PRENATAL DIAGNOSIS
Prenat Diagn 2008; **28**: 525–530.
Published online in Wiley InterScience
(www.interscience.wiley.com) **DOI:** 10.1002/pd.2018

The best approach for early prediction of fetal gender by using free fetal DNA from maternal plasma

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Objectives Detection of free fetal DNA (ffDNA) in maternal blood during pregnancy has given rise to the possibility of developing new noninvasive approaches for early prenatal diagnosis.

On a large-scale study, two protocols of real-time polymerase chain reaction (PCR) were compared in order to establish which Y-specific locus, either multicopy *DYS14* or single copy *SRY* sequence, was the most suitable for developing a test with high diagnostic efficiency for early fetal gender assessment. The second aim was to assess whether the combination of the two detection systems could increase the performance of the prenatal test.

Methods We analyzed 145 plasma samples from healthy pregnant women between 11 and 12 weeks of singleton gestation. For each sample, fetal gender was determined by using both protocols (*DYS14* and *SRY*) during the same real-time PCR run.

Results The data obtained by the *DYS14* and *SRY* assays showed an efficiency in fetal gender prediction of 97.9 and 80%, respectively. It is not advisable to combine the two protocols because this association does not help in further improvements in fetal gender prediction.

Conclusions *DYS14* assay is the best approach for early fetal gender assessment because it is more sensitive, accurate, and efficient than the *SRY* assay. Copyright © 2008 John Wiley & Sons, Ltd.

KEY WORDS: *SRY*; *DYS14*; real-time PCR; fetal gender; noninvasive prenatal diagnosis

INTRODUCTION

Since the presence of free fetal DNA (ffDNA) in maternal plasma and serum during pregnancy has been discovered, prenatal diagnosis of genetic diseases by means of noninvasive approaches has been getting increasingly closer to becoming a reality in clinical practice.

Lo *et al*. (1998) demonstrated that ffDNA circulating in maternal blood has a relatively high concentration in early pregnancy, which increases during gestation and degrades in a few hours after delivery (Lo *et al*., 1999; Angert *et al*., 2003; Benachi *et al*., 2003); therefore, it cannot interfere with prenatal diagnosis of subsequent pregnancies.

As the use of ffDNA in noninvasive prenatal diagnosis is possible only for fetal DNA sequences absent in the mother, the potential clinical applications are concerned with the prediction of RhD genotype (Singleton *et al*., 2000; van der Schoot *et al*., 2003; Clausen *et al*., 2005), Mendelian diseases (Amicucci *et al*., 2000; Chiu *et al*., 2002; Gonzalez *et al*., 2002), and fetal gender (Rijnders *et al*., 2003; Cremonesi *et al*., 2004; Birch *et al*., 2005).

Several authors have demonstrated the possibility of detecting Y-specific sequences in maternal blood during gestation using real-time quantitative polymerase chain reaction (PCR) and different types of primers and probes specific for chromosome Y, such as the multicopy *DYS14* sequence located within the *TSPY* gene (Zhong *et al*., 2001, 2002; Zimmermann *et al*., 2005; Gerovassili *et al*., 2007) and the single copy *SRY* gene (Lo *et al*., 1998; Hromadnikova *et al*., 2003).

Nevertheless, the encouraging results obtained have been generated without following a standardized and reproducible protocol and conclusions have been mostly based on a small number of samples analyzed. In addition, confounding factors that could impact routine clinical application may be undetected in small-scale studies (Chiu *et al*., 2001).

Therefore, before introducing this approach of noninvasive prenatal diagnosis of fetal gender, it is necessary to detect its accuracy and reproducibility and to understand the variables that could affect its performance.

In this study, we have analyzed a large number of samples by using two different protocols of real-time PCR, the *DYS14* and *SRY* assays, to establish which is the most suitable in terms of sensitivity, specificity, and predictability for the diagnosis of fetal gender. Moreover, we aimed to assess whether the combination of the two assays could increase the performance of the test.

MATERIALS AND METHODS

Patients

Among pregnant women who attended the Department of Obstetrics and Gynecology of the University of

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Received: 25 February 2008
Revised: 27 March 2008
Accepted: 31 March 2008

Perugia for the routine check-up of pregnancy, 145 patients were enrolled following specific criteria of selection. Gestational age was between 11 and 12 weeks of gestation, calculated from last menstruation and confirmed by ultrasound. All the women were healthy and had a singleton and physiological pregnancy.

All patients provided informed consent after they were made aware of the purpose and experimental nature of the study.

Drawing of blood samples

Peripheral blood (5 mL) was drawn and collected into tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. The samples were treated within 4 h from the sampling and until their treatment they were stored at +4 °C.

The blood samples were centrifuged at 1600 *g* for 10 min, then the supernatant was collected in a 1.5 mL tube and it was centrifuged again at 16 000 *g* for 10 min to pellet any remaining cellular debris. The plasma samples obtained were divided in aliquots of 500 μL and stored at −20 °C until their use.

The samples were blinded to all personnel involved with the preparation and analysis.

DNA extraction

Genomic DNA from 500 μL of maternal plasma was extracted by using QIAmp DSP Virus kit (Qiagen, Germany). The manufacturer's instructions were amended in that we modified the incubation time as follows: 56 °C for 20 min with the Lysis buffer, room temperature for 10 min with ice ethanol 96–100%, 56 °C for 5 min after dry centrifugation, and room temperature for 5 min before DNA nucleic acid elution. We used centrifugation, instead of vacuum system, as suggested by manufacturer's instructions, to minimize the risk of contamination.

The DNA was eluted into 60 μL of sterile and DNase-free water of which 5 μL was used as a template for the PCR. Extracted DNA was stored at +4 °C until real-time PCR analysis.

Real-time PCR

The real-time PCR analysis was performed using Real-time PCR 7300 detection system (Applied Biosystems, USA).

Extracted DNA was analyzed for *DYS14, SRY*, and telomerase loci. The multicopy *DYS14* sequence located on *TSPY* gene and the *SRY* gene were used to measure the quantity of fetal male DNA, and the telomerase sequence was used to confirm the presence and quality of total (fetal and maternal) DNA in each sample.

Primers and TaqMan probes for *DYS14* (Zhong *et al*., 2001, 2002; Zimmermann *et al*., 2005; Gerovassili *et al*., 2007) and *SRY* (Lo *et al*., 1998; Hromadnikova *et al*., 2003) sequences were selected among the most utilized and promising in literature.

Primers and TaqMan probes specific for the *DYS14* and *SRY* sequences and telomerase house-keeping gene were as follows:

DYS14 forward primer 5′-GGG CCA ATG TTG TAT CCT TCT C-3′;
DYS14 reverse primer 5′-GCC CAT CGG TCA CTT ACA CTT C-3′;
DYS14 probe 5′-(*FAM*) TCT AGT GGA GAG GTG CTC (*TAMRA*)-3′;
SRY 109 forward primer 5′-TGG CGA TTA AGT CAA ATT CGC-3′;
SRY 245 reverse primer 5′-CCC CCT AGT ACC CTG ACA ATG TAT T-3′;
SRY probe 5′-(*FAM*) AGC AGT AGA GCA GTC AGG GAG GCA GA (*TAMRA*)-3′.
Telomerase forward primer 5′-GGT GAA CCT CGT AAG TTT ATG CAA-3′;
Telomerase reverse primer 5′-GGC ACA CGT GGC TTT TCG-3′;
Telomerase probe 5′-(*FAM*) TCA GGA CGT CGA GTG GAC ACG GTG (*TAMRA*)-3′.

The singleplex reactions were set up in a total volume of 25 μL, using 12.5 μL of TaqMan Universal 2X PCR Master Mix (Applied Biosystems), 5 μL of extracted DNA and optimized primers, and TaqMan probes (Applied Biosystems). In particular, for the detection system *DYS14*, primers and probes at final concentration of 900 and 200 nM, respectively were used; and for the detection systems *SRY* and telomerase, primers and probes at final concentration of 300 and 100 nM, respectively were employed.

Cycling conditions for all reactions consisted of 2 min at 50 °C and 10 min at 95 °C, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min.

To determine the copy number of male DNA present in the plasma sample, a standard curve using serial dilutions of the reference human genomic male DNA (Promega, USA) was employed.

Levels of ffDNA were calculated according to Lo *et al*. (1998) and expressed in genome equivalents per milliliter (GE/mL) of maternal plasma by using 6.6 pg as conversion factor.

Each sample was analyzed in triplicate, multiple-negative reaction blanks, and positive controls of male DNA pool were included in every analysis. A calibration curve and each DNA sample was tested for the *DYS14* and *SRY* markers on the same reaction plate for each PCR run.

After real-time PCR runs, samples were designated as female fetus if no positive replicates were obtained, and as male fetus if they had at least two replicates with positive amplifications. When only one positive replicate occurred, the result was considered inconclusive and the analysis repeated again. After this PCR run repetition, when one positive replicate occurred, a fetus was designated as female by using the *DYS14* assay, and as male by using the *SRY* assay. This different interpretation of the results for the two assays is the consequence of the higher sensitivity of *DYS14* than the *SRY* assay, observed previously (data not shown)

DOI: 10.1002/pd

by analyzing standard curve of both systems: the lowest amount of standard male DNA was amplified by the *DYS14* assay in more than 1 well while *SRY* amplified it in none or 1 well.

Fetal gender of each sample was determined by analysis of karyotype from invasive procedures or confirmed phenotypically at birth.

Anticontamination measures

The blood sampling, plasma preparation, DNA extraction and real-time PCR analysis were carried out by female staff and in separate areas to minimize the risk of contamination.

All equipment and work areas were swabbed with fresh 10 mL/L hypochlorite. In addition, all tubes and filtertips used were sterile. UV cross-linking of pipettes was performed before starting DNA extraction and real-time PCR mixture preparation. The TaqMan Universal 2X PCR Master Mix used contained Uracil *N*-glycosilase in combination with deoxyuridine-5′-triphosphate (dUTP) to prevent contamination by the carry-over of PCR products.

Statistical analysis

The results were expressed as median values with range and interquartile range (IQR) for descriptive statistics performed by Excel software (Microsoft).

Moreover, we determined diagnostic sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), and efficiency of the two tests on the basis of true and false positive/negative results obtained.

RESULTS

DNA extraction protocol had an optimal performance as demonstrated by the presence of telomerase amplicons, detected in all samples examined.

In order to evaluate the analytical sensitivity and reproducibility of the *DYS14* and *SRY* assays, we analyzed the data obtained from the standard curve of each experiment. Our results demonstrated a higher sensitivity and reproducibility of the *DYS14* assay compared to the *SRY*'s.

While the first assay allowed reproducible amplification of the target amount as low as 0.886 GE input, the determination of the *SRY* sequence was limited up to only 8.86 GE input. The Ct values of the *DYS14* assay were at least four cycles less than the *SRY* assay. The coefficient of variation (CV), calculated on ffDNA concentration of three replicates of the same sample, showed higher reproducibility in *DYS14* in respect to the *SRY* amplification system (Table 1).

Cytogenetic analyses or phenotype at birth revealed that among 145 women enrolled in this study, 82 were bearing male fetuses and the remaining 63 patients were bearing female fetuses. By using the *DYS14* and *SRY* assays, we observed a concordance of 97.9% (142/145) and 80% (116/145), respectively, with regard to fetal gender determination.

The *DYS14* assay detected male DNA in 81 out of 82 samples from pregnant women carrying male fetuses, whereas the *SRY* assay detected male DNA in only 54 samples out of 82 showing a higher sensitivity for *DYS14* (98.8%) in respect to the *SRY* assay (65.9%).

By using the *DYS14* assay, we correctly detected 61 out of 63 samples from pregnant women carrying female fetuses showing a specificity of fetal gender detection of 96.8%; but while using the *SRY* assay the specificity was 98.4%. Thus, the specificity in fetal gender detection of the two protocols was comparable.

Among 145 samples analyzed by using the *DYS14* assay, only 18 were repeated twice after having obtained inconclusive results. PCR assay repetition showed that fetal gender was correctly determined in all samples. By using the *SRY* assay, it was necessary to repeat the test on 24 samples of which 6 samples still showed incorrect fetal gender determination.

Fetal gender detection performed by the *DYS14* protocol gave two false positive results and one false negative result, whereas the *SRY* analysis gave one false positive result and 28 false negative results. Therefore, PPV and NPV of *DYS14* analysis protocol for fetal gender determination were 97.6 and 98.4%, respectively, while for the *SRY* analysis protocol for fetal gender determination were 98.2 and 68.9%, respectively. Therefore, while PPVs of both assays were similar, NPV of *DYS14* was nearly 1.5 times higher than that of the *SRY* assay. The results of sensitivity, specificity, PPV, NPV and efficiency of the *DYS14* and *SRY* assays are summarized in Table 1.

Among the 82 samples from pregnant women bearing male fetuses, the median ffDNA concentration, detected by the *DYS14* assay was 33.44 GE/mL of maternal plasma (IQR: 17.14–103.06 GE/mL; range 0–415.95 GE/mL); instead, the median ffDNA concentration, detected by the *SRY* assay, was 5.57 GE/mL of maternal plasma (IQR: 0–19.16 GE/mL; range 0–133.66 GE/mL). These data (Table 2) evidenced how *SRY* was less sensitive in male ffDNA detection compared to the *DYS14* assay and suggested that, as early as at 11 and

Table 1—Comparison of performance in fetal gender prediction between the *DYS14* and *SRY* assays

Detection system	Gestational age (weeks)	Total No. of patients	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Efficiency (%)	CV of DNA quantity (%)
DYS14	11–12	145	98.78	96.82	97.59	98.39	97.93	23.55
SRY	11–12	145	65.85	98.41	98.18	68.89	80.00	60.54

DOI: 10.1002/pd

Table 2—Fetal DNA concentration expressed as genome equivalents of male DNA/mL of maternal blood (GE/mL) calculated on samples from male fetuses by using the *DYS14* and *SRY* detection systems

Detection system	Male DNA concentration (GE/mL)		
	Median	Interquartile range	Range
DYS14	33.44	17.14–103.06	0–415.95
SRY	5.57	0–19.16	0–133.66

12 weeks of gestation, ffDNA was present in all the samples tested except one, indicating that DNA extraction protocol worked with a high performance.

Re-examining the two false positive results obtained by the *DYS14* protocol, we observed that Ct values were at least 3 cycles higher, and consequently, ffDNA concentration was about 10 times lower for these patients than for others in which a male fetus was predicted.

The unique sample from male fetal pregnancy detected as false negative by the *DYS14* assay, did not produce any positive amplification by using the *SRY* assay. However, extracting the DNA again from another plasma aliquot of this sample, which resulted in a false negative result, the follow-up real-time PCR analysis correctly revealed a male fetus.

DISCUSSION

This study was carried out by using real-time PCR in order to assess ffDNA in maternal plasma and to verify whether two Y-amplification assays (the multicopy *DYS14* sequence and the single copy *SRY* gene) could be technically employed in fetal gender determination; and to verify whether the association of the two detection assays could improve the performance of the test. To our knowledge, this is the first study performed on a large number of patients at an early gestational age in order to compare these two assays in predicting fetal gender from ffDNA present in maternal plasma.

Our results showed the great technical performance of both protocols employing real-time PCR. In particular, the *DYS14* assay demonstrated a higher sensitivity and reproducibility in male DNA detection compared to the *SRY* assay. This evidence is in keeping with the findings observed by Zimmermann *et al*. (2005) and Zhong *et al*. (2006).

With regard to fetal gender prediction, our data obtained by the *DYS14* assay showed a high diagnostic concordance (97.9%) with the results verified by analysis of karyotype from invasive procedures, or confirmed phenotypically at birth. We have not reached 100% efficiency because we revealed two false positive results and one false negative result. By using the *SRY* detection system, we observed a concordance of 80% having incorrectly diagnosed 29 samples, of which 1 false positive result and 28 false negative results were detected.

The lower *SRY* diagnostic efficiency, in comparison with the *DYS14* assay, is due to the different number of false negative samples detected by using the two amplification systems, as also observed by Zimmermann *et al*. (2005). The smaller number of false negative results obtained from the *DYS14* assay in comparison with *SRY* could be justified by the fact that a multicopy sequence (*DYS14*) in respect to a single copy sequence (*SRY*) could be able to detect male DNA also in those samples at very low ffDNA concentration. Therefore, the data suggested that ffDNA, coming from pregnant women at 11–12 weeks of gestation, is too scarce to be detected by the *SRY* assay, but is an adequate amount to be identified by the use of multicopy sequence. On the other hand, Zhong *et al*. (2006) demonstrated that the ffDNA concentration detectable during second trimester is sufficient to be revealed by both amplification systems. This discrepancy could be due to the different gestational age in which Zhong's and our studies were carried out in the second and the first trimester of pregnancy, respectively.

The results of our study are of greater importance than those of Zimmermann *et al*. (2005) who obtained similar findings but in a much smaller population.

The data regarding the low *SRY* diagnostic efficiency could seem to be in disagreement with those of others (Lo *et al*., 1998; Guibert *et al*., 2003; Hromadnikova *et al*., 2003; Rijnders *et al*., 2003) who showed 100% accuracy and specificity; however, it should be pointed out that all the studies performed involved less than 50 patients. Authors, such as Cremonesi *et al*., 2004; Rijnders *et al*., 2004 and Birch *et al*., 2005, achieved high diagnostic accuracy and specificity of the *SRY* amplification system by analyzing a bigger population, however, they had considered a much larger gestational age interval ranging from as low as 5 up to 41 weeks. We presume that their preference for *SRY*-based PCR results could be justified by the higher concentration of ffDNA in maternal blood at advanced gestational weeks, which could enable easier detectability of ffDNA allowing the use of a lower sensitive system such as a single copy, in respect to a multicopy system.

Moreover, our results obtained by using the *DYS14* system, are of great interest in this field for those who want to use this technology in a clinical setting because they are widely intra- and interlaboratory reproducible as demonstrated by the multicenter studies (Legler *et al*., 2007) in which the researchers got similar data as ours.

For the widespread introduction of noninvasive prenatal diagnosis in clinical service, false negative results should be avoided because they are more clinically worrisome than false positives. Since the unique false negative result from the *DYS14* assay was correctly revealed as a male fetus after a second sample extraction and PCR analysis, we think that it would be advisable in carrying out two individual DNA extractions or increasing two-fold input volume of maternal plasma for extraction.

Regarding the false positives, we observed that the *DYS14* assay could reach 100% specificity by selecting a cut-off at 1.5 GE/mL of maternal plasma, while for the *SRY* assay this cut-off is not feasible because the male ffDNA concentration does not differentiate between false positive and true positive cases. Another

DOI: 10.1002/pd

possible measure to reduce the incidence of false positive results is the combination of our genetic test with early ultrasound scan in order to determine the presence of a singleton or twin pregnancy, and therefore, avoid possible bias in case of a vanishing twin.

This approach for fetal gender determination is useful for all pregnant women at risk of X-linked disorders or metabolic conditions associated with ambiguous development of external genitalia. Since a specific diagnostic test is available only for a limited number of the X-linked diseases, for all the others fetal gender is the only useful diagnostic information for first-step screening. In case of pregnancies at risk of metabolic disorders associated with ambiguous development of external genitalia, the knowledge of fetal gender is important to establish as to which of the cases needs to be pharmacologically treated.

From all these observations, we believe that 11–12 weeks of gestation is the best sampling period to achieve the optimal compromise between accuracy and precocity in test performing. Before this gestational age, the test could give a consistent number of false negative results due to the insufficient quantity or absence of ffDNA in the sample analyzed, whereas after this period, it might not be possible to assure the adequate management of pregnancy, either for the use of invasive procedures or for an optimal pharmacological treatment. For example, in case of pregnancies at risk of hemophilia or Duchenne Syndrome, if the fetus is male, the detection at 12 weeks allows the performance of conventional prenatal diagnosis by invasive procedures, whereas in case of a female fetus, the pregnant woman should not undergo any invasive procedures with the related risk of fetal loss. On the other hand, in pregnancies at risk of metabolic disorders, such as congenital adrenal hyperplasia (CAH), the early prenatal fetal gender prediction would be extremely important for carrying out steroid prophylaxis only on women with female fetuses in order to prevent their fetal virilization.

From our results, it is clear that the *DYS14* assay has been demonstrated as the most adequate and most promising to be employed in the diagnostic routine of fetal gender prediction, and to be considered the gold standard of prenatal noninvasive fetal sex diagnosis because it is sensitive, accurate, and efficient, as confirmed by Sekizawa *et al*., 2001 and Honda *et al*., 2002. It is not advisable to combine the two protocols because this association does not reach further improvements in fetal gender prediction as demonstrated by the lower sensitivity and specificity of this combination in respect to those obtained from the *DYS14* assay.

Consequently, this test could be recommended to all pregnant women with previous sons except for twin pregnancies with at least one male fetus. This exception is due to the impossibility of establishing which fetus is male, in other words, to which twin fetus the male ffDNA belongs.

We are convinced that fetal gender determination, by using real-time PCR and ffDNA from maternal plasma, represents the very near future of noninvasive prenatal diagnosis because it is feasible, rapid to perform, has a low cost, and mainly detects fetal gender at an early gestational age in a robust manner. Nevertheless, further studies on a large number of maternal plasma samples are required to define firmly cut-off values and to standardize the whole protocol.

ACKNOWLEDGEMENTS

The European Commission for the Special Non-invasive Advances in Fetal and Neonatal Evaluation (SAFE) Network of Excellence (LSHB-CT-2004-503243), from which this study was partially funded, is gratefully acknowledged.

We are deeply indebted to Prof. Antonio Tabilio, MD, Dr Mauro Di Ianni, MD, and Lorenzo Moretti, BSc, from the Department of Experimental Medicine and Oncology of University of Perugia, for providing suggestions and support in the development and application of the real-time PCR technology.

Moreover, we are also deeply indebted to Zena Baccaille and Nadia Belia, midwives, for their help in collecting blood samples and data from patients enrolled in our study.

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DOI: 10.1002/pd