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Highly Sensitive and Reliable Human Sex Determination Using Multiplex PCR

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ABSTRACT

Gender validation is indispensable in data verification for demographic information particularly in large-scale population studies and also as part of the quality assurance of biospecimen repositories. Gender validation is also critical as part of quality control processes before specimens are subjected to sequencing analysis to identify germline and somatic mutations. The SRY gene is considered a useful signature gene marker that differentiates male from female. The aim of the study was to validate the SRY gene marker for use in gender determination in large cohort studies. SRY gene-specific sequences were amplified by PCR, electrophoresed on agarose gels and the 254 bp band was visualized for male samples. A series of DNA template concentrations were tested for sensitivity determination. The reaction was validated on 48 gender-blinded samples obtained from the UMBI BioBank to determine the specificity. ATL1 gene-specific sequence on X chromosome was used as the internal control. This PCR method has demonstrated 100% gender specificity. The sensitivity of the reaction was demonstrated with as low as 0.1 ng male DNA. The findings had suggested that *SRY* analysis by Multiplex PCR is a highly sensitive and specific method for gender determination and can be extremely useful for large-scale samples.

INTRODUCTION

Gender validation is thought to be an integral part of data verification for demographic information in large-scale population studies. High quality data and highly consistent biospecimens are major requirements for biospecimen repository to ensure the highest validity in basic and translational research. Gender validation is also seen as a critical part of the quality control process for genomic application particularly in mutation studies [1]. By validating the gender data, errors that could adversely affect the sequencing results can be minimised or eliminated and the accountability of the biospecimens can be assured.

Driven by the scale of samples in biospecimen repositories, gender validation requires a highly sensitive, rapid and reliable detection method. To date, none has reported the application of genetic markers in gender validation as part of quality assurance of biospecimen repositories and quality control prior to full genetic analysis.

In humans, the sex determining region of the Y chromosome (SRY) has been reported to be responsible for the development of the male characters [2]. The *SRY* loci on the short arm of Y chromosome has been shown to be the testis-determining factor (TDF) that initiates male sex determination [3]. Hence, the detection of SRY gene is thought to be able to distinguish a male DNA sample from a female. We used a multiplex PCR method to validate the SRY gene marker for use in gender determination.

MATERIALS AND METHODS

STUDY SAMPLES

A total of 48 samples were obtained from the UMBI Biobank for the gender validation study. The gender information for each sample was only revealed after the analysis has been completed.

DNA ISOLATION AND QUANTIFICATION

Total genomic DNA from the blood samples were extracted by using an improved salt-extraction method [4]. The vacuum-dried DNA samples were then dissolved in Tris–EDTA (TE) buffer and the DNA concentrations were determined by using a Nanodrop® ND-1000 Spectrophotometer (Thermo Scientific, USA) at A260/A280.

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MULTIPLEX POLYMERASE CHAIN REACTION (PCR)

PCR reactions were prepared using QIAGEN® HotStarTaq® Master Mix (Qiagen, Germany) according to the manufacturer's protocol. The PCR mixture consisted of 50 ng DNA and 0.1 µM of each set of primers (*SRY* and *ATL1*). The ATL1 gene was used as an internal control. The oligonucleotide primer sequences were as shown in Table 1. To monitor for contamination, negative (without DNA) and positive (male and female DNA) controls were included into the PCR reactions.

Table 1: Oligonucleotide primers for amplification of the SRY and ATL1 gene specific sequences

Gene	Forward Primer (5')	Reverse Primer (3')	Base pair
SRY	CATGAACGCATTCATCGTGTGGTC	CTGCGGGAAGCAAACTGCAATTCTT	254
ATL1	CCCTGATGAAGAACTTGTATCTC	GAAATTACACACATAGGTGGCACT	301

PCR reactions were performed in a Veriti® 96 Well Thermal Cycler (Applied Biosystems, USA). The DNA template was initially denatured for 15 min at 95°C and followed by 35 PCR cycles as follows: DNA denaturation for 1 min at 94°C, annealing for 1 min at 57.1°C and elongation for 1 min at 72°C, with a final extension of the cycle at 72°C for 10 min.

To determine the sensitivity of the reaction, different concentration of DNA templates were used ranging from 0.01, 0.1, 1.0, 10 and 100 ng. For this sensitivity testing, DNA from a male individual was applied in the PCR reactions to enable the comparison between 254bp and 301bp band intensities at the varying amounts of DNA.

AGAROSE GEL ELECTROPHORESIS

Electrophoresis of the PCR products was carried out using approximately 2.5 ng of template DNA (diluted DNA from PCR product) on 2% (w/v) agarose gels in 1 X TAE buffer for 90 minutes at 50 V/cm. Subsequently, the gels were stained in GelStar® Nucleic Acid Gel Stain and visualized under ultraviolet light.

ASSESSMENT OF SENSITIVITY AND SPECIFICITY

Sensitivity of this method was assessed by the proportion of actual positives which were correctly identified (correct gender) whereas the specificity was determined by the proportion of negatives which were correctly identified. The calculation of sensitivity and specificity is described as follows:

Sensitivity (%)
= [number of true positives / (number of true positives + number of false negatives)] × 100
Specificity (%)
= [number of true negatives / (number of true negatives + number of false positives)] × 100

RESULTS

We successfully amplified and detected the *SRY* and *ATL1* amplicons with bands at 254bp and 301bp respectively. Male samples were distinguished from female samples by the presence of SRY gene-specific bands at 254bp. PCR products observed at both 254bp and 301bp indicated that the DNA originated from a male sample whereas samples from female individual showed only a single PCR product at 301bp (Figure 1). The presence of ATL1 gene-specific bands at 301bp has been consistently observed in all samples, thus demonstrates the reliability of this gene as an internal control. A representative gel image from the validation study on the gender-blinded samples (19 out of 48 samples) is shown in Figure 2.

The sensitivity and specificity of this technique established using the 48 unknown samples were both 100% (Table 2), with no false-positive or false-negative result observed. Our study had correctly identified the gender for all of the samples with 100% accuracy.

High sensitivity of this PCR reaction was achieved as demonstrated by the minimum amount of 0.1 ng of DNA template required for a successful SRY gene detection (Figure 3).

DISCUSSION

Gender determination of human DNA samples in a rapid and accurate manner is essential in several settings including clinical practice in determining sex chromosome-linked genetic disorders, prenatal sex determination, forensic investigation and basic research. The gender determination is also used as part of a quality control in large biorepositories as well as when undertaking large studies using archived biospecimens. Over the years, several techniques have been employed to determine the sex of the donor of a human DNA sample including karyotyping for chromosomal disorders [5, 6], short tandem repeat (STR) in routine forensic DNA analysis [7, 8] and multiplex-polymerase chain reaction (PCR) in prenatal [7] and ancient human skeletons [9] gender determination. Given the advancement in technology today, the screening of gender on a large number of samples, particularly in cohort studies and biorepositories, using a high-throughput, rapid and economic manner is made possible by the development of DNA-based techniques.

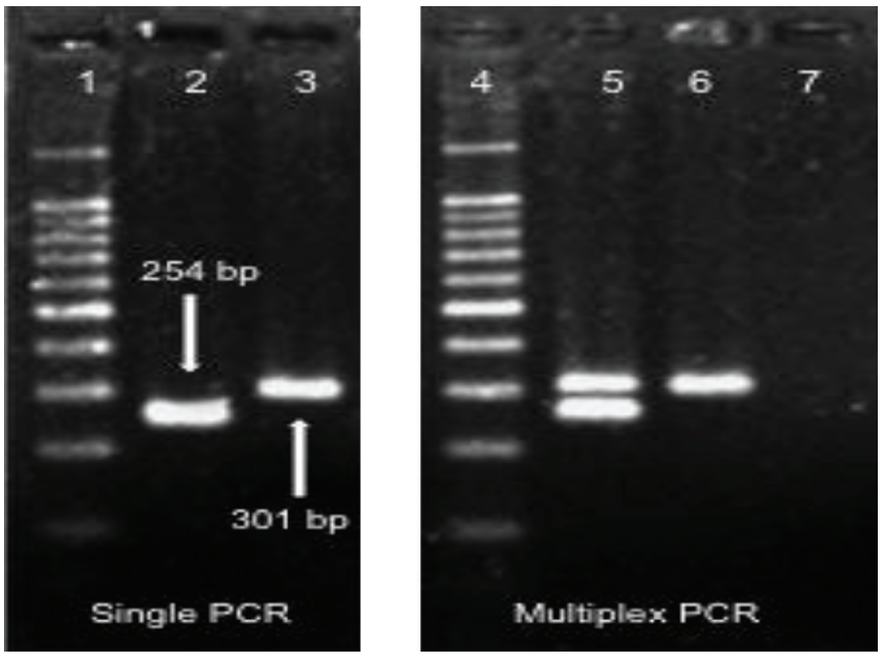

Figure 1: PCR amplification on both male and female samples. Lane 1 & 4: 100 bp marker; Lane 2: Male sample with only *SRY* primer; Lane 3: Female sample with only *ATL1* primer; Lane 5: Male sample with *SRY* and *ATL1* primers; Lane 6: Female sample with *SRY* and *ATL1* primers; Lane 7: Negative control (water). Female DNA sample showed a PCR product at 301bp whereas male DNA sample showed two PCR fragments at 254 and 301bp.

Figure 2: A representative gel image from the validation study on gender-blinded samples (19 out of 48 samples). PCR amplification was done by using *SRY* and *ATL1* primers.

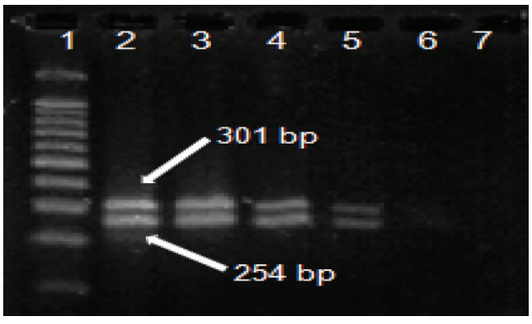

Figure 3: PCR amplification of a series of DNA template concentrations for sensitivity determination. Lane 1: 100 base-pair marker; Lane 2: 100 ng DNA template (10 ng/2 µL PCR product); Lane 3: 10 ng DNA template (1.0 ng/2 µL PCR product); Lane 4: 1.0 ng DNA template (0.1 ng/2 µL PCR product); Lane 5: 0.1 ng DNA template (10 x 10-3 ng/2 µL PCR product); Lane 6: 0.01 ng DNA template (10 x 10-4 ng/2 µL PCR product); Lane 7: Negative control (water). SRY gene specific sequence was successfully detected with DNA sample as low as 0.1ng.

Table 2: The sensitivity and specificity of SRY gene-specific sequence detection by Multiplex PCR. The gender of the blinded samples was determined with 100% accuracy.

SRY Detection	Demography		Total
	Male	Female	
Positive *SRY*	26 (true +ve)	0 (false +ve)	26
Negative *SRY*	0 (false –ve)	22 (true –ve)	22
Total	26	22	48

$$\text{Sensitivity (\%)} = \frac{\text{(true +ve)}}{\text{(true +ve) + (false -ve)}} \times 100 = \frac{26}{26} \times 100 = 100\%$$

$$\text{Sensitivity (\%)} = \frac{\text{(true -ve)}}{\text{(true -ve) + (false +ve)}} \times 100 = \frac{22}{22} \times 100 = 100\%$$

For this purpose, we have employed a simple multiplex PCR amplification of the SRY gene and the X-chromosome specific ATL1 gene and have successfully determined the sex of all unknown samples with 100% accuracy. The *ATL1*-specific fragment has been reported to be a reliable internal control for the non-invasive fetal sex determination in pregnant women [10]. This gene is located on the long arm of the X chromosome at Xq27.3 and directed the amplification of a 301 base pair fragment in all individuals [11].

We also demonstrated a higher sensitivity for this PCR reaction using a minimum DNA quantity of 0.1ng. Kastelic et al. 2009 reported the requirement of 125pg of DNA for *SRY* marker detection in male gender identification for their forensic casework [12].

In conclusion, our results have shown that we have successfully developed a very reliable, sensitive and specific method to differentiate between the male and female samples. Furthermore, the method requires only one multiplexed polymerase chain reaction in the workflow with a small amount of DNA and a minimum cost of RM10.42 per sample.

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