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# Fetal sex determination using circulating cell-free fetal DNA (ccffDNA) at 11 to 13 weeks of gestation

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**Objective** To examine the performance of a mass spectrometry-based detection platform using three Y-chromosome sequences for fetal sex determination from circulating cell-free fetal DNA (ccffDNA) in maternal blood in the first trimester of pregnancy.

**Methods** We extracted ccffDNA for the determination of fetal sex from stored maternal plasma obtained at 11 to 13 weeks' gestation from singleton pregnancies with documented fetal gender. Mass spectrometry was used to examine 236 specimens for the presence of three Y-chromosome sequences (SRY, DBY and TTTY2). The sample was classified as male, female or inconclusive depending on the detection of three, one/none and two sequences, respectively.

**Results** Three (1.3%) of the 236 cases were classified as invalid due to the absence of a well-defined spectral peak for TGIF and 22 (9.3%) were reported as inconclusive. In the 211 cases with a valid result, the fetal sex was correctly identified in 90 of 91 male babies and 119 of 120 female babies giving an accuracy of 99.1% and sensitivity and specificity for prediction of male fetuses of 98.9 and 99.2%, respectively.

**Conclusion** Fetal sex determination can be accurately determined from maternal ccffDNA in the first trimester of pregnancy using mass spectrometry analysis. Copyright © 2010 John Wiley & Sons, Ltd.

KEY WORDS: fetal sex determination; cell-free fetal DNA; first trimester; SRY; DBY; TTTY2; TGIF; mass spectrometry; laboratory-developed test

## INTRODUCTION

Non-invasive determination of fetal sex in the first trimester of pregnancy from the examination of circulating cell-free fetal DNA (ccffDNA) in maternal blood is important in the management of congenital adrenal hyperplasia (CAH) and X-linked disorders (Rijnders *et al*., 2001; Chiu *et al*., 2002; Costa *et al*., 2002; Hyett *et al*., 2005). In the case of families at risk of CAH, determination of a female fetus and subsequent administration of dexamethasone to the mother before 9 weeks' gestation can prevent the associated virilization (Pang *et al*., 1990; Speiser *et al*., 1990). In X-linked conditions, such as hemophilia and Duchenne muscular dystrophy, non-invasive determination that the fetus is female removes the need for chorionic villous sampling and the associated risk for miscarriage. The fetal sex can also be determined non-invasively by ultrasonographic examination of the fetal genitalia. Studies in the first trimester have reported that the accuracy of ultrasound ranges from 68 to 78% at 11 weeks to 83 to 100% at 13 weeks (Benoit *et al*., 1999; Efrat *et al*., 1999; Whitlow *et al*., 1999; Efrat *et al*., 2006; Hsiao *et al*., 2008; Chelli *et al*., 2009).

Non-invasive determination of fetal sex usually relies on DNA amplification, by polymerase chain reaction (PCR), and detection of Y-chromosome specific sequences in maternal plasma (Lo *et al*., 1998; Finning *et al*., 2008). Another technique for the analysis of ccffDNA is matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (the MassARRAY® system, Sequenom, Inc., San Diego, CA, USA), which combines flexibility, accuracy, automated analysis and high-throughput data generation (Ding and Cantor, 2003; Jurinke *et al*., 2004; Li *et al*., 2006; Ding, 2008). Absence of Y-chromosome sequences in maternal plasma implies that the fetus is female but this may also be the consequence of undetectable levels of cffDNA in the presence of male fetuses (Wright and Burton, 2009). Such a false-negative result can be avoided by ensuring the presence in maternal plasma of fetal-specific DNA markers (Tang *et al*., 1999; Pertl *et al*., 2000; Chim *et al*., 2005; Zhu *et al*., 2005; Wright and Burton, 2009).

The aims of our study were firstly, to examine the analytical performance of a mass spectrometry-based detection platform in specimens from a cohort of women in the first trimester of pregnancy; secondly, to perform a validation study prior to making available a laboratory-developed test (Ferreira-Gonzalez *et al*., 2006; Jennings

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*et al*., 2009), and thirdly, to evaluate the sensitivity and specificity of this test using three rather than one Y-chromosome specific marker.

## METHODS

At King's College Hospital, routine first trimester combined screening for chromosomal defects is offered. Women at 11 weeks to 13 weeks 6 days of gestation visiting for this routine appointment were invited to participate in a research study for prediction of pregnancy complications. Written informed consent was obtained from the women who agreed to participate in the study, which was approved by King's College Hospital Ethics Committee. The study included maternal blood collection and storage of plasma at −80 °C for future analysis.

In this study, 452 non-sensitized rhesus D-negative women were selected from the database and their stored plasma was used to extract ccffDNA and for subsequent determination of fetal sex and rhesus-D status. Here, we present the data on fetal sexing. The selection criteria were singleton pregnancies resulting in live birth beyond 24 weeks of gestation with documented gender of the neonate.

The 452 maternal plasma specimens were used in various phases of the analytical validation process according to the recommendations for introduction of molecular tests into clinical laboratory practice (Jennings *et al*., 2009). Once an optimized assay was available, verification was performed with a fraction of the specimens prior to committing 236 to the final phase of validation in which the laboratory personnel were blinded to the outcome of fetal sex. After the completion of analytical validation, the results of the laboratory analysis were compared with the information on fetal sex from the maternity database by a neutral third party, whose participation had been secured many weeks in advance of the generation of laboratory data.

### Sample preparation

Maternal venous blood collected in ethylene diamine tetraacetic acid (EDTA) BD vacutainer® tubes (Becton Dickinson UK limited, Oxfordshire, UK) was processed within 15 min of collection and centrifuged at 3000 rpm for 10 min to separate plasma from packed cells and buffy coat, and subsequently at 13 000 rpm for 10 min to further separate cell debris. The maternal plasma obtained after double centrifugation was then divided into 0.5 mL aliquots and stored at −80 °C until subsequent analysis.

### DNA extraction

During validation, the QIAmp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA, USA) was used to extract DNA from the plasma samples of the study population. A no-template control (NTC) was included in each run as a negative control for the test. Herring sperm DNA was used for the NTC instead of water because it better mimics a patient's sample. The negative control was composed of a standard female DNA obtained from the Coriell Institute for Medical Research (Camden, NJ, USA). The positive control was a mixture of Coriell female DNA containing 2% Coriell male DNA. This 98 to 2% ratio mimics a sample from a pregnant woman carrying a male fetus.

All samples from the study group and controls were similarly processed with controls at input levels of 2000 copies/reaction or 100 copies/μL. Briefly, 1 mL of plasma was incubated with 1 mL of buffer AL (Qiagen Inc.) and 150 μL of protease at 56 °C for 15 min. After the addition of 1 mL ethanol, the mixture was incubated for 5 min at room temperature. This mixture was applied to and drawn through a QIAvac spin column (Qiagen Inc.) as recommended by the manufacturer. The filtrate was washed sequentially with 600 μL of Buffer AW1, 750 μL of Buffer AW2 and 750 μL of ethanol. The column was centrifuged at 14 000 rpm for 3 min and subsequently incubated at 56 °C for 3 min, allowing the membrane to air dry. DNA was eluted from the membrane by applying 60 μL of nuclease-free water, incubating at room temperature for 1 min and centrifuging at 14 000 rpm for 2 min. A measured quantity of 20 μL of the final eluate served as the template for amplification reactions.

### Amplification

PCR was performed in a 96 well plate in a total reaction volume of 50 μL. A cocktail of 10× PCR buffer containing 500 mM Tris-HCl, 100 mM KCl, 50 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$, at pH 8.3 (Roche Diagnostics, Indianapolis, IN, USA), dNTPs containing 10 mM of dATP, dCTP, dGTP and dUTP each (Roche Diagnostics), allele-specific primers, 0.5 μM forward and reverse primers (IDT, Coralville, IA, USA), 1 Unit/μL Uracil N-Glycosylase (UNG, Roche Diagnostics) and 5 Units/μL Faststart Taq DNA Polymerase (Roche Diagnostics) was added to 20 μL of DNA template. Reaction conditions were 10 min incubation at 30 °C for UNG activity, followed by an initial denaturation at 94 °C for 4 min, 30 cycles of 94 °C for 20 s, 58 °C for 30 s and 72 °C for 1 min. Eleven addition cycles were performed at 94 °C for 20 s, 62 °C for 30 s and 72 °C for 1 min. A final elongation step at 72 °C for 3 min was performed prior to maintaining the reaction at 4 °C.

### Nucleotide dephosphorylation and single base extension for MassARRAY® system analysis

Following amplification, the unincorporated nucleotides were dephosphorylated by adding 1.0 unit/μL of Shrimp Alkaline Phosphatase (SensiPLEX™ Kit, Sequenom, Inc., San Diego, CA, USA) to 10 μL of amplification reaction product. The reaction was initiated at 37 °C and ran for 40 min before heat inactivating the



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enzyme at 85 °C for 5 min. Next, single base extension reaction was performed on the dephosphorylated mixture. A 4 μL cocktail containing SensiPLEX™ Kit reagents (Sequenom, Inc.) and sequence-specific primers was committed to allele-specific extension of targeted sequences. For the single base extension, an initial template denaturation was performed at 94 °C for 30 s, followed by 40 cycles at 94 °C for 5 s, 52 °C for 5 s and 80 °C for 5 s with five sub-cycles of the last two temperature profiles. A final extension at 72 °C for 3 min was performed.

## MALDI-TOF mass spectrometry analysis (MassARRAY®)

The resulting mixture was diluted with water to a final volume of 50 μL. Removal of salt cations was achieved by incubation with 15 mg CLEAN resin (Sequenom, Inc.) for 30 min. After centrifugation at 4000 rpm for 5 min, approximately 10 to 20 nL of resultant reaction product was nanodispensed onto a SpectroCHIP® II-G96 array preloaded with 3-hydroxypicolinic acid matrix. Mass spectra were obtained using a Mass Spectrometer (Sequenom, Inc.). Spectra were analyzed using MassARRAY® Typer Genotyping Software (Sequenom, Inc.). All spectra were objectively analyzed by at least two medical technologists with subsequent review by the clinical laboratory director to corroborate sex determination based on calling rules.

## Statistical analysis

Genotyping analysis software, MassARRAY®TYPER v4.0 (Sequenom, Inc.), was used to assess each spectrum individually and assign 'peak probability'. All peaks were manually reviewed in at least duplicate. The statistical software package XLSTAT-Pro 2010 (Addinsoft, USA) was used to determine sensitivity, specificity and diagnostic accuracy of the test.

## Calling rules for prediction of fetal sex

TGIFLX/Y (TGIF-like X/Y; AJ427749; subsequently called TGIF) is a human specific DNA homology block that maps to Yp11.2/Xq21.3 and comprises the largest shared region between the two sex chromosomes, spanning approximately 3.5 Mb. TGIF is transcribed into a ~2.7 kb mRNA encoded by two exons separated by a 96-bp intron (Blanco-Arias *et al.*, 2002). TGIF serves in this laboratory-developed test as an internal control for successful DNA purification from plasma. However, as TGIF is also found on the X-chromosome, it is not specific for fetal DNA. TGIF must be detected post-analytically for the assay to be considered valid.

All samples that showed the presence of a well-defined spectral peak for TGIF were then analyzed for the presence of three Y-chromosome sequences SRY (NM_003 140.1), DBY (NT_011 875.11 HsY_12 032: 1 175 399–1 275 398 homo sapiens chromosome Y contig) and TTTY2 (NC_000 024 Region:10 183 895. 10206085). If all the three Y-chromosome sequences were detected, the sample was classified as male. If one or none of the three sequences was detected, the sample was classified as female, and if only two of the sequences were detected, the sample was reported as inconclusive. The laboratory adopted these calling rules based on observations during technical feasibility and proof of concept experiments using the sensitive mass spectrometry-based detection platform. The alternative of including in the inconclusive group those with one positive Y-marker, rather than classifying them as female, would result in disqualification of many samples because spurious, non-specific and irreproducible positive results for one of the markers is common.

# RESULTS

In this analytical validation study, fetal sex determination was performed on 236 cases with a median gestational age of 12.4 weeks [interquartile range (IQR) 12.1–12.9]. The racial origin of the women was White in 182 (77.1%), Black in 45 (19.1%), Mixed in 8 (3.4%) and South Asian in 1 (0.4%). The phenotypic sex of the babies at birth was male in 110 (46.6%) and female in 126 (53.4%).

## Detection of Y-chromosome sequences

In 3 (1.3%) of the 236 cases, there was absence of a well-defined spectral peak for TGIF and these results were classified as invalid; no sex determination was made on these specimens. In the 233 remaining specimens in which a valid test result was obtained, the phenotype at birth was male in 107 and female in 126.

In the 233 cases that generated a valid test result, there were 90 (38.6%) in which all three Y-chromosome sequences were detected and these were classified as male, 119 (51.1%) in which one or none of the three sequences was detected and these were classified as female, and 22 (9.4%) in which two Y-chromosome sequences were detected and these were reported as inconclusive. Of the 119 cases classified as females, no Y-chromosome sequences were detected in 105 specimens and 14 were positive for one Y-chromosome marker: 10 for TTTY and 4 for DBY. There were six specimens from phenotypic females that generated inconclusive results in the LDT: two were positive for SRY and TTTY; three were positive for DBY and TTTY; and one was positive for SRY and DBY. There were 16 specimens from phenotypic males that generated inconclusive results in the laboratory-developed test: 8 were positive for DBY and TTTY; 7 were positive for SRY and TTTY; and 1 was positive for TTTY (by rule this last specimen should have been called female but data, not shown, from the RHD genotyping validation study suggested it was more prudent to classify it as inconclusive). Two specimens (0.86%) were incorrectly classified during the validation study. There was one discordant female result that was positive for SRY, DBY and TTTY2. There was one discordant male result that



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was negative for all the three Y-chromosome markers, though it was positive for TGIF.

## Accuracy of prediction of fetal gender

In the maternal samples from the 110 phenotypically male babies, 3 (2.7%) were invalid, 90 (81.8%) were classified as male, 1 was classified as female (0.9%) and 16 (14.6%) were inconclusive. In the maternal samples from the 126 phenotypically female babies, 1 (0.8%) was classified as male, 119 (94.4%) were classified as female and 6 (4.8%) were inconclusive.

In the 211 cases with conclusive results, the fetal sex was correctly identified in 209 cases and therefore, the accuracy of the test was 99.1% [confidence interval (CI) 97.7–100.0]. In 90 of the 91 pregnancies with male babies, the prenatal test predicted that the fetus was male and therefore the sensitivity of the prenatal test for the prediction of male fetuses was 98.9% (CI 93.3–100.0). In 1 of the 120 pregnancies with female babies, the prenatal test predicted that the fetus was male and in 119 that it was female. Therefore, the specificity of the test for the prediction of male fetuses was 99.2% (CI 94.9–100.0). In total, the prenatal test predicted that the fetus was male in 91 cases and in 90 of these the prediction was correct giving a positive predictive value of 98.9% (CI 96.8–100.0). In total, the prenatal test predicted that the fetus was female in 120 cases and in 119 of these cases the prediction was correct giving a negative predictive value for male fetuses of 99.2% (CI 97.5–100.0).

## DISCUSSION

The findings of this study demonstrate that non-invasive prediction of fetal sex from examination of ccffDNA in maternal blood can be achieved with a high accuracy in the first trimester of pregnancy. The main difference of this from previous studies is that we used three rather than one or two Y-chromosome specific markers, and during this validation of a laboratory-developed test, we employed mass spectrometry analysis as the detection platform rather than quantitative PCR for interrogation of Y-chromosome sequences.

A disadvantage of our study was that only small volumes of stored plasma samples were available for analysis. Even so, failure to extract ccffDNA occurred in only 1.3% of the samples. More significantly, the limiting plasma volumes led to our inability to reanalyze samples with inconclusive results, observed in 9.4% of our cases. The purpose of this study was to analytically validate this laboratory-developed test prior to implementation within a clinical diagnostics laboratory. Mass spectrometry was used to provide a platform amenable to interrogation of >90 DNA polymorphisms to identify candidates that are paternally inherited so as to differentiate maternal from fetal DNA. Mass spectrometry is the preferred platform for assays of density of many tens to hundreds of targets due to not only accuracy but also flexibility of panel design upstream of detection (Thongnoppakhun *et al*., 2009; Farkas *et al*., 2010). In the laboratory-developed test, male results are reported directly. Preliminary female results precipitate a reflex test on a larger volume of plasma (4 mL instead of 1 mL) in which ccffDNA is purified from plasma and maternal DNA is purified from isolated leukocytes. The two DNA sources are compared to identify paternally inherited polymorphisms thus directly demonstrating the true presence of ccffDNA and providing confidence in the subsequent laboratory test result of fetal female sex.

In all previous studies reporting results in the first trimester of pregnancy, larger volumes of maternal blood (2–20 mL) were used to extract fetal DNA and there were no reported cases of failure to achieve this (Costa *et al*., 2001; Rijnders *et al*., 2001; Sekizawa *et al*., 2001; Honda *et al*., 2002; Mazza *et al*., 2002; Guibert *et al*., 2003; Hromadnikova *et al*., 2003; Rijnders *et al*., 2003; Tungwiwat *et al*., 2003; Hyett *et al*., 2005; Zhu *et al*., 2005; Zimmerman *et al*., 2005; Chi *et al*., 2006; Ren *et al*., 2007; Picchiassi *et al*., 2008; Tungwiwat *et al*., 2008; Vainer *et al*., 2008). Additionally, there was no issue of inconclusive results because they used only one Y-chromosome specific sequence to determine the fetal sex. The Y-chromosome specific sequences used in most of the studies were either the single-copy SRY or the multi-copy DYS14. Seven early studies using the SRY sequence in a combined total of 123 pregnancies with male fetuses and 117 with female fetuses reported that prenatal testing identified correctly all female fetuses and 122 (99.2%) of the male fetuses (Costa *et al*., 2001; Rijnders *et al*., 2001; Guibert *et al*., 2003; Hromadnikova *et al*., 2003; Rijnders *et al*., 2003; Tungwiwat *et al*., 2003; Hyett *et al*., 2005). However, a study in which 100 plasma samples at 10 to 20 weeks of gestation were sent to five different laboratories using the SRY sequence reported that correct prediction of male fetuses varied from 31 to 97% (Johnson *et al*., 2004). Two studies comparing the performance of the SRY and DYS14 sequences in a combined total of 94 pregnancies with male fetuses reported that only 62 (66.0%) were correctly predicted by prenatal testing with the SRY sequence, compared to 93 (98.9%) with the DYS14 sequence (Zimmermann *et al*., 2005; Picchiassi *et al*., 2008). It was concluded that a multi-copy sequence, such as DYS14, is more sensitive, accurate and efficient than the single-copy SRY in the assessment of ccffDNA that is particularly important in the first trimester of pregnancy when the copy numbers of fetal DNA are low. The problem with the DYS14 sequence is that it has considerable homology to sequences other than Y-chromosome that could falsely classify female fetuses as male (Zimmermann *et al*., 2005). One recent study of pregnancies at 7 to 31 weeks utilized a combination of the DYS14 and SRY sequences to improve the detection of male fetuses with the former and reduce the false-positive rate with the latter (Scheffer *et al*., 2010).

Determination of fetal sex based on the detection of Y-chromosome specific markers in maternal plasma is prone to false-negative results because female fetuses are not detected directly but inferred by a negative result for Y-chromosome sequences, which could also be caused by undetectable levels of ccffDNA. In



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our study, we chose to use a combination of three Y-chromosome sequences (one of which, TTTY, is present in two copies on the chromosome) to maximize the accuracy of the test. The conservative nature of the calling rules was designed to overcome the problem of indirect inference of female fetal sex by virtue of mere absence of detection of Y-chromosome markers. Thus, the data in this validation study demonstrate that detection of three markers or less than two markers is highly accurately diagnostic for male and female sex, respectively.

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