. Short, Julio J. Mulero

Thermo Fisher Scientific Inc., 180 Oyster Point Blvd., South San Francisco, CA 94080, USA

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ABSTRACT

Y-chromosomal loci have proven useful in solving investigations where low levels of male DNA are present in a high female DNA background. 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. Thus, in an effort to increase the power of discrimination we have developed a new 6-dye, 27-plex Y-STR system that includes the 17 loci from the Yfiler® and Yfiler® Direct kits (DYS19, DYS385a/b, DYS389I/II, DYS390, DYS391, DYS392, DYS393, DYS437, DYS438, DYS439, DYS448, DYS456, DYS458, DYS635 (Y GATA C4), and Y GATA H4) plus three highly polymorphic Y-STR loci (DYS460, DYS481, and DYS533), and seven rapidly mutating Y-STR loci (DYF387S1a/b, DYS449, DYS518, DYS570, DYS576, DYS627) which allow for improved discrimination of related individuals. The Yfiler® Plus PCR Amplification Kit is a dual application assay designed to amplify DNA from extracted casework and database samples from storage cards and swab lysates via direct amplification. Compared to the Yfiler PCR Amplification Kit, the new multiplex shows increased discrimination of male lineages and also improved performance in inhibited samples, improved balance in male DNA samples mixed with female DNA at ratios >1:1000, and faster time to results. The Yfiler Plus Kit shows very high concordance to the Yfiler Kit but discordance with the PowerPlex® Y23 Kit at the DYS481 locus was observed in 2 out of 30 samples tested. This developmental validation work follows the SWGDAM guidelines and demonstrates that the assay is robust and suitable for use on forensic casework and database samples.

1. Introduction

The ability to selectively amplify male DNA in a high background of female DNA by targeting Y-STRs is well documented in the literature [1]. The resulting Y-STR haplotypes aid in the identification of persons of interest in sexual assault cases [10]. Other applications of Y-STRs include paternal lineage studies 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 [2]. Compared to autosomal STRs, the Y-STRs have reduced power of discrimination due to lack of recombination on the Y chromosome. The Yfiler Plus kit boasts increased power of discrimination when compared to the Yfiler Kit because it has additional new loci with high gene diversity values [3–8,28]. It also has the potential to improve the level of paternal lineage differentiation by including loci with high mutation rates [12,29].

The Yfiler Plus multiplex consists of 27 loci in a 6-dye configuration, that include the Yfiler loci (DYS19, DYS385a/b, DYS389I/II, DYS390, DYS391, DYS392, DYS393, DYS437, DYS438, DYS439, DYS448, DYS456, DYS458, DYS635 (Y GATA C4), and Y GATA H4) in addition to 10 new loci [9]. The ten new loci consist of three highly polymorphic loci (DYS460, DYS481, and DYS533) and seven well characterized rapidly mutating loci (DYF387S1a/b, DYS449, DYS518, DYS570, DYS576, DYS627) [12]. The inclusion of rapidly mutating Y-STRs (RM-YSTRs) with mutation rates upwards of 1% allows for discrimination of related males [12].

The Yfiler Plus Kit is a dual application kit designed for efficient amplification of extracted DNA casework samples as well as direct amplification of blood and buccal reference samples on various substrates (treated and non-treated paper, and swabs). Through primer design and master mix optimizations, the kit has been designed to specifically amplify male DNA in the presence of large amounts of female DNA background and with faster time to results than the Yfiler kit. This paper demonstrates the robustness and applicability of the Yfiler Plus Kit in casework and database applications through validation studies performed as per the

* Corresponding author.
E-mail address: siddhita.gopinath@thermofisher.com (S. Gopinath).

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guidelines published by the Scientific Working Group on DNA Analysis Methods (SWGDAM) [11,13,14].

2. Materials and methods

2.1. DNA samples

Whole human blood samples were obtained from the Interstate Blood Bank (Memphis, TN USA) and Boca Biolistics (Coconut Creek, FL USA). The donor samples were collected in the United States from randomly-selected individuals of self-reported ethnicities. DNA was extracted and purified from blood samples using an ABI® PRISM 6100 Nucleic Acid PrepStation (Thermo Fisher Scientific, Waltham, MA USA). The 2 ng/μL male Control DNA 007 supplied with the Yfiler Plus Kit was used to prepare samples for the sensitivity, models of inhibition, and mixture studies. Female Control DNA 9947A (500 ng/μL) was obtained from Coriell Cell Repositories (Camden, NJ USA). For the male specificity study, DNA was extracted and purified from Peripheral Blood Cells (All Cells, LLC, Alameda, CA USA) from 10 female donors using PrepFiler™ Express chemistry on the Automate Express™ DNA Extraction System. DNA was extracted and purified from semen samples from two donors using the PrepFiler Express™ chemistry on the AutoMate Express™ platform. Human DNAs were quantified using the Quantifiler® Duo Kit on an Applied Biosystems® 7500 Real-Time PCR System. DNA concentration of non-human DNA was determined by measuring the absorbance of the sample at 260 nm. When necessary, DNA inputs were diluted with DNA Suspension Buffer (10 mM Tris–HCl pH 8.0 and 0.1 mM EDTA) (Teknova, Hollister, CA USA).

Whatman™ FTA™ cards, and Whatman™ EasiCollect™ devices were purchased from GE Healthcare (Piscataway, NJ USA). Reference samples of blood on paper substrates were prepared by spotting 75 μL of whole blood onto the center of the sampling area on the FTA™ cards. Reference samples of buccal cells on paper substrates were prepared following the manufacturer's collection instructions using the EasiCollect™ devices.

DNA samples for 53 pairs of father-son were obtained primarily from CEPH DNA families from Coriell Cell Repositories (Camden, NJ USA).

2.2. PCR amplification and thermal cycling conditions

The Yfiler Plus Kit has been optimized in a 25 μL total reaction volume, consisting of 10 μL of Master Mix, 5 μL of Primer Set, and 10 μL of sample input volume containing 1 ng of DNA. For database samples on paper substrates run in direct amplification mode, 10 μL of DNA Suspension Buffer (10 mM Tris–HCl pH 8.0 and 0.1 mM EDTA) was added to the amplification mix to maintain a total reaction volume of 25 μL. PCR samples were amplified in MicroAmp® Optical 96-well reaction plates (Thermo Fisher Scientific, Waltham, MA USA) on the Veriti® 96-well Thermal Cyclers using the 9600 emulation mode (Thermo Fisher Scientific, Waltham, MA USA). Standard thermal cycling conditions for the Yfiler Plus Kit are: enzyme activation at 95 °C for 1 min; 30 cycles of 94 °C for 4 s, and 61.5 °C for 60 s; followed by a final extension step at 60 °C for 22 min. Design of Experiments (DOE) methodologies were used to optimize the thermal cycling parameters in conjunction with the reagent formulations to maximize assay performance while minimizing cycle time. Assay performance metrics included intracolor balance, male specificity, average peak heights, sensitivity, inhibitor tolerance, and baseline noise. The Yfiler [15] and Yfiler Direct [16] Kits (Thermo Fisher Scientific, Waltham, MA USA), used for benchmarking purposes in various studies, were run under standard conditions.

2.3. Sample electrophoresis and data analysis

Separation of PCR amplified products was performed on Applied Biosystems 3130*xl*, 3500 or 3500xL Genetic Analyzers (Thermo Fisher Scientific) using run modules and J6 6-dye variable binning modules as described in Yfiler Plus Kit User Guide [17]. The 3500xL instrument was used as the default for most studies unless mentioned otherwise. Sample setup for Capillary Electrophoresis (CE) is as follows: 9.6 μL of deionized Hi-Di™ Formamide, 0.4 μL of GeneScan™ 600 LIZ® Size Standard v2.0 (Thermo Fisher Scientific, Waltham, MA USA), plus 1 μL of PCR product/Yfiler Plus Allelic Ladder. Samples were denatured at 95 °C for 3 min then chilled on ice prior to electrophoresis. Sample injection was performed in Performance Optimized Polymer-4 (POP-4 polymer) (Thermo Fisher Scientific, Waltham, MA USA) with the following run conditions for a 3500xL: 1.2 kV for 24 s and electrophoresed at 13 kV for 1550 s. GeneMapper® *ID-X* Software v1.4 (Thermo Fisher Scientific, Waltham, MA USA) was used for data analysis using a 175 RFU peak amplitude threshold for allele calls.

2.4. PCR primer set components

The Yfiler Plus Primer Set contains primers that amplify 27 loci in a 6-dye configuration. This was achieved by combining some of the existing primers from the Yfiler Kit with new primers designed for the Yfiler Plus Kit (Supplementary Table 1). The use of an additional dye-channel enabled the addition of new loci while maintaining the marker range to less than 410 bp. The Primer Set incorporates the use of fluorescence-tagged primer oligos some of which were synthesized with mobility-modifying non-nucleotide linkers in order to optimize the spacing among loci. The five dyes used in the Yfiler Plus Kit to label amplified sample products are 6-FAM™, VIC®, NED™, TAZ™, and SID™. The sixth dye, LIZ®, is used to label the GeneScan™ 600 LIZ® Size Standard v2.0.

The underlying criteria for loci selection in the Yfiler Plus kit multiplex was to enhance the power of discrimination of the Yfiler kit multiplex by adding additional loci with high gene diversity values. Even with the addition of a new dye channel, the number of loci added was limited by the desirability to maintain the amplicon sizes to approximately less than 400 bp. This self-imposed size limitation enables effective 1 bp resolution of microvariants under most electrophoretic conditions and makes the loci less prone to the potential effects of DNA degradation. A subset of these new loci is comprised of RM-YSTRs. Amongst the 13 RM-YSTR loci characterized to date [12], loci having three copies on the Y-chromosome (DYF399S1 and DYS403S1a) were considered too challenging to reliably deconvolute male-male mixtures and therefore were not considered in our original design. Other RM-YSTR loci under consideration failed to meet specific performance criteria such as high stutter ratios (on average $\geq$30% for the trinucleotide repeat locus DYS612) while for loci such as DYS547 we were unable to identify a set of primers that would produce a male specific PCR product in mixtures where the female DNA exceeded 1 μg (data not shown).

Robustness of the primer set was determined by varying each primer pair in the multiplex in increments of 10% up to +/− 20% compared to the optimized formulation and comparing performance based on intracolor balance, peak heights, and alleles recovered.

2.5. PCR master mix components

The Yfiler Plus Master Mix includes the following components: a "hot-start" DNA polymerase, buffer, salts, dNTPs, carrier protein, and sodium azide. In order to test the robustness of the master mix formulation, the individual components were varied at increments

of 10% up to +/− 20% (v/v) from their optimized formulation in the Master Mix. The performance of the master mix at these variations from the optimized formulation was assessed by comparing intracolor balance, peak heights, and alleles recovered.

2.6. Sensitivity

For the sensitivity study, allele recovery performance was measured for male Control DNA 007 inputs ranging from 1000 to 31.25 pg and benchmarked against that of the Yfiler Kit. Samples were prepared by serially diluting male Control DNA 007 (2 ng/μL stock) in DNA Suspension Buffer. Total DNA inputs tested were: 1000, 500, 250, 125, 62.5, and 31.25 pg. Non-template controls (NTC) were included. In addition, Yfiler Plus kit sensitivity with Male:Female mixtures, comprising of male Control DNA 007 and Female Control DNA 9947A, of varying ratios was tested. The DNA input range for the male DNA was 31.25–1000 pg and for female DNA was 1–4 μg.

2.7. Male specificity

A key performance criterion for a Y-STR kit is male specificity. With Yfiler Plus, this was achieved through a combination of primer design and optimization of the Master Mix and PCR thermal cycling conditions. Female DNA from 10 donors was used to test male specificity. Amounts up to 3 μg of female DNA from each donor were amplified with the Yfiler Plus kit using the standard protocol. Amplified products were analyzed for cross-reactive artifacts in or around the read region (68–406 bp).

2.8. DNA mixtures

DNA mixtures were prepared from two male cell line donors (Control DNA 007 and Raji) in the presence of a high female DNA (Female Control DNA 9947A) background. Total male DNA input was maintained at 1 ng and total female DNA input was maintained at 200 ng in the amplification reaction. Mixture ratios between the two male DNAs ranged from 1:1 to 1:15 (which corresponds to a 500–62.5 pg DNA input range for the minor male component). Performance measured as allele recovery was benchmarked against that of the Yfiler Kit.

2.9. Species specificity

Whole blood samples for the following species were obtained from Pel-Freez Biologicals (Rogers, AK USA): Bovine, Chicken, Mouse, Pig, Rabbit, Dog, Rat, Horse, and Sheep. Genomic DNA from these whole blood samples was extracted and purified using the ABI® PRISM 6100 Nucleic Acid PrepStation. The species specificity of the Yfiler Plus Kit for human DNA was assessed by amplifying 10 ng of DNA for each of the above mentioned species as per the standard PCR protocol. Amplified products were analyzed for cross-reactive artifacts in and around the read region (68–406 bp).

2.10. PCR inhibition models

The performance of the Yfiler Plus Kit was assessed against two models of PCR inhibition. The two inhibitors selected for testing were Hematin and Humic Acid. Stock solutions of high concentration were prepared by dissolving each of the inhibitors as follows: hematin (Sigma, St. Louis, MO USA) in 0.1 N NaOH and humic acid (Sigma, St. Louis, MO USA) in DNA Suspension Buffer. Working stocks were created for both inhibitors by diluting in water. Samples containing male Control DNA 007 and each inhibitor in various concentrations were created and amplified with the Yfiler Plus Kit using standard protocol. Performance measured as allele recovery was benchmarked against that of the Yfiler Kit.

2.11. Population and concordance studies

The genotypes of 942 male individuals with self-declared ethnicities representing four major populations in the U.S (African American (257), Caucasian (237) and Hispanic (188) and Asian (260)) were determined using the Yfiler Plus Kit under standard conditions. Genotype concordance was compared to the Yfiler Kit. The 942 individuals from the same population samples were also used to calculate Haplotype Diversity and Discrimination Capacity values. Concordance between Yfiler Plus Kit and the PowerPlex Y23 Kit was performed with 30 buccal samples on FTA™ paper. Concordance between 53 pairs of male relatives was performed to test the ability of the RM-YSTRs to separate closely related males.

2.12. Performance testing

For performance testing of purified extracted DNA samples, 1 ng of genomic DNA from 42 individuals randomly selected from the above mentioned population samples, were amplified with Yfiler Plus Kit as per the standard conditions. For performance testing of Male:Female mixture samples, sample containing 1 ng of male DNA and 1 μg of female DNA, from 3 donors each, was amplified with Yfiler Plus Kit as per the standard conditions. For database samples, 55 individuals for blood and buccal samples on FTA™ paper were amplified with the Yfiler Plus Kit using standard conditions. Cycle optimization was carried out for each of the direct amplification sample types and 28 cycles was identified as optimal for both blood and buccal samples on FTA™ paper.

2.13. Sizing accuracy, precision and stutter effects

Allele sizing accuracy and precision studies were conducted on Applied Biosystems 3130*xl*, 3500 and 3500xL Genetic Analyzers. 1 ng of genomic DNA from 42 individuals, randomly selected from the above mentioned population samples, were amplified with Yfiler Plus Kit as per the standard conditions. Sizing accuracy was calculated by comparing the allele sizes observed with the genomic DNA samples to that observed in the Yfiler Plus Allelic Ladder. Sizing precision was assessed by determining observed alleles size standard deviations from multiple injections of Yfiler Plus Allelic Ladder for the different CE platforms (3130*xl*—5 injections; 3500—5 injections; 3500xL—5 injections). The GeneScan™ 600 LIZ® Size Standard v2.0 and GeneMapper® *ID-X* 1.4 software using the Local Southern method was used for all size calculations. Stutters for the Yfiler Plus Kit were calculated by dividing the stutter peak height (n+/− 1 repeat units, or n+/− 2nt) by that of the true allele peak heights (n repeats) and expressed as a percentage. 942 population samples were run on an Applied Biosystems 3500xL Genetic Analyzer using a 175 RFU peak height minimum (minimum stutter peak height of 20 RFU) to calculate the stutter values.

2.14. Statistical analysis

Intracolor balance was calculated as the lowest peak height in each dye channel divided by the highest peak height in the same dye channel and reported as a percentage. For the two multi-copy loci (DYS385 and DYF387S1a/b), the peak heights were averaged prior to calculation of the intracolor balance, and if the alleles were of the same length, the peak height were halved prior to intracolor balance calculation. Statistical analyses were performed using Minitab® (Minitab Inc., State College, PA USA) or JMP® (SAS Institute Inc., Cary, NC USA) software.

3. Results and discussion

3.1. PCR reaction—cycle number

1 ng of total Male input DNA and a 1:1000 Male:Female mixture containing 1 ng of male Control DNA 007 and 1 μg of female DNA 9947A were tested with the Yfiler Plus Kit over a range of amplification cycle numbers (29–32). As expected, an increase in cycle numbers resulted in a 1.5–2 fold enhancement in overall peak heights. 30 cycles was determined to be optimal with respect to maximizing assay sensitivity while minimizing the possible occurrence of off-scale peaks. A representative profile generated under the standard condition of 30 amplification cycles is shown in Supplementary Fig. 1 for the Male:Female mixture sample.

The amount of DNA available for direct amplification from paper substrates or swabs can vary widely, depending on the sample type (blood versus buccal), collection device, collection method, and donors' white blood cell count or buccal-shedding levels. The consequence of such variability is that each laboratory must determine the optimal cycle number based on their own samples and CE instruments. As a general rule, the optimal cycle number should generate sufficient sample peak heights while minimizing the occurrence of off-scale allele peaks or allelic drop-out events. Representative profiles generated under optimal conditions of 28 amplification cycles with in-house sample sets for blood and buccal samples on FTA™ paper are shown in Supplementary Fig. 2.

3.2. PCR reaction—denaturing, annealing, and final extension steps

The Yfiler Plus Kit thermal cycling conditions were examined for robustness by varying key parameters such as denaturing temperature, annealing/extension temperature, and final extension time over a relevant range around the optimal, with 1 ng of male Control DNA 007 by itself or in combination with 1 μg of female DNA 9947A, as well as blood and buccal samples on FTA™ paper. Results show that varying denaturing temperature by +/− 1.0 °C from the optimal 94 °C did not show any significant difference in performance for all sample types tested (data not shown). For the annealing/extension step, temperatures of 60.5, 61.0, 61.5 (optimal condition), 62.0, and 62.5 °C were tested. While full profiles were obtained at all annealing/extension temperatures tested, a significant drop in intracolor balance was observed with increasing temperatures for male Control DNA 007 and Male:Female Mixture samples (Fig. 1). Similar results were observed for the direct amplification samples (data not shown). The first loci to drop out among the 27 included in the kit were DYS385, DYS439, DYS456, and DYS627.

Non-templated terminal nucleotide addition is an activity intrinsic to the type of DNA polymerase used in the Yfiler Plus Kit [18]. This addition, typically of adenosine, results in a PCR product that is one nucleotide longer than the predicted amplicon, often referred to as the "+A" form. In STR multiplexes, a post-amplification final extension step is commonly included to ensure that nucleotide addition goes to completion on all amplified products in order to maintain uniform electrophoretic sizing.

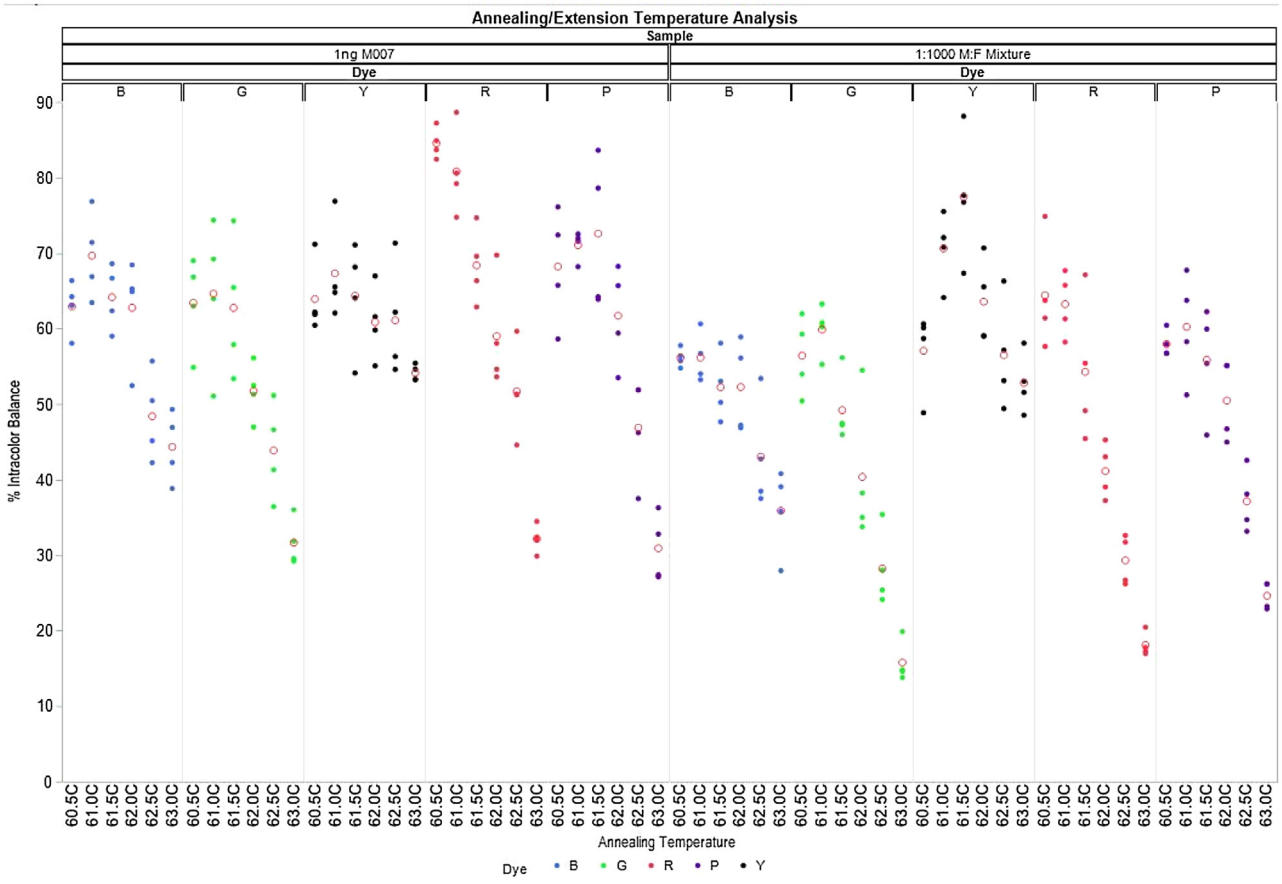

Fig. 1. Annealing/extension temperature analysis using 1 ng male Control DNA 007 and 1:1000 Male:Female Mixture. 1 ng of male Control DNA 007 by itself or in combination with 1 μg of female DNA 9947A, was amplified with the Yfiler Plus Kit for 30 cycles over the indicated range of annealing/extension temperatures. Reaction products were analyzed on an Applied Biosystems 3500xL Genetic Analyzer. Red circles indicate the mean.

Results from varying final extension times over a range of 7–22 min show that shortening the extension times to 7 min results in "split" and "shoulder" peaks due to incomplete +A addition and the loci most affected were DYS19, DYS390 and DYS438 (data not shown). Optimal final extension time was determined to be 22 min.

3.3. PCR reaction—master mix and primer mix

The Yfiler Plus Kit uses the same 6-dye system as the GlobalFiler™ Kit. The inclusion of a 6th dye enabled a design with 11 mini Y-STR loci (defined as those falling below 220 bp), and the rest of the loci to be less than 410 bp in size, thereby facilitating amplification of degraded DNA samples.

'Design of Experiments (DoE)' methodologies were used to optimize the Yfiler Plus Primer Set and Master Mix components in order to maximize robustness and performance. Key performance criteria used during development were maximal intracolor balance, maximal sensitivity, maximal inhibitor tolerance, minimal reaction time and most significantly minimal cross-reactivity in the presence of high female DNA background. The robustness of the Yfiler Plus Primer Set and Master Mix was verified by varying reagent input volumes over a range of +/− 20% (v/v), in 10% increments, for each of the individual primer sets (data not shown) and master mix components. Intracolor balance results for each dye channel at the various magnesium concentration's tested are presented as an example for the sample type 1:1000 Male:Female Mixture (Fig. 2), as well as blood and buccal samples on FTA™ paper (Supplementary Fig. 3) amplified in direct amplification mode. Full profiles were obtained under all conditions tested, but a significant drop in intracolor balance was observed with decreasing concentrations of magnesium, and was thus identified as the most sensitive component in the master mix. The magnesium concentration in the master mix was optimized to maximize performance while minimizing variability through DoE methodologies. The most significant response to magnesium was observed in the +20% (P20) samples and the loci most affected were DYS392, DYS438, DYS518 and DYS627. Results from the testing of the remaining master mix components showed no significant differences in performance with respect to intracolor balance, peak heights, and alleles recovered resulting from varying concentrations of each component (data not shown).

3.4. Sensitivity

The sensitivity of the Yfiler Plus Kit was tested with a range of DNA inputs, and benchmarked against the Yfiler Kit. Amplified products from all reactions were analyzed on the same 3500xL instrument to control for potential instrument-to-instrument variability in signal intensity. Results show that full profiles were obtained with both chemistries consistently for DNA inputs greater than or equal to 125 pg (data not shown). While allele drop-outs were observed at the stochastic lower DNA inputs, the Yfiler Plus Kit yielded a greater number of allele calls compared to the Yfiler Kit [24].

For a Y-STR kit, sensitivity in the presence of a high background of female DNA is a critical performance criterion. In order to test this, a range of Male:Female mixture ratios with varying amounts of male and female DNA were amplified with the Yfiler Plus Kit, and performance with respect to allele recovery was measured. Results show that even in the presence of high female DNA background of

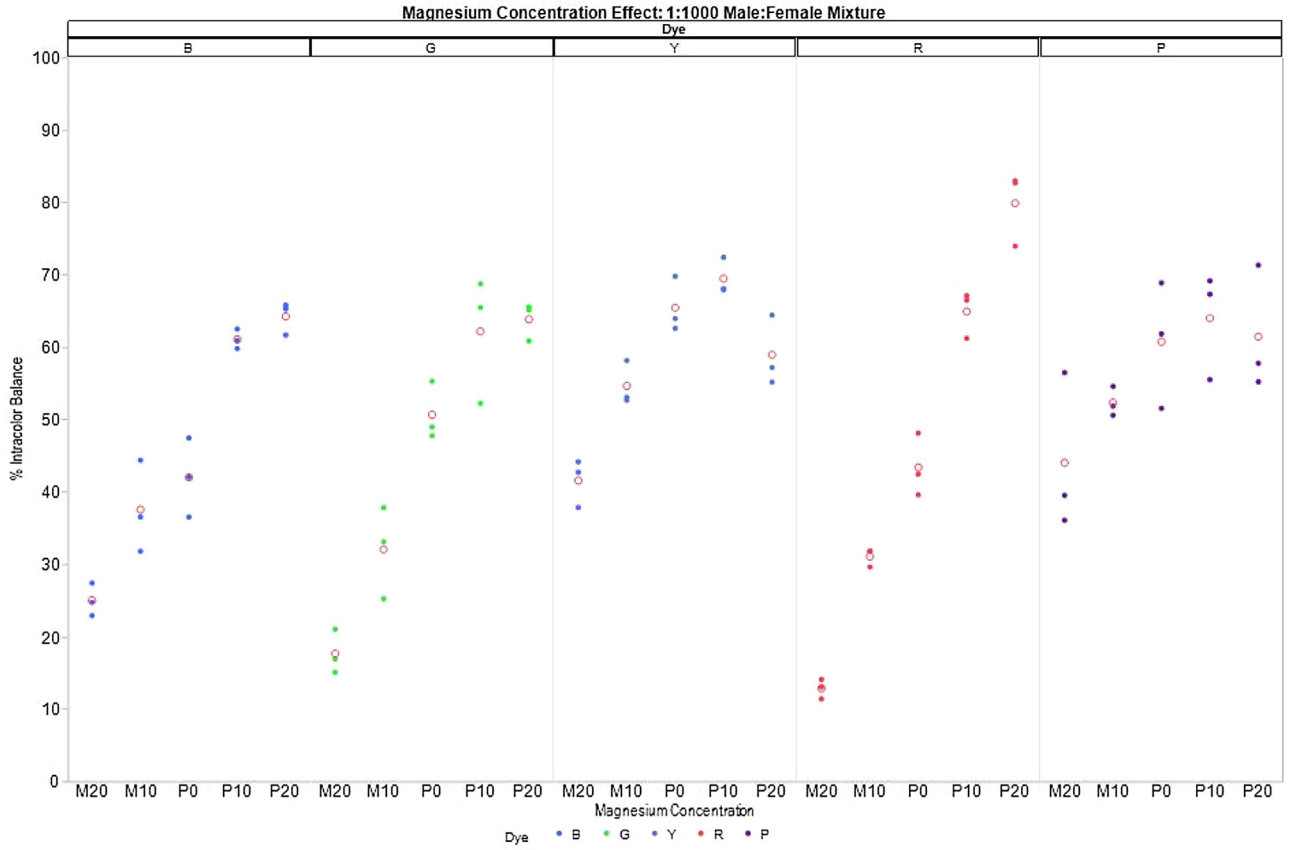

Fig. 2. Effect of magnesium concentration on amplification of 1:1000 Male:Female mixture containing 1 ng of male Control DNA 007 and 1 μg of female DNA 9947A. The figure shows the average intracolor balance ratios over all dye channels expressed as percentages from reactions (n = 3 replicates for each condition) in which magnesium concentration in the master mix was varied +/− 20%, in 10% increments from the standard optimal input (P0). M = minus and P = plus. Red circles indicate the mean.

up to 3 μg, full profiles were obtained with male DNA inputs down to 250 pg (Fig. 3A and B).

An important complement to sensitivity (signal) is the absence of substantial background (noise) in a given assay system. In addition to the sensitivity study above, 12 replicates non-template controls (NTCs) were run with the Yfiler Plus Kit. Overlay electropherograms from 4 replicates are presented (Supplementary Fig. 4). Results show that the Yfiler Plus kit generates a clean baseline with no artifacts in the read region (68–406 bp) above the peak amplitude threshold of 175 RFU. It is important to note that absolute peak height values and background levels will vary substantially, depending upon the instrument and experimental setting.

3.5. Male specificity

Casework samples typically processed with Y-STRs may contain high levels of female DNA background. In such situations, it is imperative that the Y-STR chemistry produce minimal female cross-reactive amplification products in the read region that can interfere with accurate profile interpretation. Small reproducible artifacts were observed in the read region in the presence of female DNA inputs of 1 μg or higher, but the chemistry was optimized to minimize these artifact peak heights to be well below the 175 RFU peak amplitude threshold. However, one reproducible artifact observed in the TAZ dye channel at 412 bp consistently crosses the 175 RFU peak amplitude threshold, but this does not affect profile interpretation as it lies outside the read region (68–406 bp).

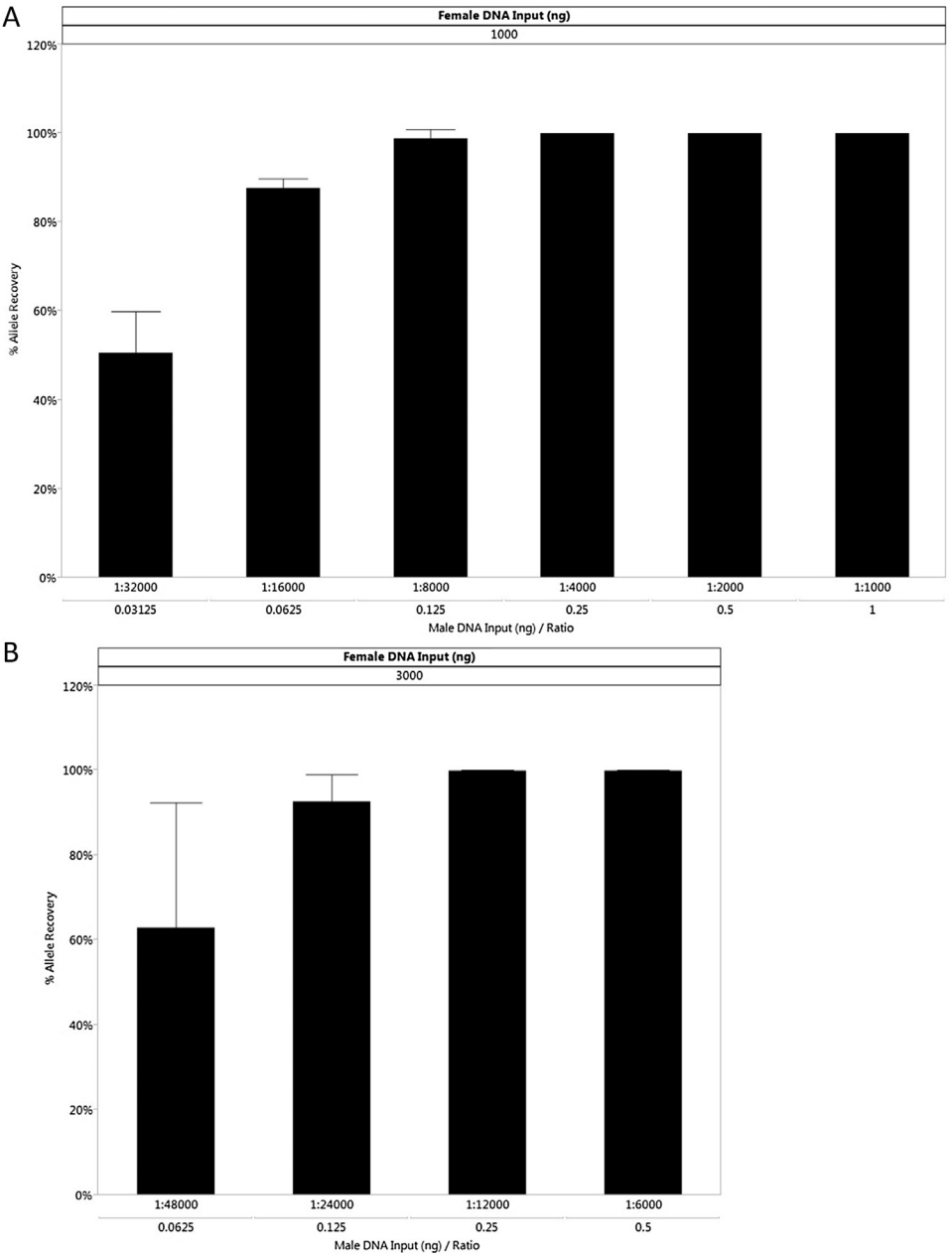

Fig. 3. Sensitivity study using Male:Female Mixture. Male:Female mixtures at varying ratios and DNA inputs (N = 3 each condition) were amplified with the Yfiler Plus Kit. Allele recovery expressed as a percentage is presented. Results are shown for 1000 ng (A) and 3000 ng (B) of female DNA input. Each error bar represents the standard deviation from the mean.

Supplementary Table 2 lists all of the reproducible female cross-reactive artifacts observed during development.

3.6. DNA mixture studies

Mixtures of male DNA are frequently encountered in forensic casework samples. For a Y-STR multiplex assay, it is very important to be able to distinguish the minor and major male contributors in the presence of a high female DNA background. In order to model this scenario, DNA isolated from two male donors, selected to have minimal allele overlap at all loci, was mixed at varying ratios in the presence of a high female DNA background to generate mixture samples containing 1 ng combined male DNA input plus 200 ng of female DNA input. These mixtures were then tested for allele recovery performance with the Yfiler Plus Kit and benchmarked against the Yfiler Kit. Results show that as the input amount of DNA from the minor contributor decreased its peak heights decrease accordingly. All unique minor profiles were obtained for the minor contributor at non-overlapping and non-stutter positions from a 1:1 to a 1:8 ratio (125 pg minor contributor DNA input) (Fig. 4). At a 1:15 ratio an average loss of 1 allele in 12 was observed for the Yfiler Plus Kit (>90% of the unique minor profile recovered).

3.7. Species specificity

10 ng input DNA of several non-primate species namely, bovine, chicken, dog, horse, mouse, pig, rabbit, rat, and sheep was amplified with the Yfiler Plus Kit. Results show that no cross-reactive peaks were observed above the peak amplitude threshold of 175 RFU with all the species tested (Supplementary Fig. 5).

3.8. Models of inhibition

Extracted DNA from forensic casework samples often contains substances known to inhibit PCR reactions. Most common inhibitors encountered are carry-through of small amounts of the upstream extraction chemistries (such as phenol/chloroform), or from elements intrinsic to the sample itself (such as hematin from blood and humic acid from soil) [19–21]. The presence of such inhibitors at high concentrations result in inefficient PCR amplification and failure to produce full profiles, thereby reducing the amount of probative information returned. Mock inhibition samples with 1 ng of male Control DNA 007 containing varying concentrations of hematin and humic acid were prepared to test inhibitor tolerance of the Yfiler Plus Kit chemistry. Full profiles were obtained at hematin concentrations up to 400 μM and humic acid concentrations up to 100 ng/μL (Fig. 5A and B). Allele drop-outs were observed at humic acid concentrations of 250 ng/μL. In contrast, performance benchmarking against the Yfiler Kit shows significantly improved inhibitor tolerance of the Yfiler Plus Kit as the Yfiler Kit fails to return any alleles beyond hematin levels of 20 μM and humic acid levels of 10 ng/μL.

3.9. Population studies and genotype concordance

A population study was conducted with the Yfiler Plus Kit using 942 male samples, spanning 4 ethnicities, namely, African American, Caucasian, Hispanic, and Asian. The Yfiler kit was used to confirm genotypes. As shown in Table 1, a 100% concordance was achieved between genotypes produced using Yfiler Plus and the Yfiler ones with the exception of a well characterized case of discordance in Hispanic samples [22,26] owing to a null allele at the DYS448 locus. The DYS448 locus in these samples has an internal 84 bp deletion that results in a shifted allele that falls in an adjacent smaller locus. Depending on the Y-STR kit used, the shifted allele falls in different loci: In the Yfiler kit the shifted allele falls into the DYS437 locus, in the Yfiler Plus kit the shifted allele falls into the DYS19 locus and in the PowerPlex Y23 kit the shifted allele falls into the DYS576 locus (Fig. 6). The population data was also used to calculate haplotype diversity and discrimination capacity values. The Yfiler Plus Kit showed improved haplotype diversity and discrimination capacity compared to the Yfiler Kit within each individual ethnic group (Table 2). The number of unique haplotypes observed for each ethnic group was higher with Yfiler Plus compared to Yfiler.

Two cases of discordance (out of 30 buccal samples on FTA paper tested) were observed at the DYS481 locus using the PowerPlex Y23 kit (Fig. 7). The self-declared ethnicities of the two discordant individuals were Chinese and Indian. Sequence analysis of both discordant alleles found a G/A SNP sixteen nucleotides outside the repeat region compared to the reference sample, but no evidence of a deletion/insertion microvariant was found

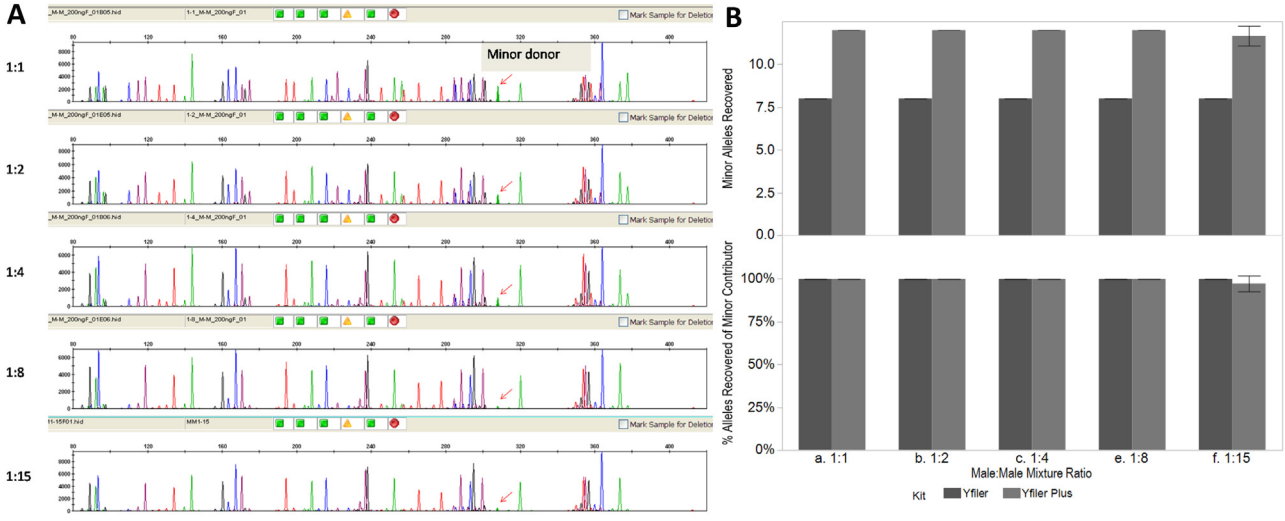

Fig. 4. Male:Male Mixture analysis. Male:Male mixture samples (total of 1 ng input DNA) from two different donors known to have minimal genotype overlap were prepared in the presence of 200 ng female DNA and amplified using the Yfiler Plus kit for 30 cycles. Representative profiles for DNA mixtures (different ratios) are presented in Panel A with the red arrow pointing to a minor contributor allele. The percentages of allele recovery for the minor contributor (alleles located in non-overlapping and non-stutter positions) are shown in Panel B. Each error bar represents the standard deviation from the mean.

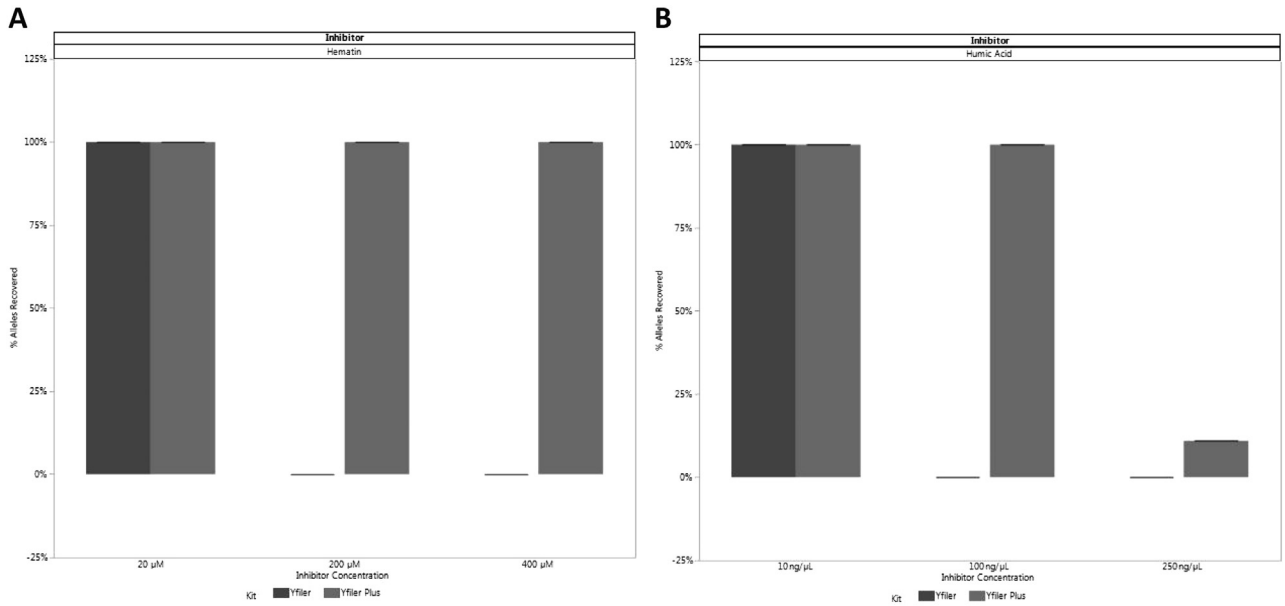

Fig. 5. Models of Inhibition. Using the standard protocol, 1 ng of male Control DNA 007 was amplified with the Yfiler Plus and Yfiler Kits in the presence or absence of inhibitory substances (hematin, humic acid), over a range of concentrations (n = 3 replicates). Results for allele recovery expressed as a percentage are presented for hematin in Panel A and humic acid in Panel B.

Table 1
Concordance study with the Yfiler Plus Kit. Concordance study was performed comparing genotypes obtained with the Yfiler Plus and Yfiler Kits for four population groups.

Population	N	Yfiler Plus Concordance
Caucasian	237	100%
African American	257	100%
Hispanic	188	100%[a]
Asian	260	100%
Total	942	

[a] Full concordance was observed between Yfiler Plus and Yfiler except in 2 samples with a well characterized deletion in DYS448.

(Supplementary Fig. 6). This G to A base change could be affecting a secondary structure leading to a mobility shift. A shift of 0.78 nucleotides from the center of the bin was observed on the 3130xl instrument which could be misinterpreted as a 0.1 microvariant. The Yfiler Plus Kit did not identify any microvariants in the DYS481 locus with the 942 population samples tested on the 3500xL platform.

In studies performed with father-son pairs, Yfiler Plus was able to separate 19% (10/53) of the pairs due to a mutation event compared to 8% (4/53) for Yfiler as a result of the inclusion of the RM-YSTRs in the Yfiler Plus kit. Fig. 8 shows an example of a father-son pair where, a discordance observed at the DYS449 locus resulted in different haplotypes for the relative pair.

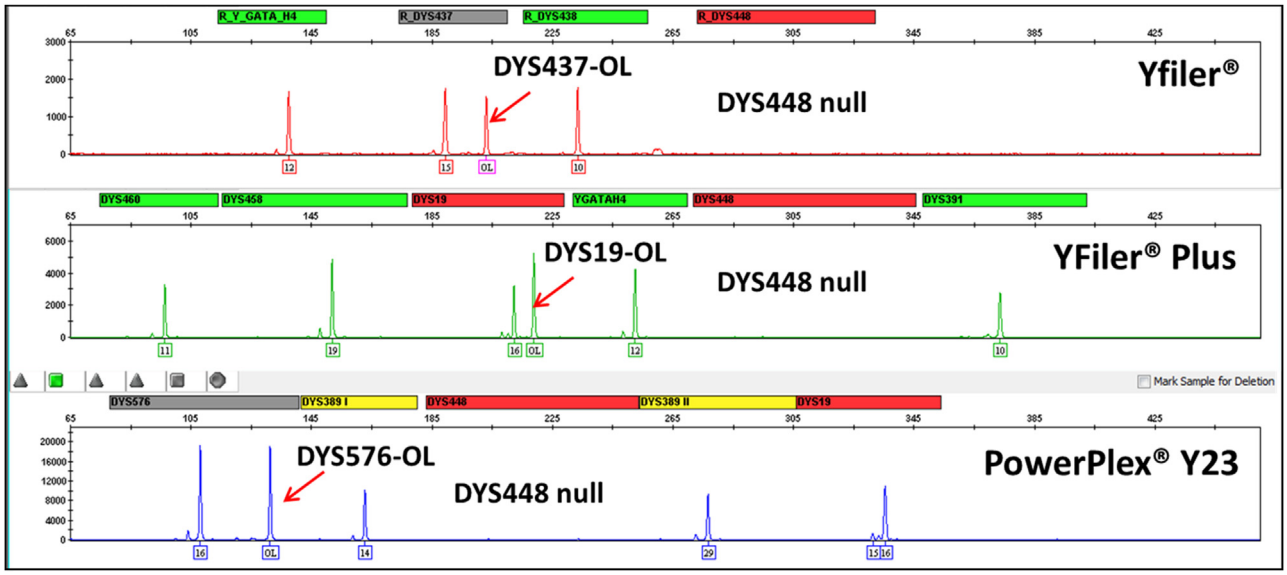

Fig. 6. Electropherogram of profiles showing the null DYS448 locus. The same Hispanic sample was amplified with the Yfiler, Yfiler Plus and the PowerPlex Y23 kits resulting in three discordant haplotypes at the DYS437, DYS19 and the DYS576 loci.

Table 2
Yfiler Plus Haplotype Diversity and Discrimination Capacity. The genotypes of 942 individuals, with approximately equal representation among African American (257), Caucasian (237), Hispanic (188), and Asian (260) donors, were determined using the Yfiler Plus Kit, and haplotype diversity and discrimination capacity values were calculated and compared to the Yfiler Kit.

	Yfiler® Plus (27 loci)				Yfiler® (17 loci)			
Ethnicity	African American	Caucasian	Hispanic	Asian	African American	Caucasian	Hispanic	Asian
Number of Males	257	237	188	260	257	237	188	260
Haplotype Diversity	0.99611	0.99578	0.99446	0.99553	0.99605	0.99567	0.99429	0.99536
Discrimination Capacity	100.00%	100.00%	97.87%	94.23%	99.22%	98.73%	96.28%	91.92%
# times haplotype observed								
1	257	237	180	233	253	231	174	221
2	0	0	4	11	2	3	7	17
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	1	0	0	0	1

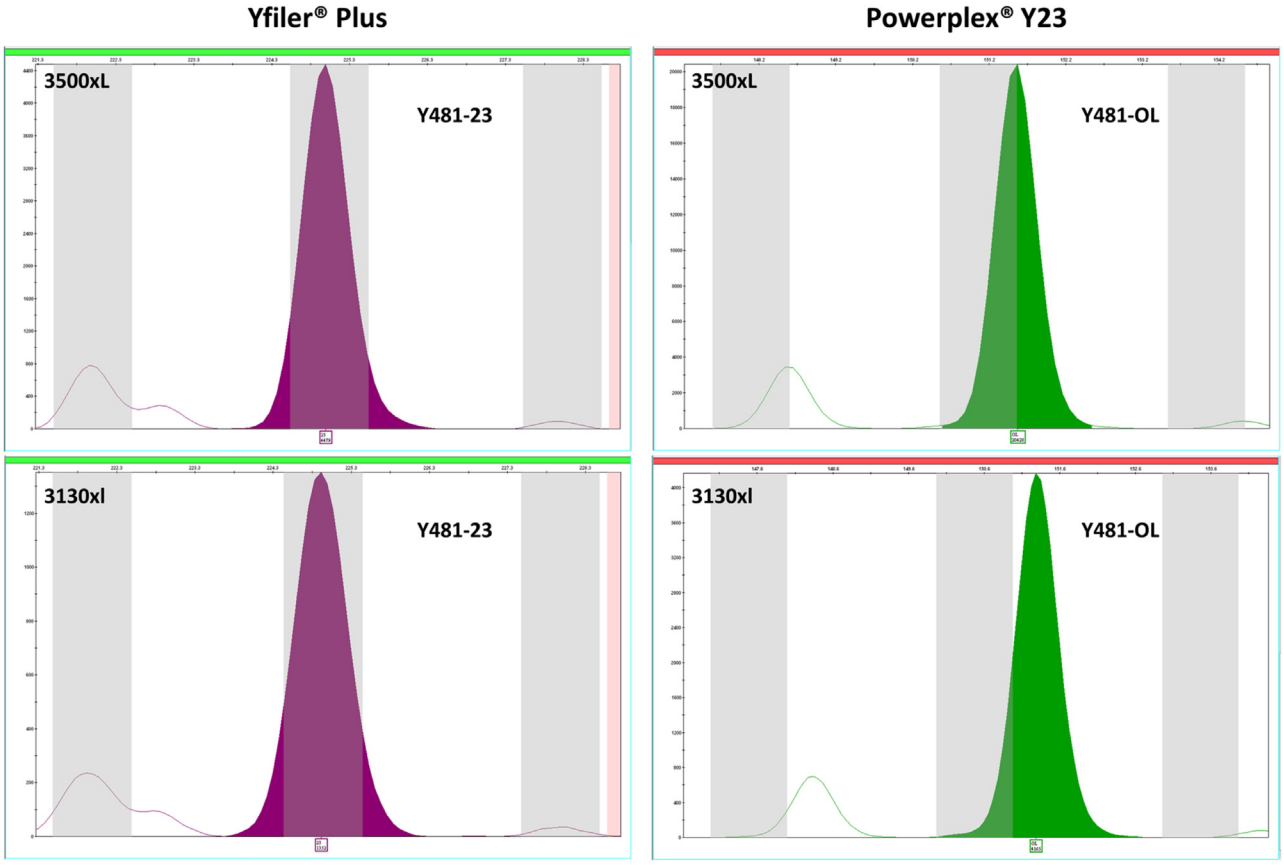

Fig. 7. Electropherogram showing the mobility shift in the DYS481 locus when amplified using the PowerPlex Y23 Kit. A genotype comparison was made with the Yfiler Plus Kit and the PowerPlex Y23 Kit.

3.10. Performance testing

Performance of the Yfiler Plus Kit with purified extracted DNA, Male:Female DNA mixture, and samples on storage cards was measured with respect to intracolor balance and average peak heights with a larger sample set. Performance between the Veriti® Thermal Cycler and the GeneAmp® PCR System 9700 cycler was compared with purified extracted DNA samples. The results show that with purified DNA samples, the peak heights within each dye channel are very well balanced for the Yfiler Plus Kit, with average intracolor balance (expressed as a percentage) values ranging from 62% (VIC®) to 70% (TAZ™) with the Veriti® Thermal Cycler (Supplementary Fig. 7). Results also show that the performance between the Veriti® Thermal Cycler and the GeneAmp® PCR System 9700 was comparable with respect to average peak heights and intracolor balance. With Male:Female mixture samples, the performance of the Yfiler Plus Kit was benchmarked against that of the Yfiler Kit. Results show that the Yfiler® Plus Kit shows significantly higher intracolor balance (expressed as a percentage) compared to the Yfiler Kit (Fig. 9). The performance of the Yfiler Plus Kit was benchmarked against that of the Yfiler Direct Kit with blood and buccal samples on FTA cards. Results show that for both

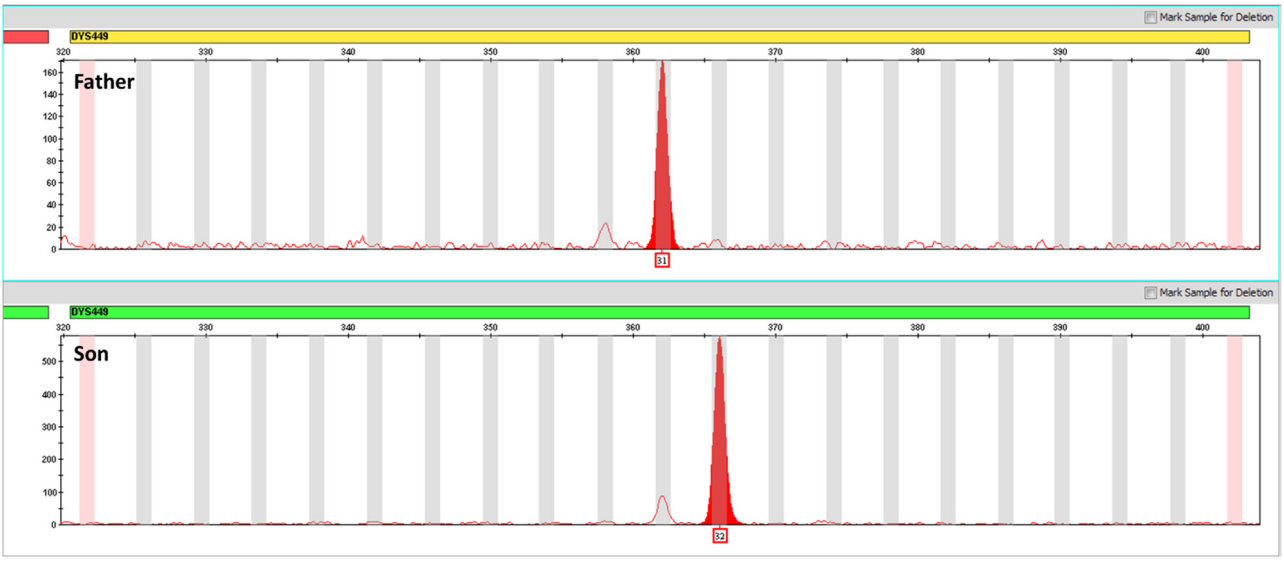

Fig. 8. Electropherogram showing a mutation at a rapidly mutating Y-STR (DYS449) between a father-son pair. Reaction products were analyzed on an Applied Biosystems 3500xL Genetic Analyzer.

sample types, the Yfiler Plus Kit generates comparable peak heights and intracolor balance (expressed as a percentage) to the Yfiler Direct Kit (Fig. 10).

3.11. Accuracy, precision and stutter

The sizing accuracy and precision for the Yfiler Plus Kit was determined empirically by comparing size differences between 42 randomly selected population samples and the alleles generated by the Yfiler Plus Kit Allelic Ladder on all supported CE instruments (3130*xl*, 3500 and 3500xL). All samples were electrophoresed as per the standard CE conditions described in the Yfiler Plus Kit user guide [17]. All sample alleles were within ±0.5 nt of a corresponding allele in the Allelic Ladder, irrespective of the CE platform used (Supplementary Fig. 8). Sizing precision was assessed by calculating the standard deviation in the size values obtained for Yfiler Plus Kit Allelic Ladder alleles over multiple injections on supported CE instruments (3130*xl*, 3500 and 3500xL). The targeted standard deviation less than 0.15 nt was met for all alleles on all CE platforms (data not shown). These results demonstrate accuracy and precision levels sufficient for the discrimination and sizing of microvariants and off-ladder peaks.

Stutters are minor by-products of the PCR amplification due to slippage at the polymerase/strand interface [23,25]. Typically they are one repeat unit (n-1) smaller than the target allele (n). Depending on the repeat sequence of the given STR locus, other less common forms of stutter, such as a plus-stutter which is one repeat unit (n + 1) larger than the target allele (n), may form. These stutter peaks can confound profile interpretation and are therefore accounted for in the analysis methods. This is done by characterizing each of the commonly observed stutters at each locus in the multiplex as a percentage value relative to the true adjacent allele peak. 942 extracted samples were evaluated for stutter and the mean stutter percentage values plus three standard deviations were used for the stutter filter in the Yfiler Plus Stutter file for GeneMapper ID-X v1.4 (Supplementary Table 3). The trinucleotide repeat DYS481 locus has the highest minus-stutter (n − 1 repeat unit) filter value, and some loci have stutter filters for plus-stutters (n + 1 repeat unit) as well as n-2 and n + 2 nt stutters.

4. Conclusion

The Yfiler Plus Kit when compared to the Yfiler Kit offers higher power of discrimination and enhanced chemistry for improved performance with challenging samples, such as sexual assault mixtures. The new 6-dye chemistry format enhances our multiplexing capability by enabling the addition of additional informative loci. The Yfiler Plus Kit contains seven new rapidly mutating Y-STRs (DYS576, DYS627, DYS518, DYS570, DYS449, and DYF387S1a/b) that increase haplotype resolution and the potential to distinguish male relatives.

The most noteworthy characteristic of the Yfiler Plus Kit is its performance with mixture samples containing a high level of female DNA. The Yfiler Plus Kit provides well balanced profiles of male DNA in the presence of up to 1 μg female DNA and a clean background with minimal female cross-reactive artifact peaks in the read region (68–406 bp). None of the female artifacts documented during development crossed the peak amplitude threshold of 175 RFU when multiple female donors were tested at 1 μg inputs. The Yfiler Plus kit also boasts improved sensitivity even with mixture samples containing high levels of female DNA with full profiles obtained in the majority of times with male DNA inputs down to 125 pg. In studies performed with Male:Male mixtures, all the minor contributor alleles in non-overlapping and non-stutter positions could be distinguished at minor DNA inputs down to 125 pg. Allele drop-outs are observed at DNA inputs of 63 pg or lower, but owing to an expanded locus set, more alleles are recovered compared to the Yfiler Kit [24]. Despite high sensitivity, a clean baseline has been demonstrated.

In addition to the above mentioned improvements, the Yfiler Plus Kit demonstrated improved inhibitor tolerance, faster PCR cycle time of <95 min, and ability to amplify samples on storage cards in direct amplification mode. This current work demonstrates good concordance, allele sizing accuracy and precision for the Yfiler Plus Kit, as well as good intracolor balances for all dye channels used for sample detection. All of these results have been reproduced by various external labs involved in beta-testing of the Yfiler Plus Kit.

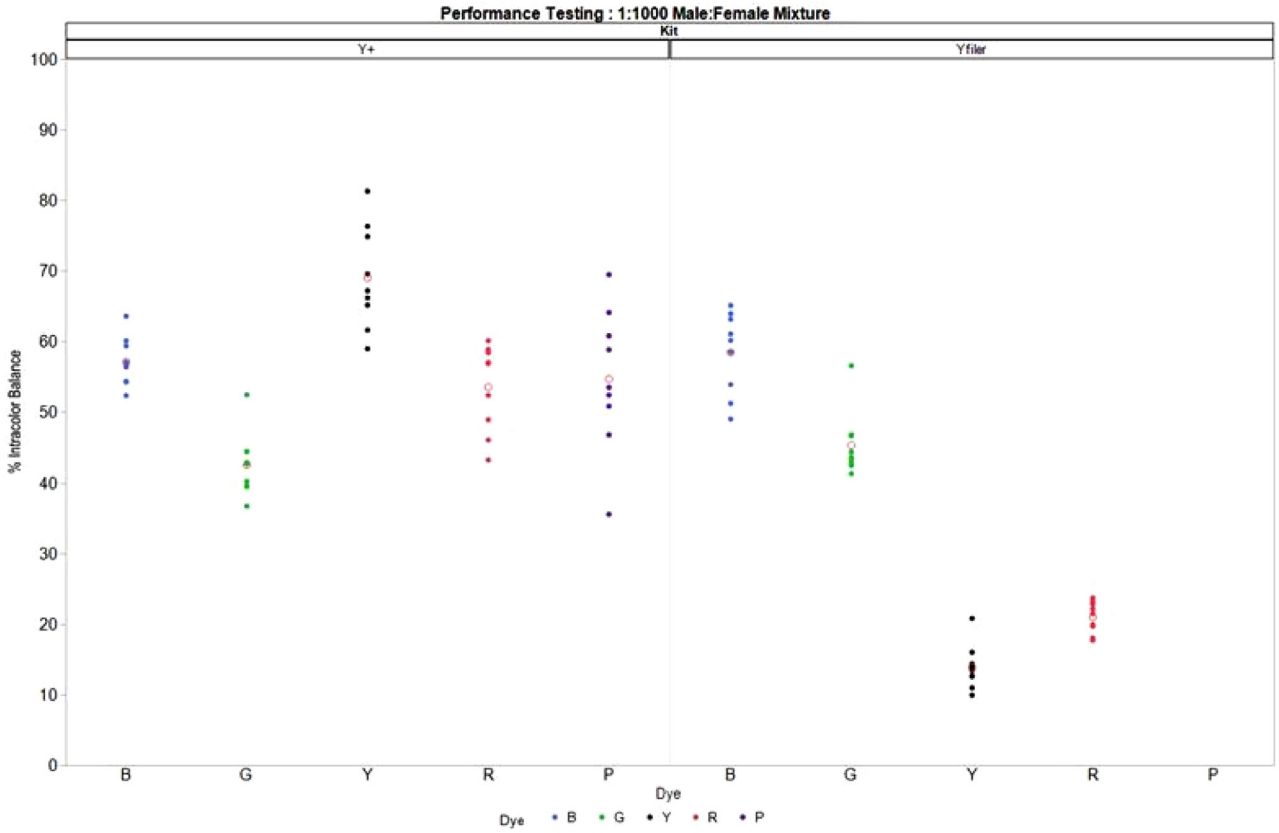

Fig. 9. Performance comparison of Male:Female mixture samples between Yfiler Plus and Yfiler Kits. Samples containing 1 ng of male DNA and 1 μg of female DNA from three donors each were amplified with the Yfiler Plus Kit and benchmarked against the Yfiler Kit. The figure shows the average intracolor balance ratios over all dye channels expressed as percentages from reactions (n = 3 male and female donors, n = 3 replicates for each donor). Red circles indicate the mean.

A case of discordance was observed in 2 out of 30 samples (buccal sample on FTA™ paper) amplified with the PowerPlex Y23 kit at the DYS481 locus due to a mobility shift relative to the kit's allelic ladder. The only change in the nucleotide sequence of both samples was a G/A SNP located 16 nucleotides away from the repeat region (Fig. 9). This mobility shift with the PowerPlex Y23 could result in misinterpretation of the DYS481 locus genotype as a 0.1 microvariant. The DYS481 mobility shift, which could be due to a change in secondary structure, has not been observed with the Yfiler Plus kit. It is worth noting that the amplicon size of the DYS481 locus in the Yfiler Plus kit is almost 50% longer than the PowerPlex Y23 kit amplicon, perhaps making it less sensitive to

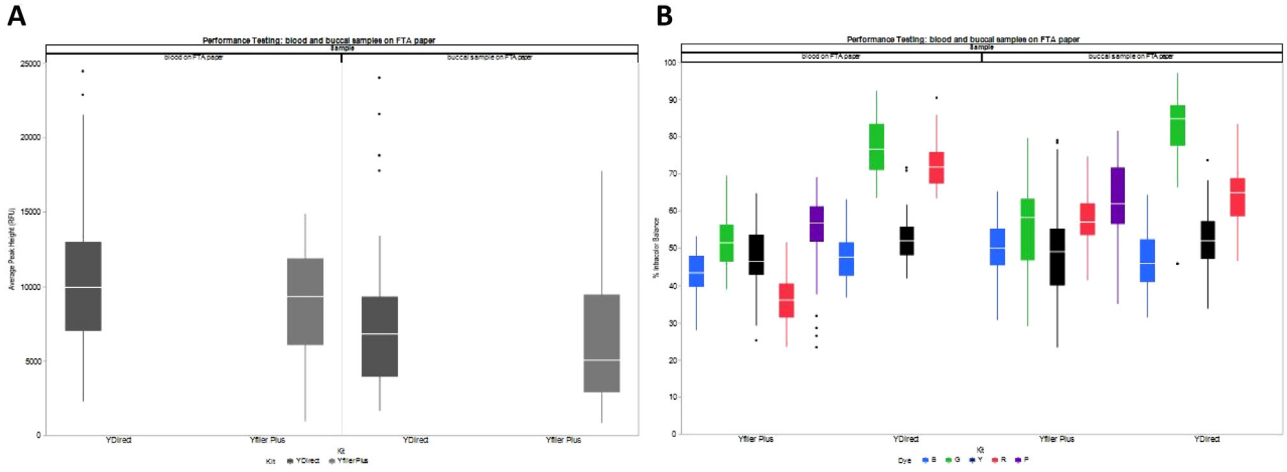

Fig. 10. Performance comparison on 55 blood and buccal samples on FTA™ paper. Blood on FTA™ paper was amplified for 28 cycle using the Yfiler Plus Kit and for 26 cycles using the Yfiler Direct Kit. Buccal samples on FTA™ paper were amplified for 28 cycles using the Yfiler Plus Kit and for 27 cycles using the Yfiler Direct Kit. Each box represents the 25th through the 75th percentile of the observed values, with the center line indicating the median. Whiskers indicate the range of the data, with presumed outliers (points that are different from the mean by more than twice the pooled standard deviation) shown as black dots. Electrophoresis for all samples was conducted on the same 3500xL Genetic Analyzer.

secondary structure differences. Mobility of the fragment may also be affected by the strand of DNA that is labeled in the PowerPlex Y23 kit for the DYS481 locus compared to that in the Yfiler Plus kit [27].

The experiments performed in this developmental validation follow the SWGDAM guidelines and the results demonstrate the robustness and validity of the Yfiler Plus Kit for use on forensic casework and database samples. It is recommended that each lab conduct their own internal validation studies in accordance with SWGDAM guidelines [13,14].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fsigen.2016.07.006.

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