

# Accuracy of fetal gender determination in maternal plasma at 5 and 6 weeks of pregnancy

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**Objective** To assess the viability of the early diagnosis of fetal gender in maternal plasma before 7 weeks of pregnancy by real-time polymerase chain reaction (real-time PCR), starting at 5 weeks of pregnancy.

**Method** Peripheral blood was collected from pregnant women, starting at 5 weeks of gestation. After centrifugation, plasma was separated for fetal DNA extraction. DNA was analyzed by quantitative real-time PCR for two genomic regions, one on the Y chromosome (DYS-14) and the other shared by both sexes ( $\beta$ -globin), by the TaqMan Minor Groove Binder (MGB) probe assay. The results of the examinations were compared to fetal gender determined after delivery.

**Results** A total of 79 examinations of fetal DNA in maternal plasma were performed for 52 pregnant women. Accuracy according to gestational age was 92.6% (25 of 27 cases) at 5 weeks, and 95.6% (22 of 23 cases) at 6 weeks. These results also demonstrate that fetal DNA is present at low concentrations in maternal plasma at 5 weeks (8.5 genome equivalents (GE)/mL) and 6 weeks (34.1 GE/mL) of pregnancy.

**Conclusion** Quantitative real-time PCR and TaqMan MGB probes specific for the detection of fetal gender in maternal plasma starting at 5 weeks of gestation have good sensitivity and excellent specificity. Copyright © 2006 John Wiley & Sons, Ltd.

KEY WORDS: real-time PCR; TaqMan Minor Groove Binder; fetal DNA; maternal plasma; fetal sexing

## INTRODUCTION

The passage of fetal cells into maternal blood is a well-known phenomenon that was first determined at least 35 years ago. This source of fetal material is thought to be a possible tool for the detection of fetal abnormalities (Walknowska *et al.*, 1969). Almost 10 years ago, researchers described the presence of fetal DNA in maternal plasma and serum for the first time (Lo *et al.*, 1997). In addition, several investigators emphasized the importance of this finding as a source of fetal material in maternal blood, which might provide the opportunity for future noninvasive examination for the possible diagnosis of fetal disorders such as chromosome diseases (fetal aneuploidies), paternally inherited genetic disorders, or genetic diseases of autosomal recessive inheritance. The detection of fetal DNA was confirmed by the presence of a region of Y chromosome. Pregnant women with a male fetus had a positive examination and those with a female fetus had a negative examination. Approximately one year after researchers first described

the presence of fetal DNA in maternal plasma and serum, the same investigators succeeded in quantifying fetal DNA in relation to maternal DNA by means of a new molecular biology technique denoted as *real-time quantitative polymerase chain reaction* (PCR) (Lo *et al.*, 1998). Fetal DNA was extracted from the plasma of pregnant women and this DNA was later analyzed by real-time PCR for the detection of two genomic regions, one on Y chromosome and the other in a region common to both sexes called  $\beta$ -globin. Amplification of the  $\beta$ -globin region serves as a positive control of the reaction, and the region of Y chromosome is detected when the pregnant woman carries a male fetus. On this basis, a male fetus is diagnosed when the signal of amplification occurs in this region of Y chromosome, whereas a female fetus is diagnosed by exclusion of its occurrence. The methodology applied to quantify the fetal DNA was based on a 'standard curve' in which a quantified DNA known to be male is used. This DNA is diluted and, in the case of the present study, the dilutions ranged from 1000 to a single copy of Y chromosome. Thus, the standard curves and the DNA extracted from the plasma of pregnant women, which presents its curve among the 'standard' ones, were run simultaneously and the DNA was then quantified. Using this methodology, the authors were able to demonstrate that during the first and second trimester of pregnancy,

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fetal DNA corresponds to a mean 3.4% concentration of all DNA present in plasma, and this concentration increases to 6.2% during the third trimester of pregnancy (Lo *et al.*, 1998).

Most of the current studies using real-time PCR have shown 100% sensitivity and specificity for the diagnosis of fetal gender during the first trimester of gestation (Lo *et al.*, 1998; Costa *et al.*, 2001; Sekizawa *et al.*, 2001). However, almost all these investigations studied the detection of fetal DNA starting in the 7th week of gestation, with doubts existing about whether it would be possible to detect it at earlier gestational ages. For this reason, the objective of the present study was to evaluate the viability of an early diagnosis of fetal gender at 5 weeks and 6 weeks of gestation.

## MATERIAL AND METHODS

### Patients and samples

Pregnant women carrying a single fetus, starting at 5 weeks of pregnancy, and who had conceived by *in vitro* fertilization were invited to participate in the study. After being informed about the research, the women interested in participating signed a term of consent, which was approved by the Research Ethics Committee of the University of Ribeirão Preto (N. 024/05). Multiple samples were collected from patients in the 5th and 6th week of gestation and the reactions were performed without knowledge of the result of the previous examination. Also, in order to confirm the high accuracy of the assay, we blindly retested some of the first-trimester samples after 7 weeks of pregnancy (average of 12.2 weeks). All plasma samples were re-analyzed without knowledge of the previous result. The abortion cases were excluded. Result of the examination was confirmed by the gender of the neonates as they were born. To calculate the sensitivity and specificity of the method, we used the system of comparison with the newborn's sex, which we considered to be the 'gold standard'.

### Plasma samples and DNA extraction

Ten milliliters of peripheral blood was collected into EDTA-containing tubes. Blood was centrifuged for plasma separation at 1800 g for 10 min within 24 h of collection, and the plasma was placed in a PCR tube and frozen at  $-20^{\circ}\text{C}$  or stored in a refrigerator for a maximum of 48 h. The PCR tubes containing maternal plasma were then centrifuged again at 6000 g for 10 min. The supernatant obtained was placed in new PCR tubes from which 0.4 mL was removed for maternal-fetal DNA extraction. DNA extraction was performed using a commercial kit (QIAamp Blood Micro Kit- Qiagen, GmbH, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted from the column with 50  $\mu\text{L}$  of the buffer AE in order to increase the concentration. The entire process was carried out by

a female operator to avoid possible exogenous contamination with male DNA.

### Fetal DNA detection by real-time quantitative PCR

The reactions were carried out using real-time PCR equipment (7500 Real Time PCR System, Applied Biosystems, Foster City, CA, USA) and the TaqMan system for the detection of the amplification product (Lee *et al.*, 1993; Livak *et al.*, 1995). Maternal-fetal DNA was submitted to the analysis of two genomic regions, one common to both sexes ( $\beta$ -globin) and the other specific for a region of Y chromosome (DYS-14). PCR was set up in a final volume of 25  $\mu\text{L}$  using 12.5  $\mu\text{L}$  of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), with 400 nM of each primer (300 nM for  $\beta$ -globin), 150 nM of each TaqMan Minor Groove Binder (MGB) probe, and 10  $\mu\text{L}$  of extracted DNA. After an initial 2-min incubation at  $50^{\circ}\text{C}$  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  $95^{\circ}\text{C}$  was started. Amplification was then performed for 45 cycles of denaturation ( $95^{\circ}\text{C}$ , 15 s) followed by annealing ( $60^{\circ}\text{C}$ , 60 s). Continuous fluorescence was monitored in the annealing step for each sample. For better safety and efficiency, the two regions were analyzed in separate reactions and in duplicate. Identical thermal profiles were used for both the DYS-14 and the  $\beta$ -globin TaqMan MGB systems. In addition, two positive controls were used in each reaction, that is, DNA from the gestation of a male fetus, as well as two negative controls (DNA of a female fetus) and the no template control (NTC), which is used to determine whether there was contamination of some reagent during the reaction procedure. The mean time required for the procedure, from blood centrifugation to the result, was about 3 h.

### Construction of the calibration curves

A standard dilution curve using a known concentration of male genomic DNA (1000 to 1 genome equivalents (GE)) was run in parallel and in triplicate with each analysis in order to determine the number of genome copies of male DNA present in the plasma sample. The conversion factor of 6.6 pg of DNA per cell was used, and the concentration, expressed as genome copies/mL, was calculated as described previously (Lo *et al.*, 1998). The standard curves were run under ideal conditions in which the plot of Ct vs log of the copy number had a gradient (slope) of  $\sim -3.3$ , a y-intercept of about 38, and an  $R^2$  greater than 0.98. (Figure 1)

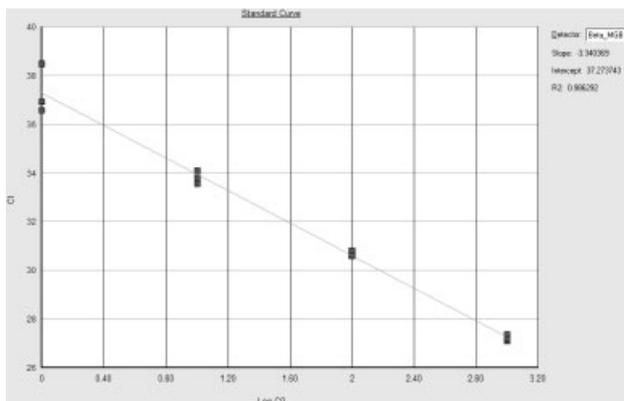


Figure 1—Plot of the threshold cycle (Ct) against the input target quantity (triplicate tenfold dilution series of DNA of 1000 to one copy)

RESULTS

Fetal gender determination by real-time PCR

Seventy-nine examinations of maternal plasma fetal DNA from 52 pregnant women were performed

(Table 1). Five patients who aborted were excluded since there was no way to confirm the fetal gender in those cases. The rate of accurate determination varied according to gestational age (Figure 2); however, there was agreement of the results in 100% of the cases in which male gender was diagnosed, regardless of gestational age. Only 3 of 79 examinations showed discordance of the diagnosis of fetal gender, all of them being false-negative results, that is, a female was diagnosed while the fetal sex was male.

Thirty examinations were performed during the 5th week of gestation; of these, three cases progressed to abortion and two yielded discordant results regarding the gender of the child. Thus, the accuracy was 92.6% (25 of 27), sensitivity was 87% (95% confidence interval: 0.61–0.98), and the negative predictive value was 0.84 (95% CI: 0.54–0.98). During the 6th week of gestation, 2 of 25 cases progressed to abortion and only 1 of the 23 cases was discordant, corresponding to 95.6% accuracy, 92% sensitivity (95% CI: 0.66–0.99), and a negative predictive value of 0.90 (95% CI: 0.55–0.99). It should be pointed out that 100% of the samples collected, starting at 7 weeks of gestation, were confirmed to be right (29 of 29). In addition, the specificity of the examination was 100%, regardless of gestational age, corresponding to a positive predictive value of 1.0.

Table 1—Correlation of fetal gender with gestational age and accuracy of the determination

Gestational age	Female	Male	male GE/mL <sup>a</sup>	Accuracy (%)	Sensitivity (%)	Specificity (%)	PPV <sup>b</sup> (%)	NPV <sup>c</sup> (%)
5 weeks	13	14	8.5	92.6	87	100	100	84
6 weeks	10	13	34.1	95.6	92	100	100	90
≥7 weeks (12.2 weeks)	17	12	186.5	—	—	—	—	—
Total	40	39	—	—	—	—	—	—

<sup>a</sup> GE/mL, Y genome equivalents/mL of maternal plasma.

<sup>b</sup> PPV, Positive predictive value.

<sup>c</sup> NPV, Negative predictive value.

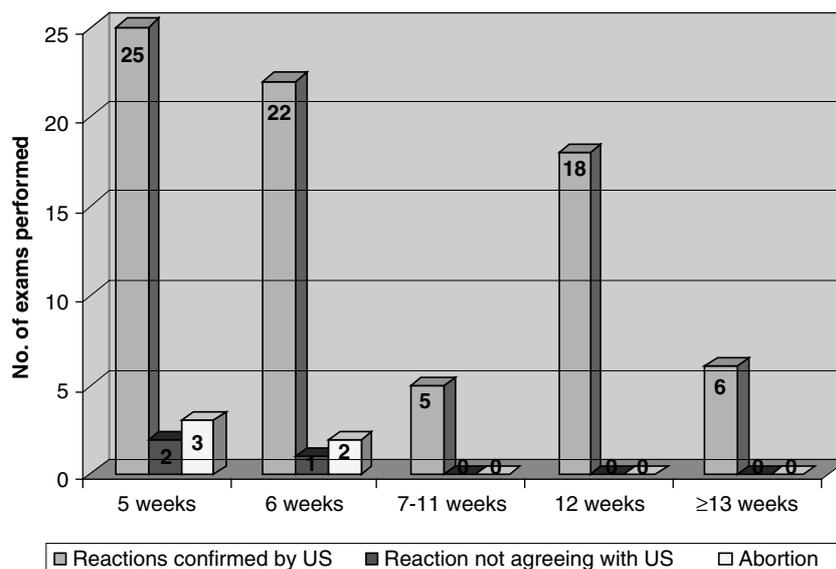


Figure 2—Number of examinations performed according to gestational age

## Quantitative analysis of fetal DYS-14 and $\beta$ -globin gene from maternal plasma

The actual amounts of total fetal DNA detected in the 79 samples examined are shown in Table 1. The mean amount of  $\beta$ -globin DNA detected was 1024 GE/mL (range, 106–5823 GE/mL). The  $\beta$ -globin concentration in maternal plasma samples was used as a measure of the total amount of extracted DNA (maternal and fetal) and served as a control of amplification in cases of female gender results. There was no correlation between gestational age and the total amount of DNA detected.

The absolute concentrations of male DNA detected in the maternal plasma with male DNA were 8.5 GE/mL (range, 0.17–49.1) at 5 weeks and 34.1 GE/mL (range, 5.6–79.1) at 6 weeks of pregnancy. The mean amount after 6 weeks (average of 12.2 weeks) was 186.5 GE/mL (range, 30.9–493.4). These concentrations correspond to 1.0% (range, 0.17–5.6%) and 6.8% (range, 0.34–23.4%) of the total plasma DNA at 5 and 6 weeks of pregnancy, respectively.

### DISCUSSION

The present data coincide with the literature reports showing that the accuracy of the technique is higher than 97% when the examination is performed from the 7th week of pregnancy (Lo *et al.*, 1998; Costa *et al.*, 2001; Honda *et al.*, 2001; Sekizawa *et al.*, 2001). Sekizawa *et al.* (2001), who analyzed the same genomic sequence that was used by us in the present study (DYS-14), evaluated 302 pregnant women with a gestational age of 7 to 16 weeks and obtained 97.2% accuracy for the determination of male gender (139 of 143 women). The reason for not detecting Y chromosome in four of these pregnant women who were carrying a male fetus is unclear. It is known that the concentration of fetal DNA in maternal blood increases with gestational age (Lo *et al.*, 1998), and therefore, the older the gestational age, the easier the diagnosis of fetal gender. However, for reasons still unknown, there seem to be periods during pregnancy when there is no minimum concentration of fetal DNA for detection. Among all our cases, the cases that had no agreement between the examination results and the fetal gender of the newborn were of early gestational age (5 and 6 weeks), yielding only false-negative result. This led us to believe that no circulating fetal DNA was as yet present in the maternal blood of these cases at the detection level of the technique. However, all these cases were confirmed by a new examination carried out at a more advanced gestational age.

Since only a few studies have been performed for the detection of fetal gender before 7 weeks of pregnancy (Thomas *et al.*, 1995; Birch *et al.*, 2005; Galbiati *et al.*, 2005), the novelty of the present study was the possibility of detecting fetal gender at 5 and 6 weeks of pregnancy, with a wide confidence margin and the subsequent quantification of the amount of male DNA in maternal plasma. In another recent report, the authors

performed a study to evaluate the earliest gestational age when fetal DNA can be reliably detected in maternal plasma, and they concluded that the SRY region can be detected in maternal plasma in 50% of the cases (one of two patients) at 5 weeks of pregnancy (Rijnders *et al.*, 2003). We used a different region for detecting the Y chromosome (DYS-14) in maternal plasma, and detected male fetal DNA in 92.6% of the cases (25 of 27). The mechanism that explains the accuracy of our result is that the DYS-14 is a multicopy region in the male human genome and differs from the SRY, which has only one copy (Kostiner *et al.*, 1998). Another reason could be the designing of primer–probe sets using the MGB technology. MGB probes offer high sensitivity and accuracy due to their short length, which increases the sensitivity of probe–sequence complexes to single base changes and their stability in the presence of such changes (Kutyavin *et al.*, 2000).

One of the most significant observations made in the present study is the very low concentration of fetal DNA in maternal plasma at 5 weeks of pregnancy, corresponding to 1% of the total plasma DNA, on average. After 6 weeks of pregnancy, the fetal DNA reaches a 'standard' concentration for the first or the second trimester. In addition, our data regarding the quantification of Y chromosome at 5 weeks of gestation showed that the mean is less than 10 genomic copies in 1 mL maternal plasma. This means that less than a single copy is being detected per reaction, which shows the extreme sensitivity of the method applied.

The definition of fetal gender can be very useful for the management of X-linked genetic diseases, as in the case of fragile X and hemophilia. The early diagnosis of fetal gender can be especially of help with respect to the anxiety of parents who are at genetic risk for transmitting these X-linked diseases. The possibility of confirming the feminine gender of a fetus during the first weeks of gestation can be of great benefit. Similarly, the examination can be a valuable tool for assisted reproduction in clinics that perform embryo sexing when there is a risk of inheriting an X-linked disease. It is possible to confirm the fetal gender at the very beginning of pregnancy by simply using a blood test, with no requirement for any invasive late examination, such as those involving the collection of a villus or amniotic fluid. Another useful feature of the examination concerns the risk of recurrence of congenital adrenal hyperplasia, which is treated with dexamethasone at the very beginning of pregnancy to prevent the possible virilization of the affected female fetuses. There is no need for any treatment in the male fetuses, whether affected or not, consequently, knowledge of the fetal gender can avoid unnecessary use of medication that may have side effects for the mother (Rijnders *et al.*, 2001; Bartha *et al.*, 2003). Recently, some investigators were able to demonstrate the detection of fetal RNA in addition to DNA in maternal plasma (Lo, 2005; Chiu *et al.*, 2006). This discovery may represent a valuable tool for the study of fetal gene expression related to different phases of intrauterine life, that is, the study of certain gene types that are expressed and silenced according to gestational age.

Using maternal plasma, it is also possible to determine, in addition to fetal sex, the fetal RhD status (Clausen *et al.*, 2005), paternally inherited gene disorders, or an autosomal recessive disorder for which each parent has a different mutation (Pertl *et al.*, 2000; Saito *et al.*, 2000; Li *et al.*, 2004). Theoretically, any paternal sequence not shared by the maternal DNA sequence can be detected in the plasma of pregnant women from the 7th week of gestation, provided that such sequences are not too large, because this DNA is in frank degradation and has small fragments.

In conclusion, the diagnosis of fetal gender by real-time PCR starting from 5 weeks of pregnancy has good sensitivity and excellent specificity. However, for cases in which the female gender is diagnosed during the 5th and 6th week of gestation, we recommend a repeat examination from the 7th week of gestation for a definitive diagnosis.

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