PRENATAL DIAGNOSIS
*Prenat Diagn* 2011; **31**: 802–808.
Published online 31 May 2011 in Wiley Online Library
(wileyonlinelibrary.com) **DOI:** 10.1002/pd.2770

# Fetal RHD genotype detection from circulating cell-free fetal DNA in maternal plasma in non-sensitized RhD negative women

**Allan T. Bombard[1,5]*, Ranjit Akolekar[2], Daniel H. Farkas[3], Anna L. VanAgtmael[3], Frank Aquino[1], Paul Oeth[1] and Kypros H. Nicolaides[2,4]**

[1]*Sequenom Center for Molecular Medicine, 3595 John Hopkins Ct, San Diego, CA, USA*
[2]*Harris Birthright Research Centre, Kings College Hospital, London, UK*
[3]*Sequenom Center for Molecular Medicine, 301 Michigan St., Grand Rapids, MI, USA*
[4]*Department of Fetal Medicine, University College Hospital, London, UK*
[5]*Sequenom, Inc., 3595 John Hopkins Ct, San Diego, CA, USA*

**Objective** To examine the performance of the SensiGene Fetal RHD Genotyping Laboratory Developed Test (RHD Genotyping LDT) using circulating cell-free fetal DNA (ccff DNA) extracted from maternal plasma.

**Methods** ccff DNA was extracted from maternal blood from non-sensitized women with singleton pregnancies in two cohorts, one with a serotype reference (11–13 weeks' gestation) and one with the reference source (6–30 weeks' gestation). The presence of three *RHD* exon sequences (exons 4, 5, 7), the psi-pseudogene, three Y-chromosome sequences (SRY, DBY and TTTY2), and the X/Y-chromosome TGIF gene control were determined using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry—the RHD Genotyping LDT.

**Results** The cohort with a serotype RhD reference showed correct classification in 201 of 207 patients, a test accuracy of 97.1%, with a sensitivity and specificity for prediction of RhD serotype of 97.2 and 96.8%, respectively. The cohort with a genotype RHD reference showed correct classification in 198 of 199 patients, indicating a test accuracy of 99.5% with a sensitivity and specificity for prediction of RHD genotype of 100.0 and 98.3%, respectively.

**Conclusion** Fetal RHD genotyping can accurately be determined using ccff DNA in the first and second trimesters of pregnancy. Copyright © 2011 John Wiley & Sons, Ltd.

KEY WORDS: fetal RHD determination; cell-free fetal DNA; psi-pseudogene; SRY; DBY; TTTY2; TGIF; Sequenom Center for Molecular Medicine Laboratory Developed Test

## INTRODUCTION

Noninvasive prenatal determination of fetal Rhesus D (RHD) genotype from analysis of circulating cell-free fetal DNA (ccff DNA) in maternal plasma can be useful in the management of pregnancies of RhD negative women with RHD heterozygous partners (ACOG Practice Bulletin #75, 2006). Identification of the pregnancy at increased risk for isoimmunization early in the course of pregnancy can set the stage for optimal pregnancy management using middle cerebral artery Doppler velocimetry to identify the need for fetal transfusion, or, if later in gestation, early delivery. Using birth as the ultimate outcome, approximately half of previously sensitized women could be identified as not being at increased risk, thereby reducing the need for unnecessary examinations and parental concern. For that reason, a noninvasive determination of fetal RHD genotype can be a useful tool for all sensitized women, reducing the iatrogenic risk of increasing maternal sensitization due to chorionic villus sampling (CVS) or amniocentesis. Additionally, in isoimmunized women requiring invasive testing for prenatal diagnosis of chromosomal defects or genetic abnormalities, knowledge of the fetal RHD genotype would be useful in deciding whether to conduct first trimester CVS because of the risk of feto-maternal hemorrhage, and therefore worsening of the severity of alloimmunization and fetal hemolysis would be higher than with second trimester amniocentesis (Brambati *et al*., 1986; Tabor *et al*., 1987). In the case of women with no RhD hemolytic antibodies, knowledge that the fetus is RHD negative is useful in determining the need for prenatal and postnatal immunoprophylaxis with anti-D. In the case of isoimmunized patients, knowledge that the fetus is RHD negative may avoid the need for intensive prenatal monitoring to predict and treat fetal anemia.

In these studies, we examine the feasibility and accuracy of the RHD Genotyping Laboratory Developed Test (RHD Genotyping LDT) utilizing ccff DNA extracted from maternal plasma and analyzed using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Two clinical cohorts were used for evaluation, the first using as a reference

*Correspondence to: Allan T. Bombard, Sequenom Center for Molecular Medicine, 3595 John Hopkins Court, San Diego, CA 92106, USA. E-mail: Allan@SCMMLab.com



*Received: 7 December 2010*
*Revised: 24 March 2011*
*Accepted: 28 March 2011*
*Published online: 31 May 2011*

point the clinical RhD serotype obtained from neonatal cord blood at the time of delivery (cohort 1), and the second using an RHD genotype previously determined by the source laboratory as the reference for accuracy (cohort 2).

## METHODS

Sample collection and ethics committee approval of cohort 1 has been described previously (Akolekar *et al*., 2010). Cohort 2 represents clinical samples submitted to the Sequenom Center for Molecular Medicine in Grand Rapids (SCMM-GR), a Clinical Laboratory Improvement Amendment (CLIA)-certified, College of American Pathology-accredited, specialty reference laboratory, for analysis of RhD negative pregnant women using the RHD Genotyping LDT. All women were non-sensitized and properly consented to participate in this study prior to blood draw. In both cohorts 1 and 2, the laboratory performing the assays for this study received blinded samples without foreknowledge of either fetal RhD genotype or sex.

Ccff DNA was purified for determination of fetal RHD genotype in two subset cohorts, each obtained from singleton pregnancies. The first set of blinded samples contained 1.0 mL of maternal plasma from 236 singleton, non-sensitized, pregnancies with documented fetal RhD serology. These samples were obtained at 11–13 weeks' gestation, and the results of noninvasive fetal sexing in this cohort were reported previously (Akolekar *et al*., 2010). The second subset was composed of 205 blinded, 1.0 mL maternal plasma samples, at 6–30 weeks' gestation, that were processed originally at the SCMM-GR; this was used for a clinical test validation performed in the Sequenom Center for Molecular Medicine in San Diego (SCMM-SD). The reference authority information for this second cohort of clinical samples (available 1.0 mL maternal plasma aliquots) was the SCMM-GR. Results for a noninvasive reflex test for determination of ccff DNA presence (Fetal Identifier Test, van den Boom *et. al*., 2006) in this cohort were not available because of sample volume limitation.

## Sample preparation

The steps for sample preparation of the initial validation samples (cohort 1) were as previously described (Akolekar *et al*., 2010). For the clinical samples (cohort 2) whole blood samples were centrifuged at 1600 × *g* for 10 min at 4 °C then placed in pre-labeled conical tubes. The majority of plasma from the initial spin was re-centrifuged at 15 500 × *g* for 10 min at 4 °C and made ready for DNA extraction.

## DNA extraction, amplification, nucleotide dephosphorylation and single-base extension for RHD Genotyping LDT

These steps were performed as previously described (Akolekar *et al*., 2010). Briefly, 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 sample. The RHD positive and negative controls were provided by SCMM-GR. All samples from the study group and controls were similarly processed with controls at input levels of 2000 copies/reaction, or 100 copies/μL. The presence of three RHD exon sequences (exons 4, 5, 7), the psi-pseudogene, three Y-chromosome sequences (SRY, DBY and TTTY2), and the X/Y-chromosome TGIF gene control were determined using MALDI-TOF MS—the RHD Genotyping LDT. Details can be found in the companion article (Akolekar *et al*., 2010). The primer sequences for this assay have been published and are available for review (Oeth *et al*., 2008).

## Statistical analysis

Genotyping analysis software was used to assess each spectrum individually and assign genotype calls based on a proprietary peak-identification and calling algorithm (Oeth *et al*, 2009). All peaks were manually reviewed in at least duplicate. The statistical software package XLSTAT-Pro 2010 (Addinsoft, USA) was used to determine sensitivity, specificity, positive and negative predictive values and overall accuracy of the test.

## Calling rules for prediction of fetal RHD genotype

As reported in the companion publication (Akolekar *et al*., 2010), TGIFL X/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 as an internal control for successful DNA purification from plasma in the RHD Genotyping LDT. However, as TGIF is also found on the X-chromosome, it cannot be assumed to be specific for fetal DNA. TGIF must be detected post-analytically for the assay to be considered valid.

All samples which showed the presence of a well-defined spectral peak for TGIF were then analyzed for presence of the three RHD exons 4, 5 and 7 and for a 37-base pair insertion found in exon 4 which is indicative of the presence of the psi-pseudogene as well as three Y-chromosome sequences (SRY, DBY and TTTY2). If all three RHD exon specific markers were detected, the sample was classified as RHD positive; if one or none of the three exon sequences was detected the sample was classified as RHD negative; and if only two of the sequences were detected the sample was reported as inconclusive. Samples positive for the psi-insertion were reported as psi (+)/RHD variant. The calling



DOI: 10.1002/pd

Table 1—Fetal RHD assay calling rules

| Criteria | Call |
|---|---|
| No *TGIF* detected | INVALID test |
| Three Y markers and *TGIF* | Male (M) |
| ≤1Y marker and *TGIF* | Female (F) |
| Two Y markers and *TGIF* | Inconclusive (INC) |
| Three *RHD* markers and *TGIF* | *RHD* Positive (POS) |
| ≤1 *RHD* marker and *TGIF* | *RHD* Negative (NEG) |
| Two *RHD* markers and *TGIF* | Inconclusive (INC) |

RHD, Rhesus D.

rules for both the RHD and Y-based control assays are summarized in Table 1.

## RESULTS

In the first clinical validation study (cohort 1), fetal RHD genotyping was performed on 236 maternal plasma samples with a median gestational age of 12.4 weeks (interquartile range, 12.1–12.9). The ethnic origin of the women was 182 Caucasian (77.1%), 45 African (19.1%), 8 mixed race (3.4%) and 1 South Asian (0.4%). The neonatal RhD phenotype, determined by serology at the time of birth, was positive in 163 samples (69.1%) and negative in 73 samples (30.9%).

In 2 (0.9%) of the 236 samples, there was absence of a well-defined spectral peak for TGIF, and these results were classified as invalid (Figure 1). In the 234 (99.1%) samples with sufficient DNA extraction, the result was conclusive in 207 samples (88.5%); inconclusive in 16 samples (6.8%); and psi (+)/RHD variant in 11 samples (4.7%; a buffy coat was not available for study so maternal vs paternal psi (+)/RHD variant status could not be determined). In the 207 samples with sufficient DNA extraction and a conclusive result, the neonatal RhD phenotype was positive in 142 samples (68.6%) and negative in 65 samples (31.4%). The prenatal sample was classified as RHD positive in 140 samples, including 138 samples in which the neonatal RhD type was RHD true positive and 2 samples in which it was false positive. The prenatal sample was classified as RHD negative in 67 samples, including 63 samples in which the neonatal RhD type was true negative and 4 samples in which it was false negative (Table 2).

The RHD Genotyping LDT correctly predicted the neonatal RhD phenotype in 201 of 207 samples and therefore the accuracy of the test was 97.1% (95% CI, 93.5–98.8). In 138 of the 142 samples with RhD positive fetuses, the test predicted that the fetus was positive and in 4 that it was negative and, therefore, the sensitivity of

Table 2—Clinical cohort 1—RHD genotyping results

| | RhD clinical positive | RhD clinical negative |
|---|---|---|
| Positive RHD result | True positive (138) | False positive (2) |
| Negative RHD result | False negative (4) | True negative (63) |

RHD, Rhesus D.

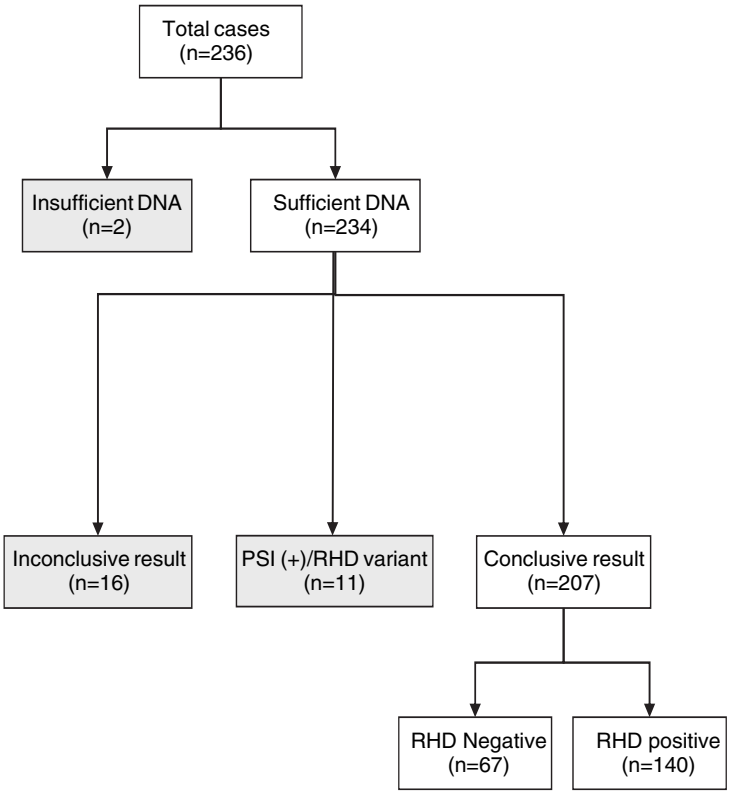


Figure 1—Flow chart summarizing cohort 1 fetal RHD genotyping results



DOI: 10.1002/pd

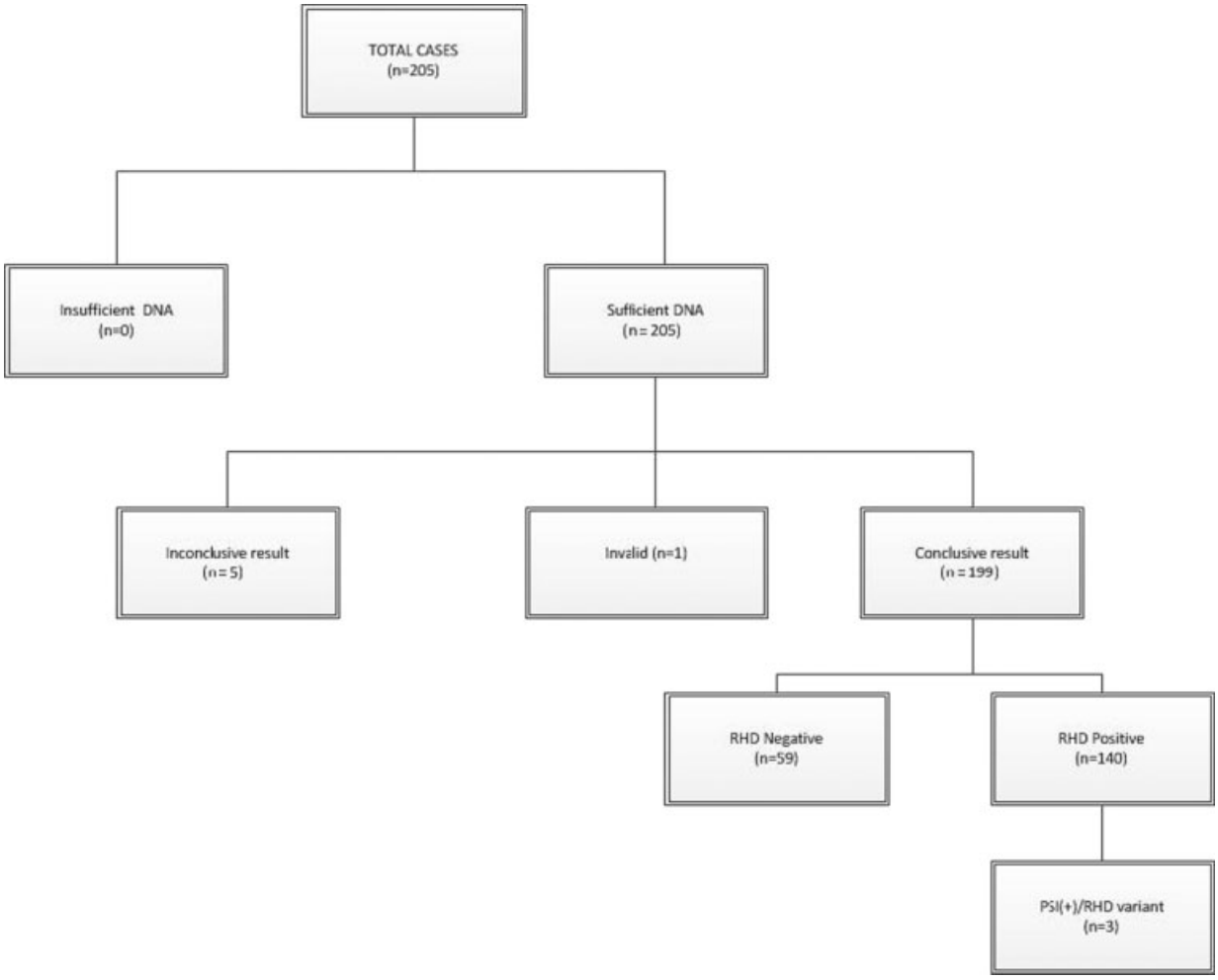


Figure 2—Flow chart summarizing cohort 2 fetal RHD genotyping results

the test for the prediction of RhD positivity was 97.2% (95% CI, 93.0–98.9). In 63 of the 65 samples with RhD negative fetuses, the RHD Genotyping LDT predicted that the fetus was negative and, in 2, that it was positive; therefore, the specificity of the test for the prediction of RhD positivity was 96.9% (95% CI, 89.5–99.1). The test predicted that the fetus was RhD positive in 140 samples and in 138 of these the prediction was correct, giving a positive predictive value of 98.6% (95% CI, 94.9–99.6). The test predicted that the fetus was RhD negative in 67 samples and in 63 of these the prediction was correct, giving a negative predictive value for RhD positive fetuses of 94.0% (95% CI, 85.6–97.6) (Table 2).

In the second study (cohort 2), fetal RHD genotyping was performed on 205 maternal plasma samples (Figure 2). The samples were provided by the reference laboratory and all demographics, as well as results, were blinded to the testing laboratory. Fetal gestational age was available in only 90 samples (43.9%), with gestational ages ranging from 5 weeks 5 days to 30 weeks 1 day and a median gestational age of 12.1 weeks (32% second trimester). The ethnic origin of 181 women who provided clinical samples was declared (88.3%) and included 144 Caucasian (79.6%), 13 African (7.2%), 14 Hispanic (7.7%) and 10 other (5.5%) women. Five (2.4%) of the 205 samples were excluded from analysis because of inability to conclusively genotype the sample (inconclusive) and one (0.5%) blinded sample was excluded because the SCMM-GR reference laboratory ultimately generated no RHD result (the patient's physician later cancelled the RHD genotyping order). Thus, there was an overall inconclusive rate of 2.9% (6/205). Three samples showed a psi (+)/ RHD variant (1.5%; buffy coat analysis on these samples determined that the mother also had a psi (+)/RHD variant). In the 199 samples with a valid result, the fetal RHD genotype was correctly identified in 198 giving an accuracy of 99.5% (95% CI, 97.2–99.9) and sensitivity and specificity for prediction of male fetuses of 100.0% (95% CI, 97.3–100.0) and 98.3% (95% CI, 91.0–99.7), respectively, as well as positive and negative predictive values of 99.3% (95% CI, 96.1–99.9), and 100% (95% CI, 93.8–100.0), respectively (Table 3). In as much as this was a clinical validation using samples provided by a reference laboratory, neonatal serologic outcome was not available for secondary validation of results. A summary of the overall performance of each of the seven individual markers employed in this assay (RHD: exon 4, exon 5, exon 7, and the psi-pseudogene; sex determination: TTTY, DBY and SRY), including the error rate for each



DOI: 10.1002/pd

Table 3—Clinical cohort 2—RHD genotyping results

| | RhD clinical positive | RhD clinical negative |
|---|---|---|
| Positive RHD result | True positive (140) | False positive (1) |
| Negative RHD result | False negative (0) | True negative (58) |

RHD, Rhesus D.

Table 4—Performance of cell-free fetal DNA markers in the maternal circulation for the determination of RHD status and fetal sex in RHD negative and female fetuses

| Marker error summary by assay | | | |
|---|---|---|---|
| RHD | Cohort 1 | Cohort 2 | Overall accuracy |
| Exon 4 | 0 | 0 | 100% |
| Exon 5 | 2 | 1 | 99.82% |
| Exon 7 | 1 | 1 | 99.88% |
| Psi-pseudogene | 0 | 0 | 100% |
| Total assays | 828 | 796 | 1624 |
| Fetal sex | | | |
| TTTY | 1 | 1 | 99.84% |
| DBY | 1 | 0 | 99.92% |
| SRY | 1 | 1 | 99.84% |
| Total assays | 621 | 597 | 1218 |

RHD, Rhesus D.

marker in the case of RHD negative female fetuses, is detailed in Table 4.

## DISCUSSION

Several studies examined the prenatal accuracy of fetal RHD genotyping from analysis of ccff DNA in maternal plasma which ranged from 32 to 100% (Geifman-Holtzman *et al*., 2006; Daniels *et al*., 2009; Grill *et al*., 2009; Legler *et al*., 2009). Most of the studies examined samples from all three trimesters of pregnancy but the majority were from the second and third trimesters. The results of the eight studies providing data derived from first trimester samples are summarized in Table 5 (Lo *et al*., 1998; Zhang *et al*., 2000; Costa *et al*., 2002; Finning *et al*., 2002; Randen *et al*., 2003; Brojer *et al*., 2005; Al-Yatama *et al*., 2007; Machado *et al*., 2006; Minon *et al*., 2008). The number of samples in individual studies varied between 12 and 102 and in the combined total of 269 cases, the assay used in these studies correctly predicted the neonatal RhD phenotype in 256 cases (95.2%), and the sensitivity for the prediction of RhD positivity was 94.7% with a specificity of 96.0%.

Noninvasive determination of fetal RHD genotype usually relies on DNA amplification, by PCR, and detection of chromosome 1-specific sequences in maternal plasma (Lo *et al*., 1998; Finning *et al*., 2008). To examine the performance of fetal RHD genotyping as a routine test in all RhD negative women, high-throughput methods with robotic isolation of fetal DNA have been described that offer a cost-effective alternative to routine administration of anti-D (van der Schoot *et al*., 2006; Finning *et al*., 2008; Legler *et al*., 2009). Another technique for analysis of ccff DNA is MALDI-TOF MS which combines flexibility, accuracy, automated analysis and high-throughput data generation (Ding and Cantor 2003; Jurinke *et al*., 2004; Li *et al*., 2006; van der Schoot *et al*., 2008; Ding 2008; Oeth *et al*., 2009; Thongnoppakhun *et al*., 2009; Farkas *et al*., 2010; Tynan *et al*., 2010). In a companion publication we have also showed that fetal sexing can reliably be performed using the MALDI-TOF MS technology (Akolekar *et al*., 2010).

When the fetal RHD genotype is reported to be negative, like the mother's, an RHD negative result cannot be used to identify the presence of non-maternal DNA so fetal sex determination must be considered. 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 ccff DNA in the presence of male fetuses (Wright and Burton, 2009). False negative results 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). This same risk, namely that insufficient or no ccff DNA was extracted

Table 5—Studies reporting use of cell-free fetal DNA in maternal circulation for determination of fetal RHD status in the first trimester of pregnancy

| | | | | RHD positive | | RHD negative | |
|---|---|---|---|---|---|---|---|
| Author | Gestation (weeks) | Gene | Control | *n* | Correct (%) | *n* | Correct (%) |
| Lo *et al*. (1998) | 7–14 | Exon 10 | $\beta$-Globin | 9 | 7 (77.8) | 3 | 3 (100) |
| Zhang *et al*. (2000) | <13 | Exon 7 | None | 12 | 11 (91.7) | 8 | 8 (100) |
| Costa *et al*. (2002) | 8–14 | Exon 10 | GALT | 62 | 62 (100) | 40 | 40 (100) |
| Finning *et al*. (2002) | <14 | Exons 4, 5, 6, 10 | CCR5, SRY | 13 | 13 (100) | 6 | 6 (100) |
| Randen *et al*. (2003) | 6–14 | Exon 7 | $\beta$-Globin, SRY | 9 | 6 (66.7) | 9 | 7 (77.8) |
| Brojer *et al*. (2005) | <14 | Exons 7, 10, Intron 4 | $\beta$-Actin, SRY, polymorphisms | 20 | 19 (95.0) | 10 | 8 (80.0) |
| Al-Yatama *et al*. (2007) | 6–13 | 7 | $\beta$-Globin, SRY | 8 | 7 (87.5) | 6 | 6(100) |
| Machado *et al*. (2006) | <14 | Exon 10, Intron 4 | None | 11 | 10 (90.9) | 4 | 4 (100) |
| Minon *et al*. (2008) | 10–13 | Exons 4, 5, 10 | CCR5, SRY | 26 | 26 (100) | 13 | 13 (100) |

RHD, Rhesus D.



DOI: 10.1002/pd

from a particular sample for RHD detection, existed for cohort 1 with no recourse as only single 1.0 mL aliquots were available for this retrospective study. The study did show four potential false negatives. Given the very high analytical sensitivity of this RHD genotyping assay (limit of detection = 5.5 copies/reaction, data not shown) it is highly likely that lack of detectable ccff DNA was indeed the case for these observations. Other possible explanations include chain-of-handling errors, technical errors during ccff DNA extraction or incorrect RhD serotype assignment of the newborn. To address the deficiency of insufficient or no ccff DNA for a particular sample in routine medical practice, the protocol for clinical samples calls for the availability of at least 4.0 mL of maternal plasma, which allows for repeat genotyping as well as the addition of a control assay for paternally inherited SNPs if necessary (van den Boom *et al*., 2006). This enables an important control, the assessment of paternally derived SNPs, the presence of which documents the existence of non-maternal DNA—essential for confirmation of a diagnosis when the fetus is predicted to be an RhD negative, female fetus. In the second sample cohort, both sensitivity and negative predictive value were 100%. The results of the second sample cohort enabled the validation and implementation of the RHD Genotyping LDT within a CLIA-certified laboratory (Jennings *et al*., 2009). The MALDI-TOF MS provides a platform that enables interrogation of >90 DNA polymorphisms that may be used to clarify the diagnosis in fetuses predicted to be RHD negative and female (van den Boom *et al*., 2006), improving both accuracy and excluding the samples that had a failed extraction or insufficient ccff DNA for analysis. By identifying candidate SNPs that are paternally inherited so as to differentiate maternal from fetal DNA, the prediction of an RhD negative female fetus can reliably be discerned from a failed assay (which would also result in a negative result).

The findings of this study show that the RHD Genotyping LDT using MALDI-TOF MS technology for the detection of the RHD gene exons 4, 5, and 7 located on chromosome 1 in the prediction of fetal RHD genotype from examination of ccff DNA in maternal blood can be achieved with a high accuracy in both the first and second trimesters of pregnancy. There are several clinical implications of this highly performing test for fetal RHD genotype. Firstly, for RhD negative pregnant patients with confusing or unclear antibody titers, direct fetal genotyping can aid in the clinical management of patients. Secondly, for sensitized patients in need of invasive prenatal diagnosis for genetic indications, noninvasive testing of fetal RHD genotype can identify those RHD negative fetuses that could benefit from invasive first trimester prenatal diagnosis by CVS. Third, for those non-sensitized RhD negative patients who are opposed to the administration of vaccines or other human blood products during pregnancy, the identification of an RHD positive fetus provides additional information to the clinician for determining the need for antepartum administration of RhD immune globulin. Lastly, as noted in the SAFE network Framework Six report, RHD NonInvasive Prenatal Diagnosis (NIPD) is currently used routinely in the European Union for the management of sensitized women. NIPD, however, has the potential to complement prevention (Freeman *et al*., 2006). For non-sensitized RhD negative pregnant women who have RHD negative fetuses (approximately 40% of cases), the decision *not* to administer RhD immune globulin can be discussed with the patient.

ACKNOWLEDGEMENTS

The study was supported by a grant from The Fetal Medicine Foundation (UK Charity No: 1037116). The funding for sample collection, processing and storage of samples was sponsored by the Sequenom Center for Molecular Medicine, 3595 John Hopkins Ct., San Diego, CA, USA. We thank L. Rawlings, K. Ingersoll, M. Dobb, T. Paladino, J. Stoerker, C. Koessel, S. Fitzgerald and N. Miltgen for excellent technical assistance and Dr Arnold Cohen for serving as the neutral third-party for comparison of laboratory results with the reference data.

## REFERENCES

American College of Obstetrics and Gynecology. 2006. Management of alloimmunization during pregnancy. ACOG Practice Bulletin #75. *Obstet Gynecol* **108**: 457–464.

Akolekar R, Farkas DH, VanAgtmael AL, Bombard AT, Nicolaides KH. 2010. Fetal sex determination using circulating cell-free fetal DNA (ccffDNA) at 11 to 13 weeks of gestation. *Prenat Diagn* **30**: 918–923.

Al-Yatama MK, Mustafa AS, Al-Kandari FM, *et al*. 2007. Polymerase-chain-reaction-based detection of fetal rhesus D and Y-chromosome-specific DNA in the whole blood of pregnant women during different trimesters of pregnancy. *Med Princ Pract* **16**: 327–332.

Blanco-Arias P, Sargent CA, Affara NA. 2002. The human-specific Yp11.2/Xq21.3 homology block encodes a potentially functional testis-specific TGIF-like retroposon. *Mammalian Genome* **13**: 463–468.

Brambati B, Guercilena S, Bonacchi I, Oldrini A, Lanzani A, Piceni L. 1986. Feto-maternal transfusion after chorionic villus sampling: clinical implications. *Hum Reprod* **1**: 37–40.

Brojer E, Zupanska B, Guz K, Orziñska A, Kaliñska A. 2005. Noninvasive determination of fetal RHD status by examination of cell-free DNA in maternal plasma. *Transfusion* **45**: 1473–1480.

Chim SS, Tong YK, Chiu RW, *et al*. 2005. Detection of placental epigenetic signature of maspin gene in maternal plasma. *Proc Nat Acad Sci* **102**: 14753–14758.

Costa JM, Giovangrandi Y, Ernault P, *et al*. 2002. Fetal RHD genotyping in maternal serum during the first trimester of pregnancy. *Br J Haematol* **119**: 255–260.

Daniels G, Finning K, Martin P, Massey E. 2009. Noninvasive prenatal diagnosis of fetal blood group phenotypes: current practice and future prospects. *Prenat Diagn* **29**: 101–107.

Ding C. 2008. Maldi-TOF mass spectrometry for analyzing cell-free fetal DNA in maternal plasma. *Methods Mol Biol* **444**: 253–267.

Ding C, Cantor CR. 2003. A high-throughput gene expression analysis technique using competitive PCR and matrix-assisted laser desorption ionization time-of-flight MS. *Proc Natl Acad Sci USA* **100**: 3059–3064.

Farkas DH, Miltgen NE, Stoerker J, *et al*. 2010. The suitability of matrix assisted laser desorption/ionization—time of flight mass spectrometry in a laboratory developed test using cystic fibrosis carrier screening as a model. *J Mol Diagn* **12**: 611–619.

Finning K, Martin P, Summers J, Massey E, Poole G, Daniels G. 2008. Effect of high throughput RHD typing of fetal DNA in maternal plasma on use of anti-RhD immunoglobulin in RhD negative pregnant women: prospective feasibility study. *BMJ* **336**: 816–818.

Finning KM, Martin PG, Soothill PW, Avent ND. 2002. Prediction of fetal D status from maternal plasma: introduction of a new noninvasive fetal RHD genotyping service. *Transfusion* **42**: 1079–1085.

Freeman K, Osipenko L, Clay D, Hyde J, Szczepura A 2006. Initial Report on NIPD Evidence Base: Prepared by Socio-Economic Group, University of Warwick for Workpackage 6, SAFE Network of Excellence, Special Noninvasive Advances in Fetal and Neonatal Evaluation (SAFE) CONTRACT No LSHB-CT-2004-503243.

Geifman-Holtzman O, Grotegut CA, Gaughan JP. 2006. Diagnostic accuracy of noninvasive fetal Rh genotyping from maternal blood—a meta-analysis. *Am J Obstet Gynecol* **195**: 1163–1173.

Grill S, Banzola I, Li Y, *et al*. 2009. High throughput noninvasive determination of foetal Rhesus D status using automated extraction of cellfree foetal DNA in maternal plasma and mass spectrometry. *Arch Gynecol Obstet* **279**: 533–537.

Jennings L, Van Deerlin VM, Gulley ML, College of American Pathologists Molecular Pathology Resource Committee. 2009. Recommended principles and practices for validating clinical molecular pathology tests. *Arch Pathol Lab Med* **133**: 743–755.

Jurinke C, Oeth P, van den Boom D. 2004. MALDI-TOF mass spectrometry: a versatile tool for high-performance DNA analysis. *Mol Biotechnol* **26**: 147–164.

Legler TJ, Muller SP, Haverkamp A, Grill S, Hahn S. 2009. Prenatal RhD testing: a review of studies published from 2006 to 2008. *Transfus Med Hemother* **36**: 189–198.

Li Y, Holzgreve W, Kiefer V, Hahn S. 2006. Maldi-tof mass spectrometry compared with real-time PCR for detection of fetal cell-free DNA in maternal plasma. *Clin Chem* **52**: 2311–2312.

Lo YM, Hjelm NM, Fidler C, *et al*. 1998. Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. *N Engl J Med* **339**: 1734–1738.

Machado IN, Castilho L, Pellegrino J Jr, Barini R. 2006. Fetal RHD genotyping from maternal plasma in a population with a highly diverse ethnic background. *Rev Assoc Med Bras* **52**: 232–235.

Minon JM, Gerard C, Senterre JM, Schaaps JP, Foidart JM. 2008. Routine fetal RHD genotyping with maternal plasma: a four-year experience in Belgium. *Transfusion* **48**: 373–381.

Oeth P, del Mistro G, Marnellos G, Shi T, van den Boom, D. 2009. Qualitative and quantitative genotyping using single base primer extension coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MassARRAY®). *Methods Mol Biol* **578**: 307–343.

Oeth P, Ehrich M, Lee M. 2008. Nucleic acid-based tests for RHD typing, gender determination, and nucleic acid quantification; United States Patent Application Publication; Pub. No.: US *2008/0299562;* Pub. Date: December 4, 2008; Filed: February 7, 2008. Inventors: Paul A. Oeth, San Diego, CA (US); Mathias Ehrich, San Diego, CA (US); Min S. Lee, San Diego, CA (US).

Pertl B, Sekizawa A, Samura O, Orescovic I, Rahaim PT, Bianchi DW. 2000. Detection of male and female fetal DNA in maternal plasma by multiplex fluorescent polymerase chain reaction amplification of short tandem repeats. *Hum Genet* **106**: 45–49.

Randen I, Hauge R, Kjeldsen-Kragh J, Fagerhol MK. 2003. Prenatal genotyping of RHD and SRY using maternal blood. *Vox Sang* **85**: 300–306.

Tabor A, Bang J, Nørgaard-Pedersen B. 1987. Feto-maternal haemorrhage associated with genetic amniocentesis: results of a randomized trial. *Br J Obstet Gynaecol* **94**: 528–534.

Tang NL, Leung TN, Zhang J, Lau TK, Lo YM. 1999. Detection of feta-derived paternally inherited X-chromosome polymorphisms in maternal plasma. *Clin Chem* **45**: 2033–2035.

Thongnoppakhun W, Jiemsup S, Yongkiettrakul S, *et al*. 2009. Simple, efficient, and cost-effective multiplex genotyping with matrix assisted laser desorption/ionization time-of-flight mass spectrometry of homoglobin beta gene mutations. *J Mol Diagn* **11**: 334–346.

Tynan JA, Angkachatchai V, Ehrich M, Paladino T, van den Boom D, Oeth P. 2010. Mulitiplexed analysis of circulating cell-free fetal nucleic acids for noninvasive prenatal diagnostic RHD testing. *Am J Obstet Gynecol* **204**(3): 251.e1–e6.

Van den Boom D, Oeth P, Ehrich M, *et al*. 2006. "Fetal Identifiers" as a universal approach for noninvasive prenatal diagnostics. *Am J Obstet Gynecol* **195**: S714, #570.

Van der Schoot CE, Hahn S, Chitty LS. 2008. Non-invasive prenatal diagnosis and determination of fetal Rh status. *Semin Fetal Neonatal Med* **13**: 63–68.

Van der Schoot CE, Soussan AA, Koelewijn J, Bonsel G, Paget-Christiaens LG, de Haas M. 2006. Noninvasive antenatal RHD typing. *Transfus Clin Biol* **13**: 53–57.

Wright CF, Buton H. 2009. The use of cell-free fetal nucleic acids in maternal blood for non-invasive prenatal diagnosis. *Human Reproduction Update* **15**: 139–151.

Zhang J, Fidler C, Murphy MF, *et al*. 2000. Determination of fetal RhD status by maternal plasma DNA analysis. *Ann N Y Acad Sci* **906**: 153–155.

Zhu B, Sun Q-W, Lu Y-C, Sun M-M, Wang L-J, Huang X-H. 2005. Prenatal fetal sex diagnosis by detecting amelogenin gene in maternal plasma. *Prenat Diagn* **25**: 577–581.