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# Noninvasive fetal sex determination in maternal plasma: a prospective feasibility study

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**Purpose:** To prospectively validate a protocol for noninvasive fetal sex determination in maternal plasma and demonstrate its applicability to clinical practice.

**Methods:** Peripheral blood from 404 pregnant women undergoing prenatal invasive testing was collected from 6 to 23 weeks of gestation. Real-time PCR was performed for the *SRY* gene and multicopy DYS14 marker sequence located within the *TSPY* gene by the TaqMan minor groove binder probe assay as a first-line test. Owing to a false-positive result, amplification of repetitive motifs of the *DAZ* gene region was also tested as a second-line test performed in the last 232 patients enrolled in our series. A diagnostic algorithm was designed using a combination of these three markers. Fetal gender determined by noninvasive prenatal diagnosis (NIPD) was compared with that diagnosed by quantitative fluorescent PCR after invasive testing or ultrasound.

**Results:** A single false-positive result was obtained in the first 172 pregnancies. Reporting criteria were modified in the subsequent 232 pregnancies, giving an overall sensitivity and specificity of 100% (95% CI 99.8–100%) and 99.5% (95% CI 98.1–100%), respectively. Pregnancy outcome was obtained in all cases, including 221 male-bearing and 183 female-bearing pregnancies.

**Conclusion:** NIPD for fetal sex determination in maternal plasma is highly accurate and clinically applicable if robust reporting criteria are applied.

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**Key Words:** fetal sex determination; noninvasive prenatal diagnosis; TaqMan minor groove binder; X-linked genetic disorder

## INTRODUCTION

Early determination of fetal sex is of paramount importance in the management of congenital adrenal hyperplasia (CAH) and X-linked disorders. Traditionally, fetal sex determination in the first half of pregnancy has been performed either using invasive techniques, which carry a procedure-related miscarriage rate of around 1.0%,[1,2] or noninvasively by ultrasound. However, reliable sonographic determination of fetal sex cannot be made in the first trimester because development of the external genitalia is not complete.[3]

In X-linked conditions, such as hemophilia and Duchenne muscular dystrophy, early and reliable noninvasive determination of the fetal sex by examining circulating cell-free fetal DNA (cffDNA) in maternal blood might eliminate the need for invasive testing.[4] Apoptotic syncytiotrophoblasts from the placenta are the primary source of cffDNA.[5] cffDNA can be detected in the maternal circulation at 5 weeks of gestation and clears within several hours after birth.[6,7] Fetal DNA molecules represent 10%–20% of the total DNA circulating in the maternal plasma[8,9] and therefore clinical applications are currently limited to the detection of alleles arising de novo or those found in the father but not in the mother.

Several reports on fetal sex determination in maternal plasma have been published by targeting Y-chromosome-specific sequences. Use of both the single-copy *SRY* gene sequence[10–12] and the multicopy DYS14 marker sequence of the *TSPY* gene on the Y chromosome have been reported, using real-time PCR (RT-PCR).[13–15] However, other simple and sensitive tests for fetal gender determination using a unique multicopy region of the Y chromosome, such as the *DAZ* family, have been developed.[16,17] Although results are encouraging, the diagnostic accuracy varies widely depending on the protocols and methods used, with sensitivity and specificity ranging from 31% to 100%.[18,19] Moreover, most data have been obtained in a research setting rather than from clinical practice.[20]

Here we report the successful implementation of fetal sex determination using cffDNA in our laboratory and show that these tests are highly accurate when performed after 6 weeks using stringent reporting criteria that includes a third Y-chromosome-specific sequence for confirmation.

## MATERIALS AND METHODS

### Study specimens

Between January 2009 and December 2010, all women undergoing amniocentesis or chorionic villus sampling at our center with a high risk for fetal chromosomal defects ($n$ = 390) as well as couples with clinical history of gender-associated disorders ($n$ = 14) were invited to participate in the noninvasive prenatal

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diagnosis (NIPD) study. Approximately 10 ml of maternal blood for NIPD testing was collected at a single time point, between 6 and 23 weeks of gestation, and always before the invasive prenatal procedure. In all cases, fetal gender was unknown at the time of blood sampling and was later confirmed by quantitative fluorescent PCR (QF-PCR)[21]—in case of invasive procedure—or by prenatal ultrasound beyond the first trimester of pregnancy. All specimens were coded to facilitate blind testing. Written informed consent was obtained from all subjects. Twin pregnancies and spontaneous miscarriages were not included in the study. The study was approved by our Hospital Ethics Committee.

### Sample preparation and cffDNA extraction from plasma

Maternal peripheral blood was collected in EDTA vacutainer tubes, immediately aliquoted, and processed within a few hours. After centrifuging at 1,600*g*, plasma was carefully removed and recentrifuged at 14,500*g*[22,23] The supernatant was collected and stored at −20 °C until further processing. cffDNA was extracted from 500 µl of maternal plasma using the QIAamp DSP Virus Kit (Qiagen, Hilden, Germany) as described previously.[24] DNA was eluted from the column with 50 µl of buffer AE to increase the concentration. Whenever the first test yielded inconclusive or invalid results, a second aliquot of plasma from the same blood sample was used. Strict precautions against PCR contamination were taken and only female operators performed the procedures.

### Real-time PCR

The reactions were carried out using real-time PCR equipment (StepOne Real Time PCR System, Applied Biosystems, Foster City, CA) and the TaqMan minor groove binder (MGB) system for the detection of the amplification product.

Two Y-chromosome-specific regions—a single-copy gene (*SRY*) and a multicopy marker (DYS14)—were systematically analyzed in all cases. Because of a false-positive result in the first 172 studies, and to increase the sensitivity of the assay, an additional multicopy sequence (*DAZ*) was implemented as a second-line test.

PCR was set up in a final volume of 20 µl using 10 µl of TaqMan Universal PCR Master Mix (Applied Biosystems), with 300 nM of each primer, 200 nM of each TaqMan MGB probe, and 9 µl of extracted DNA. After an initial 2-min incubation at 50 °C to allow the activity of AmpErase uracil *N*-glycosylase to cleave the contaminant PCR products from previous reactions, a first denaturation step of 10 min at 95 °C was started. Amplification was then performed for 45 cycles of denaturation (95 °C, 15 s) followed by annealing (60 °C, 60 s). Continuous fluorescence was monitored in the annealing step for each sample. The number of amplification cycles required to reach a fixed threshold signal intensity is referred to as the cycle threshold ($C_t$). For better safety and efficiency, all regions were analyzed in separate reactions and in duplicate. Identical thermal profiles were used for *SRY*, DYS14, and the *DAZ* TaqMan MGB systems.

In addition, a positive control was used in each reaction (cffDNA from the gestation of a male fetus) as well as a negative control (cffDNA of a female fetus) and the no-template control, which was used to determine whether there was contamination of any reagent during the reaction procedure. The time required to complete the whole procedure, from maternal blood centrifugation to the result, was about 4 h.

### Interpretation

Amplification was labeled as positive, negative, or inconclusive according to $C_t$ as outlined in **Table 1**. Interpretation was routinely performed considering the combined results for DYS14 and *SRY*. Singleplex *DAZ* PCR was used as confirmation when discordant results between DYS14 and *SRY* PCR were obtained. In this way, the primer pair used for the *DAZ* assay produces five amplicons with 100% identity, mapping on the 2.4-Kb repetitive motifs of the *DAZ* gene region (one for each of *DAZ*1, *DAZ*2, and *DAZ*3 and two for *DAZ*4).[25] Thus, the utilization of this sequence as confirmation could give a higher sensitivity compared with the *SRY* assay when a negative result for this gene is obtained. Likewise, a positive result for *SRY* in combination with negative or inconclusive results for multicopy sequences, as a result of their better sensitivity,[26] was considered a technical failure. Samples were reported as male or female as shown in Figure 1.

### Data analysis

After all pregnancy outcomes had been collected, descriptive statistics were generated using Prism 5 software (GraphPad Software, San Diego, CA). The Fisher exact test (two-sided) was used to determine sensitivity and specificity with 95% confidence intervals (CIs). The positive predictive value (PPV), negative predictive value (NPV), overall sensitivity, and failure rate of the fetal sex determination by NIPD test were evaluated and compared with QF-PCR and/or ultrasonographic examination (see **Table 2**).

## RESULTS

### Patient demographics

In this analytical validation study, fetal sex determination was performed in 404 cases with a median gestational age of 14.6 weeks (range: 6 weeks, 2 days to 23 weeks, 0 days). Fourteen tests (3.5%) were performed for X-linked conditions (see Figure 2 for distribution). In these cases, the median gestational age was 9 weeks (range: 8 weeks, 0 days to 13 weeks, 0 days). Pregnancy, as determined by QF-PCR or ultrasonography, was ascertained in all cases, including 221 male-bearing and 183 female-bearing pregnancies.

### NIPD results

Results were conclusive upon first testing in 388 of 404 samples (96%). In the first 171 studies, PCR had to be

**Table 1** Cycle threshold cutoff points for DYS14, *SRY*, and *DAZ*

| | $C_t$ DYS14 | $C_t$ *SRY* | $C_t$ *DAZ* |
|---|---|---|---|
| Positive | ≤32 | ≤40 | ≤39 |
| Negative | ≥36 | >40 | >39 |
| Inconclusive | >32 and <36 | | |

$C_t$, cycle threshold.

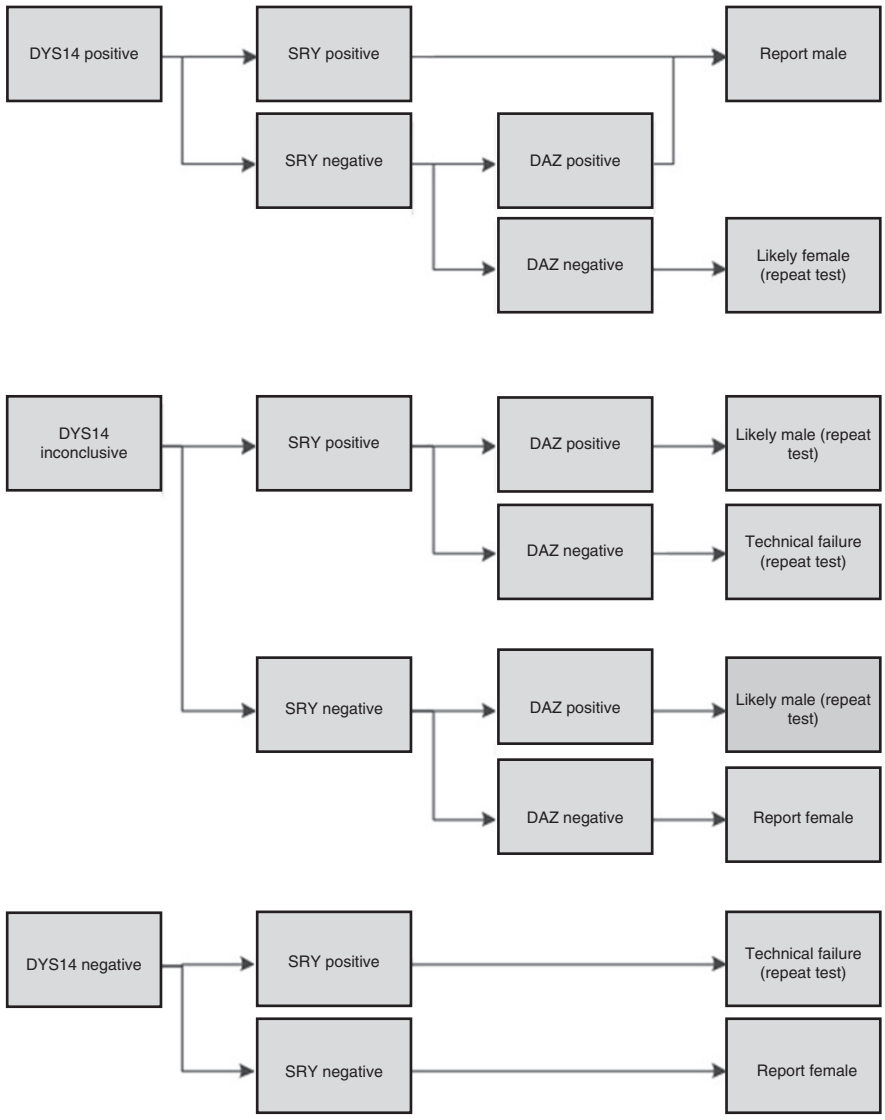


**Figure 1 Algorithm used for noninvasive fetal sex determination using three Y-chromosome-specific TaqMan minor groove binder probes.**

**Table 2** Statistical parameters of the study

| | Fetal sex by noninvasive prenatal diagnosis |
|---|---|
| Samples analyzed | 404 |
| Female fetuses | 183 |
| Male fetuses | 221 |
| False positives | 1 |
| False negatives | 0 |
| Sensitivity (%) | 100 (95% CI 99.8–100%) |
| Specificity (%) | 99.5 (95% CI 98.1–100%) |
| Positive predicted value | 99.5 (95% CI 98.4–100%) |
| Negative predicted value | 100 (95% CI 99.7–100%) |

CI, confidence interval.

repeated in two cases because of inconclusive results with the combined use of DYS14 and *SRY*. Case 172 resulted in a false positive and confirmation by *DAZ* PCR was carried out in 14 of the following 232 samples as described above.

As shown in **Table 3**, the mean cycle threshold value for *SRY* in male-bearing pregnancies was 38.49 (SD 1.55). The mean cycle threshold value for DYS14 in male-bearing pregnancies was 30.82 (SD 1.28). As cited above, a positive result was found for DYS14 in a female fetus (*SRY* negative and retrospectively confirmed by *DAZ* PCR); in addition, amplification was observed in 63.9% of the samples from female-bearing pregnancies (mean cycle threshold value 37.43, SD 1.47). Regarding the *DAZ* PCR results for confirmation (Figure 1), it should be pointed out that in all cases of positive results for DYS14 and negative results for *SRY* ($n = 2$), a positive result

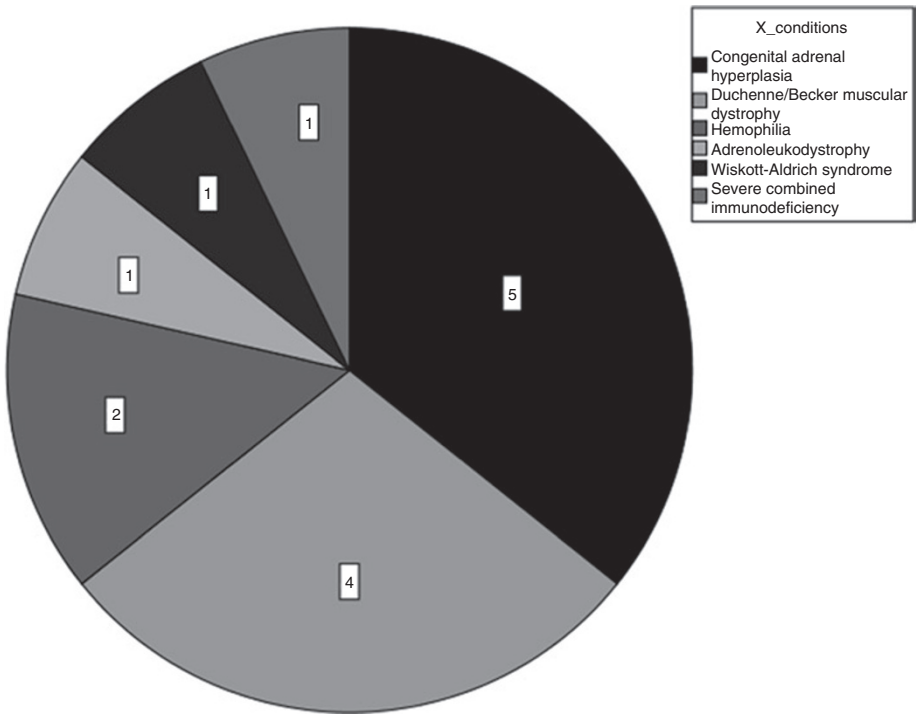


**Figure 2 X-linked conditions referred for noninvasive fetal sex determination.**

**Table 3** Cycle threshold results for DYS14, *SRY*, and *DAZ* real-time PCRs

| | XY | | | XX | |
|---|---|---|---|---|---|
| | $C_t$ *SRY* | $C_t$ DYS14 | $C_t$ *DAZ* | $C_t$ *SRY* | $C_t$ *DYS14* |
| Number of values | 221 | 221 | 5 | 3 | 117 |
| Mean ($C_t$) | 38.49 | 30.82 | 37.5 | 40.67 | 37.43 |
| SD | 1,551 | 1,285 | 1,243 | 1,155 | 1,476 |
| SE | 0.1048 | 0.08684 | 0.3589 | 0.6667 | 0.1364 |
| Lower 95% CI of mean | 38.29 | 30.65 | 36.71 | 37.8 | 37.16 |
| Upper 95% CI of mean | 38.7 | 30.99 | 38.29 | 43.54 | 37.70 |

$C_t$, cycle threshold; CI, confidence interval.

for *DAZ* was obtained and accordingly a male fetus was ascertained. In addition, when the DYS14 result was inconclusive and the *SRY* result was negative, if the *DAZ* result was positive ($n = 3$), fetal sex was confirmed as male, whereas if a negative result was obtained for *DAZ* ($n = 9$), a female fetus was verified.In 42.8% (6/14) of referrals for risk of X-linked conditions (Figure 2), a female was predicted by NIPD, whereas in 57.2% (8/14) of referrals a male was predicted by NIPD. Prediction of a female by NIPD meant that invasive testing was avoided in three cases of X-linked disorder (Duchenne/Becker muscular dystrophy, hemophilia, and Wiskott–Aldrich syndrome). Likewise, it was possible to initiate early treatment with steroids in three cases of CAH, where a female was predicted by NIPD.

In general, test results were reported within 24–48 h after blood sampling.

## DISCUSSION

The findings of this study demonstrate that noninvasive prediction of fetal sex from examination of cffDNA in maternal blood can be achieved with a high accuracy. The main difference in comparison with previous studies is that we used a specific protocol for cffDNA extraction combined with the application of TaqMan MGB probes for the detection of Y-chromosome single-copy and multicopy sequences following a stringent algorithm.

Amplification technique and gestational age have been recently found to be significant predictors of test performance.[27] Real-time PCR assays using MGB probes allow better sequence specificity and lower fluorescent background in comparison with no-MGB probes. Using this technology, fetal sex can be reliably determined in maternal plasma from the 7th week of gestation.

False-negative and false-positive results in sex determination using cffDNA are still a matter of concern. First, false-negative results due to failure to detect the Y-chromosome sequences may be associated with significant differences in yield of cffDNA between different DNA extraction methods. It is important to keep in mind that cffDNA is fragmented into <300-bp segments.[28,29] Hence, for manual reference methods, it has been demonstrated that the QIAamp DSP Virus Kit might be the optimal tool because it extracts mainly small fragments of DNA, finally providing larger amounts of cffDNA.[30,31] Second, regarding false-positive results, it has been observed that a multicopy sequence, such as DYS14, is more sensitive, accurate, and efficient than the single-copy *SRY* in the assessment of cffDNA, which is particularly important early in the first trimester of pregnancy when the copy numbers of fetal DNA are low.[32] The drawback of the DYS14 sequence is that it has considerable homology to sequences other than the Y chromosome that could falsely classify female fetuses as male.[33] In comparison with previous studies,[34] we have obtained a high frequency of amplification signals with DYS14 PCR in female fetuses (35% vs. 63.9%). In this sense, the use of centrifugation has been pointed out as a critical step to avoid contamination by residual circulating cells from previous pregnancies. We believe that not only a second microcentrifugation step but also the *g*-force and time used are essential to obtain truly cell-free plasma.[35] In order to distinguish between the true- and false-positive data, this study incorporated a second multicopy probe of the Y chromosome. *DAZ* PCR amplifications have much better sensitivity than SRY but lower compared with DYS14, probably owing to the twofold higher number of copies of the DYS14 versus the five amplicons produced by the *DAZ* primers.[26] However, the region detected by this probe has a negligible risk of a false-negative result in male fetal sex detection in maternal plasma because of de novo *DAZ* deletions, which arise with a frequency of 1/4,000; this may preclude the application of *DAZ* PCR assay as a first-line test for fetal sex determination.[36]

Some recently published studies used a combination of the DYS14 and *SRY* sequences to improve sensitivity and specificity of the assay.[34,37] 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 instead are inferred by a negative result for Y-chromosome sequences, which could also be caused by undetectable levels of cffDNA. Because of the higher sensitivity of DYS14, testing for only DYS14 has been suggested.[32,33] However, the inclusion of two multicopy Y-chromosome-specific assays increases the sensitivity and specificity of the test as a whole.

In this study, a case first analyzed with *SRY* and DYS14 and later confirmed by *DAZ* PCR was a false positive. False positives can result from technical issues such as contamination or clinical abnormalities such as the presence of a nonidentical vanishing twin or a confined placental mosaicism/chimerism.[38]

It has recently been shown that some false positives are due to the presence of a vanishing (male) twin, although this is only expected to cause a false-positive result in around 0.3%–0.7% of cases. To minimize this, it has been suggested that all cffDNA testing for fetal sex should be accompanied by an ultrasound scan, which should be done in the first 7 weeks.[39]Despite using a very low volume of plasma (500 μl), no false-negative results were found in our study and we were able to issue results before the 10th week of gestation and without the need for a second maternal blood sample, as distinguished from other groups.[34,40] In our approach, female fetuses are not detected directly but only inferred by a negative result for Y-chromosome-specific sequences. We chose to use a combination of three Y-chromosome sequences to maximize the accuracy of the test. A robust algorithm for reporting fetal sex was designed to overcome this problem. Thus, the data in this validation study demonstrate that the detection of three markers is highly accurate for clinical use.

In conclusion, noninvasive fetal sex determination in maternal plasma can be translated into clinical practice for gender-specific inherited disorders. The need for invasive diagnostic testing in women with a high risk of sex-linked genetic disease can be reduced by up to 50%. Ultrasonographic examination should be used to check for twin pregnancies before blood sampling and to confirm the fetal sex reported by NIPD.

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## DISCLOSURE

The authors declared no conflict of interest.

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