

Determination of Fetal RhD Status by Maternal Plasma DNA Analysis

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The prenatal determination of fetal rhesus D (RhD) status is useful for the management of sensitized RhD-negative women. Conventional methods for prenatal RhD status determination involve invasive methods such as amniocentesis.¹ The discovery of circulating cell-free fetal DNA in maternal plasma and serum opens up a new source of fetal genetic material for prenatal RhD status determination.² Indeed, this possibility has recently been realized by three groups who demonstrated the presence of fetal-derived *RHD* gene sequences in RhD-negative women bearing RhD-positive fetuses.^{3–5} The detection system used by our group is a homogenous assay based on real-time PCR analysis^{3,6} using exon 10 of the *RHD* gene. However, as it has been advocated that at least two independent PCR systems should be used for the clinical diagnosis of fetal RhD status, we describe, in this communication, a second real-time PCR assay for RhD status determination using sequences derived from exon 7 of the *RHD* gene.

RESULTS AND DISCUSSION

Primers for the exon 7 real-time *RHD* PCR system were as described by Faas *et al.*⁴ A fluorogenic probe (RD7T), internal to the primers, was designed. The sequence of RD7T was 5'-FAM AGC TTG CTG GGT CTG CTT GGA GAG ATC TAMRA-3'. FAM and TAMRA were the fluorescent reporter and quencher as previously described.³

Real-time PCR was carried out in duplicate in a final volume of 50 μ L using components provided in a TaqMan PCR Core Reagent Kit (Perkin-Elmer, U.S.A.). A sample was scored as RhD-positive when at least one of these duplicate reactions gave a positive result. The reaction mixture contained 1 $\times$ reaction buffer A; 200 μ M each of dATP, dCTP, and dGTP; 400 μ M of dUTP; 4 mM of MgCl₂; 300 nM of each

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TABLE 1. Fetal RhD status detected by real-time PCR analysis of exon 7 of the *RHD* gene

Trimester	RhD-positive fetus ^a		RhD-negative fetus ^a	
	Number	PCR positive	Number	PCR negative
First	12	11 (91.7%)	8	8 (100%)
Second	12	12 (100%)	13	13 (100%)
Third	8	8 (100%)	5	5 (100%)

^aRhD status as determined by serologic typing of fetal cord blood for first- and third-trimester samples, and RhD PCR analysis using amniotic fluid for second-trimester samples.

primer; 25 nM of the RD7T TaqMan probe; 1.25 U of AmpliTaq Gold; and 0.5 U of AmpErase uracil *N*-glycosylase. Five μ L of plasma DNA extracted using a QIAamp spin column (Qiagen, Germany) was used for amplification as described.⁷ The amplification reaction was carried out in an ABI Prism 7700 Sequence Detector as previously described.⁷ The thermal profile consisted of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

The initial evaluation of the exon 7 real-time *RHD* PCR system involved the application of this system to peripheral blood DNA extracted from 42 blood donors (16 RhD-positive and 26 RhD-negative), collected by the Department of Hematology at John Radcliffe Hospital (Oxford, UK). Real-time PCR results were in complete concordance with serology. The application of the PCR system to serial dilutions of DNA from an RhD-positive individual indicated that the system was sensitive enough to detect the DNA equivalent from a single cell.

This real-time PCR system was then applied to plasma samples from 58 RhD-negative pregnant women recruited from the Nuffield Department of Obstetrics and Gynecology at John Radcliffe Hospital (Oxford, UK). First-trimester samples were obtained during a routine antenatal checkup. Second-trimester samples were obtained prior to amniocentesis, when 10 mL of amniotic fluid was taken for confirmation of fetal RhD status by PCR. Third-trimester samples were collected just prior to parturition. Cord blood samples from the fetuses recruited from the first and third trimesters were obtained at delivery and fetal RhD status was confirmed by serology. The results of maternal plasma PCR analysis are listed in TABLE 1, indicating that accurate prenatal fetal RhD genotyping was achieved in the great majority of cases.

These results thus confirmed previous observations by our group and others that fetal RhD genotyping is possible by maternal plasma/serum analysis.^{3–5} Our present work has provided a second real-time homogenous *RHD* PCR assay suitable for this type of analysis. It is likely that the combination of the current exon 7 assay and our previously described exon 10 system will allow even more robust prenatal analysis to be undertaken. The ability of the TaqMan system to accommodate multiple fluorescent labels⁸ potentially allows further development of the system into a time-efficient multiplex format.

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