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TECHNICAL NOTE

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Validation of *SRY* Marker for Forensic Casework Analysis

ABSTRACT: Determining the gender of the source of forensic DNA evidence is based on the amelogenin test. However, at times the assay may not be indicative of gender assignment, because of deletions at the amelogenin site. Previously, we described successful coamplification of a marker residing within the *SRY* gene with the short tandem repeat markers from two commercially available human identification kits. The study herein addresses the validation of primers for the target *SRY* gene regarding specificity, sensitivity, and robustness. Among 115 unrelated male Slovenians no null allele was observed. Repeatable and reliable results were obtained from as little as 25 pg of template DNA, indicating a high sensitivity of detection for the assay. No polymerase chain reaction product was observed even at a concentration of 10 ng/μL of template female DNA. Additionally, the male specific marker could be detected in mixed male and female samples down to a ratio of 1:16.

KEYWORDS: forensic science, DNA typing, validation, sex determination, amelogenin gene, *SRY* gene, mutation

Determining the gender of the source of a forensic DNA sample at times can be informative in various forensic investigations, especially in sexual assault cases. Sex determination is routinely performed by amplification by the polymerase chain reaction (PCR) of a region of the amelogenin. The assay typically generates a 106 bp long fragment from the X chromosome and a 112 bp long fragment from the Y chromosome (1). In the forensic field, the amelogenin (*AMEL*) gender test is carried out as part of a multiplex assay using commercially available identification kits, such as AmpFlSTR® SGM™ Plus kit (Applied Biosystems, Foster City, CA) and PowerPlex® 16 System (Promega Corp., Madison, WI).

However, several studies have shown that the amelogenin gender test may not always be concordant with true male gender in forensic casework or in prenatal diagnosis (2–5). This discrepancy is because of the structural variability within the Y chromosome (6–9). Deletions of *AMELY* can result in no amplification product and these null *AMELY* alleles can occur up to about 8.0% in some population groups (3,5,7,8,10–17). In the Slovenian male population *AMELY* null alleles are infrequent occurring in one out of 8300 male individuals (17). Using the YFiler™ kit (Applied Biosystems), this *AMELY* null male also was null at the DYS458 locus. Thus, the data support that the null allele is likely the result of a larger deletion on the short arm of the Y chromosome.

To reduce the potential interpretation difficulties in the few cases where gender misinterpretation may be problematic, some authors have investigated using genetic markers lying in the sex-determination region Y (*SRY*) on the Y chromosome (6,7,9). In these studies, the *SRY* assay was performed as an additional singleplex PCR or in combination with primers for *AMEL*. However, this approach requires an additional assay subsequent to *AMEL* and short tandem repeat (STR) typing, thus consuming more evidentiary material, as well as being laborious and time consuming.

Drobnič (17) reported successful amplification of a novel marker residing in the *SRY* gene which results in a 96 bp long PCR product and be incorporated into either the AmpFlSTR® SGM™ Plus (Applied Biosystems) or PowerPlex® 16 System (Promega Corp.) identification kits. Thus, it is feasible to incorporate the *SRY* gene assay into any routine *AMEL* and STR analysis. Moreover, the small size of the *SRY* amplicon provides two benefits for forensic DNA testing. First, male gender determination can be successful when typing degraded forensic samples, at least as successful as that for *AMEL*. Second, because of its short length, the *SRY* amplification product does not migrate with any of the *AMEL* or STR alleles in the multiplex STR kits.

The present study was undertaken to perform some validation studies on the *SRY* marker for use in forensic cases. Validation of the *SRY* marker was performed in accordance with the recommendations of the SWGDAM revised validation guidelines (18). The validation studies included repeatability, sensitivity, gender specificity, and mixture studies.

Materials and Methods

Quantification and PCR Amplification

Quantification of DNA was conducted using the Quantifiler™ Human DNA Quantification Kit (Applied Biosystems) with 2.0 μL of DNA extract on the ABI Prism 7000 Sequence Detection System (Applied Biosystems).

Amplification of DNA was performed using the *SRY* primers under AmpFlSTR® SGM™ Plus (Applied Biosystems) manufacturer's recommendations as reported previously (17). A singleplex DNA amplification was carried out in a total volume of 25 μL containing 5 U/μL AmpliTaq Gold® Polymerase, 0.2 μM forward *SRY* primer, 0.24 μM reversed *SRY* primer and 10.0 μL 10× Gold® STR buffer (Applied Biosystems) in a Perkin-Elmer 9600 thermal cycler (Applied Biosystems). A singleplex DNA amplification was

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used in all studies except for the mixture study. Mixture studies were carried out as multiplex amplification. The same amount of *SRY* primers as used in a singleplex reaction was coamplified with the AmpFlSTR® SGM™ reaction mixture in a total volume of 25 μL following the procedures described in the technical manual. Ten microliters of appropriately diluted DNA were added to each tube so that the final template input range was 0.1–1.0 ng.

DNA Typing

Amplified product was combined with the Genscan-500 ROX internal line standard and loaded on an ABI Prism® 310 Genetic Analyzer as described by the manufacturer (Applied Biosystems). Samples were injected for 5 sec at 15 kV and electrophoresis was conducted at 15 kV and 60°C with Performance Optimized Polymer 4 (POP™ 4, Applied Biosystems). Data from samples amplified using AmpFlSTR® SGM™ Plus PCR Amplification kit were collected using ABI Prism Collection software version 3.7 with virtual filter set F. Results were analyzed using GeneScan® 3.7 analysis software. Using Genotyper® version 3.7 analysis software (Applied Biosystems), STR allele designations were made based on comparison with the allelic ladder. The *SRY* allele calls were made manually using the amplicon length determined using GeneScan® 3.7 analysis software.

Repeatability

DNA samples were obtained from buccal swabs taken from 115 unrelated male individuals from our casework. The samples were extracted using the chelex extraction method (19). The samples were prepared by serial dilution from samples of known concentration. DNA was added in each PCR at a concentration range of 0.5 to 1.0 ng/μL and amplified under AmpFlSTR® SGM™ Plus manufacturer's recommendations. The samples were analyzed three times by the same operator using the same ABI Prism® 310 Genetic Analyzer (Applied Biosystems).

Sensitivity Studies

Varying amounts of male control DNA 007 (Applied Biosystems) (ranging from 0.025 to 1.0 ng/μL) and of male DNA casework samples (ranging from 0.0625 to 1.0 ng/μL) were amplified to determine the minimum amount of input DNA that could be used to obtain a *SRY* profile. Each quantity of control and DNA casework sample were tested three times.

Gender-Specificity Studies

A male control cell line DNA (9948), female cell line control DNA (9947A) (Promega Corp.) and one male and one female DNA sample from our casework were amplified at a concentration of 1.0 ng/μL, respectively.

Singleplex reactions using our designed primer set for the *SRY* locus were performed with female control DNA 9947A (Promega Corp.) at concentrations of 1, 5, and 10 ng/μL.

Mixture Studies

Male–female mixture studies were performed on five different sets of male and female DNAs from our casework at ratios of 1:1, 1:2, 1:4, 1:8, and 1:16. Each mixture was tested twice. The amount of female casework DNA was held constant at 1.5 ng, while the amount of male casework DNA varied from 1500 down to 93.7 pg.

Results and Discussion

A validation study was carried out to define some limitations of *SRY* typing of forensic specimens using the *SRY* marker developed by Drobnič (17). The validation studies included repeatability, sensitivity, gender specificity, and mixture analyses.

Repeatability

The amplification and typing of the *SRY* marker was successful for all 115 male samples and there were no discrepancies with gender assignment. The amplification was carried out in a singleplex reaction. All 115 male samples were tested at three different capillary electrophoresis conditions but with the same ABI Prism® 310 Genetic Analyzer (Applied Biosystems) and yielded sizes of 94.44 ± 0.07 bp, 94.47 ± 0.18 bp, and 94.36 ± 0.06 bp. These lengths in bp are slightly lower than the known 96 bp size of the amplicon. Such differences are well known when using capillary electrophoresis (20). However, the results demonstrate the more important factor for genetic typing that the precision is exceedingly high. The new primer set for the *SRY* marker enables precise and repeatable results for male gender determination as a single system. Because no null allele was observed there is strong support that the primer binding sites are conservative and that the *SRY* marker is a good candidate for a reliable male gender test for forensic purposes. However, with a sample of 115 males a null allele can occur at a frequency as high as 3% (at 95% confidence level) and not be detected. Further testing should be done on a larger number of male samples to obtain a better assessment of potential primer binding site variants with the *SRY* marker region.

Sensitivity Studies

Amplification and correct typing was achieved at all concentrations ranging from 0.125 to 1.0 ng/μL of the male control DNA 007 (Applied Biosystems) and the male DNA casework sample. The amplification was carried out in a singleplex reaction. While the optimal quantity of template DNA was 1.0 ng, conclusive typing of the *SRY* marker was effective over a wide range of input template DNA, making the test applicable for challenged forensic DNA samples. The lower limit of template male control DNA 007 (Applied Biosystems) that yielded *SRY* PCR product with a peak height over 260 relative fluorescent units (RFU) was 50 pg, and as little as 25 pg of template DNA were sufficient to generate a *SRY* peak with a height of 80 RFUs (Fig. 1). The data were consistent over most replicates and support that the assay for the *SRY* marker is very sensitive for pure, nondegradated DNA. However, at 62.5 pg of casework male DNA some drop-out was observed (Fig. 2). Therefore, reliable male gender determination for routine forensic samples could be observed at a DNA quantity as low as 125 pg.

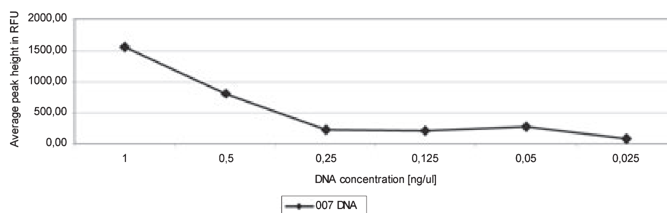

FIG. 1—*Sensitivity studies using consecutive dilutions of genomic of male control DNA 007 (Applied Biosystems) from 1000 pg down to 25 pg, analyzed on the ABI Prism® 310 Genetic Analyzer.*

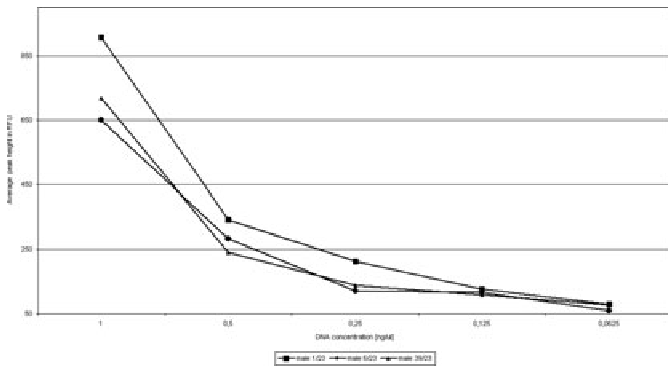

FIG. 2—*Sensitivity studies using consecutive dilutions of three unrelated DNA casework samples from 1000 pg down to 62.5 pg, analyzed on the ABI Prism® 310 Genetic Analyzer. The figure shows the amplification of three different male DNA casework samples.*

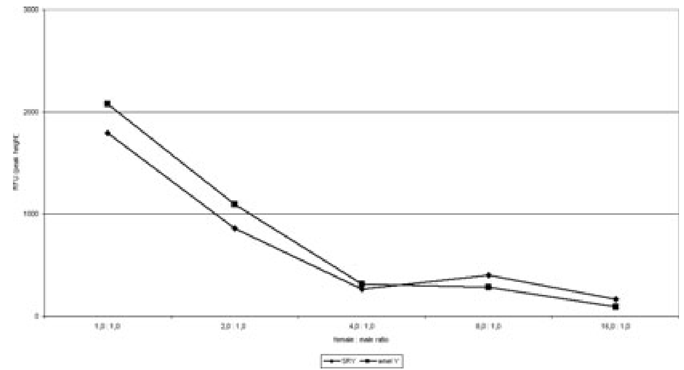

FIG. 4—*Mixture studies—comparison of peak heights of* SRY *gene profile and* AMELY *gene profile in admixed samples with constant female DNA concentration and decreasing concentration of male DNA.*

Gender-Specificity Studies

Two female DNA samples were tested for cross-reactivity with the *SRY* marker assay in a singleplex amplification. The assay failed to produce detectable *SRY* product from female control DNA even at concentrations of 5 and 10 ng/μL. The results of the failure of amplification of *SRY* product from female control DNA 9947A (Promega Corp.) and a female DNA casework sample at the concentration 1.0 ng/μL are shown in Fig. 3. The data support that the new set of *SRY* primers is highly specific for the *SRY* gene on the Y chromosome.

Mixture Studies

The mixture study was carried out in a multiplex reaction. *SRY* primers were coamplified with STR primers from the AmpFlSTR® SGM™ kit. The presence of a high background of female DNA in a sample had no impact on amplification of *SRY* marker down to the tested ratio of 1:16 (93.7 pg male DNA:1.5 ng female DNA). The decrease in peak height from approximately 1800 RFU to approximately 160 RFU of the *SRY* component is concomitant with a reduction of male DNA concentration in the mixed samples (Fig. 4). These results are consistent with those observed for the

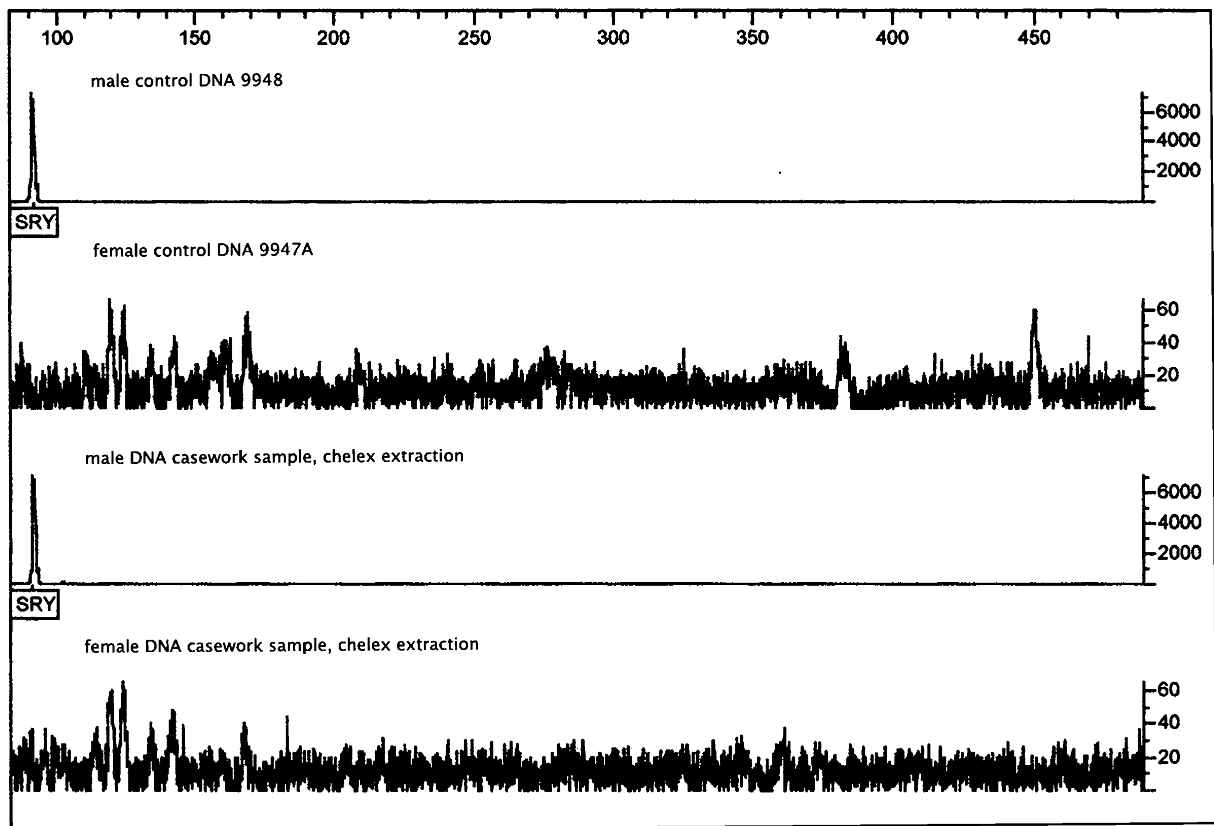

FIG. 3—*Electropherograms of male control DNA 9948—1 ng/μL, of female control DNA 9947A—1 ng/μL (Promega Corp.) and of male—1 ng/μL and female—1 ng/μL DNA sample from our casework.*

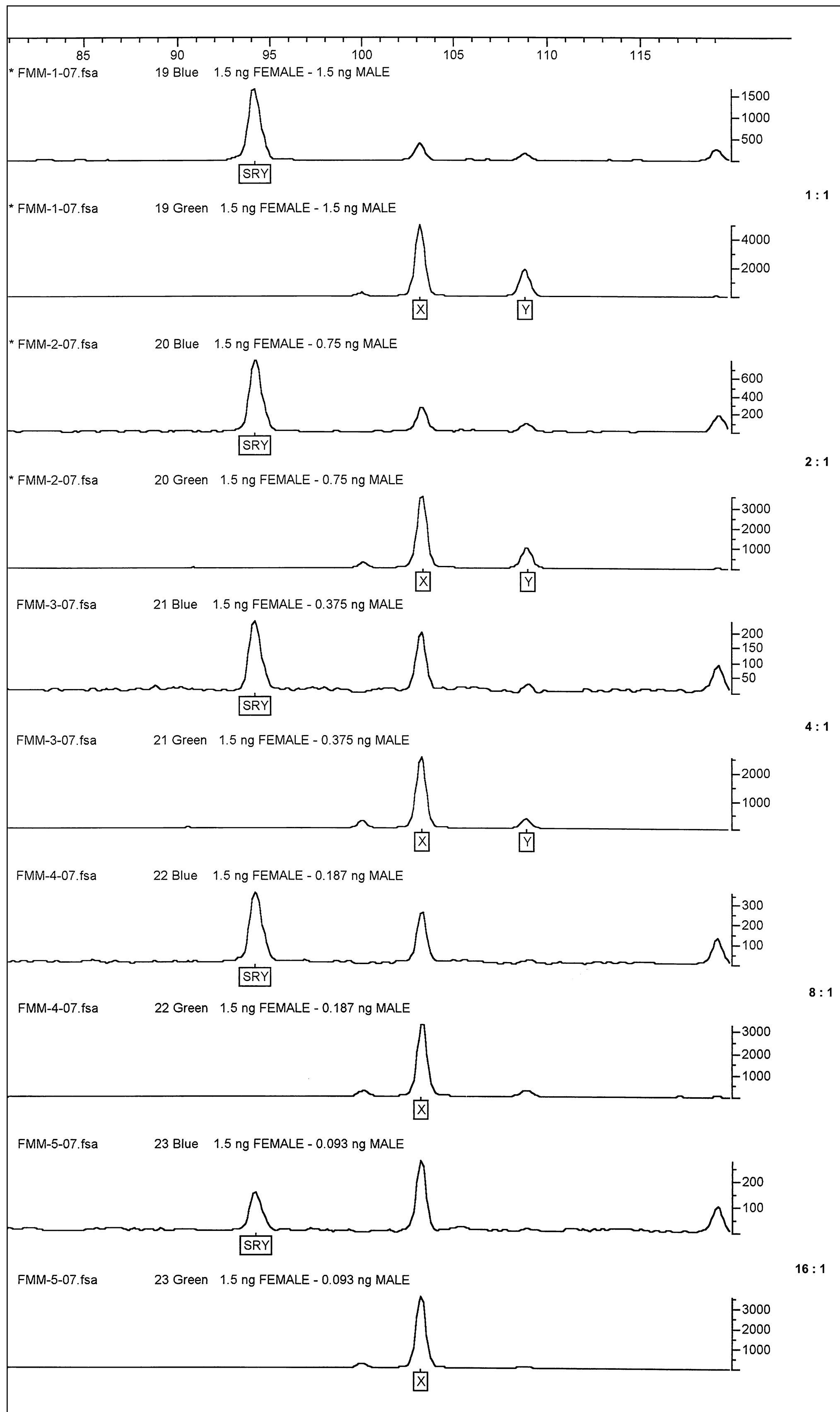

FIG. 5—*Mixture studies—amplification of female and male DNA casework sample. Partial electropherogram that capture amelogenin (AmpFlSTR® SGM™ Plus kit [Applied Biosystems]) and* SRY *genes—profiles are shown from top to bottom, with constant female concentration of DNA (1.5 ng) and decreasing concentration of male DNA: 1:1 (1.5 ng male DNA), 2:1 (750 pg male DNA), 4:1 (375 pg male DNA), 8:1 (187.5 pg male DNA), and 16:1 (93.7 pg male DNA).*

AMELY (data not shown). The robustness and sensitivity of the *SRY* assay in mixed samples is demonstrated at mixture ratios of 1:16 with a total of 93.7 pg male DNA in comparison with the *AMELY* allele assay (Fig. 5). *AMELY* was only detected successfully at a mixture ratio of 4:1 with 375 pg male DNA. Therefore, mixtures with low amounts of male DNA amidst high concentrations of female DNA can be typed with the *SRY* male gender marker assay.

Conclusions

The validation studies reported herein support that the *SRY* male gender marker developed by Drobnič (17) is sensitive, reliable, and can be used in concert with commercially available human STR identification kits to successfully type DNA derived from forensic samples. The *SRY* marker assay as a singleplex or included in multiplex kits can serve as an adjunct to standard gender typing. Future studies should include large population scale *SRY* marker analyses to determine whether drop-out is sufficiently low for forensic gender typing.

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