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A Multiplex PCR for Non-invasive Fetal *RHD* Genotyping Using Cell-free Fetal DNA

GEORGIA TOUNTA[1], CHRISTINA VRETTOU[1], AGGELIKI KOLIALEXI[1], NIKOLAS PAPANTONIOU[2], ASPASIA DESTOUNI[1], GEORGE T. TSANGARIS[3], ARIS ANTSAKLIS[1], EMMANUEL KANAVAKIS[1] and ARIADNI MAVROU[1]

[1]*Department of Medical Genetics, and* [2]*First Department of Obstetrics and Gynecology, Athens University School of Medicine, Athens, Greece;*
[3]*Division of Biotechnology, Centre of Basic Research, Foundation of Biomedical Research of the Academy of Athens, Athens, Greece*

Abstract. *Aim: To design a protocol for non-invasive prenatal diagnosis of fetal Rhesus D (RhD) status. Materials and Methods: A total of 112 single lymphocytes were used to test the efficiency of the assay. The protocol was validated using blood samples from 84 RhD-negative pregnant women at 7-24 weeks of gestation. Cell-free DNA (cfDNA) was enzymatically digested using AciI and analyzed by a polymerase chain reaction (PCR) that allowed simultaneous amplification of RHD exons 7 and 10, SRY, RASFF1A and ACTB. Results: On the one genome-equivalent level, the efficiency of the protocol was ≥94.6% for each locus amplified. Conclusive results from the first set of PCRs were obtained for 79 cases with one false-positive. In five cases the analysis was repeated and, subsequently, all cases were accurately diagnosed. Conclusion: The proposed protocol is rapid, applicable in most molecular diagnostic laboratories and provides the basis for non-invasive examination of fetal RhD with 96.7% specificity and 100% sensitivity*

Prenatal determination of the fetal Rhesus D (RhD) status in RhD-negative women at risk of carrying a RhD-positive fetus can be achieved by amniocentesis or chorionic villus sampling (CVS) (1, 2). Both procedures, however, carry a risk not only for miscarriage but also for maternal sensitization (3, 4). Non-invasive prenatal detection of fetal *RHD* sequences in maternal plasma was first shown to be feasible in 1998 (5). Presently, technical improvements in cell-free fetal DNA (cffDNA) isolation and analysis have achieved a sensitivity of 99% and specificity of 99.88% in *RHD* fetal genotyping (6-10). However, a drawback of these genotyping assays is the possibility of false-negative results, which may be due to low levels of cffDNA in maternal blood (especially if the sample is acquired early in pregnancy), loss of cffDNA during processing and/or use of an insufficiently sensitive genotyping assay (11). The availability, therefore, of a fetal-specific marker which can confirm the presence of cffDNA in the sample is a useful safeguard measure.

Several strategies have been proposed in order to verify the presence of cffDNA in maternal plasma. Detection of *SRY* sequences is the obvious one, but is only applicable in pregnancies with male fetuses (7, 12). Other approaches rely on the detection of repeat sequences or bi-allelic polymorphisms (13). This strategy, however, is limited by the fact that the robustness of *RHD* assays does not match the assays directed towards some bi-allelic markers and the process is labor intensive. Most importantly, bi-allelic polymorphic markers are not informative in more than 60% of cases due to low heterozygosity rates between the parents (14, 15). A different approach includes the use of universal fetal DNA markers that identify epigenetic differences between fetal and maternal DNA sequences (16).

It has been shown that the DNA methylation profile of CpG islands in the tumor suppressor gene mammary serine protease inhibitor (maspin, *SERPINB5*) is hypomethylated in the placenta but hypermethylated in maternal blood cells (17). Methylation analysis, however, of *SERPINB5* requires bisulfite conversion which can lead to DNA degradation, thus decreasing the efficiency of hypomethylated *SERPINB5* detection as a positive control for the presence of fetal DNA in a routine clinical setting (18).

More recently, the promoter region of the tumor suppressor gene Ras association (RalGDS/AF-6) domain family member 1 (isoform A) gene (*RASSF1A*) was proposed as a possible epigenetic fetal marker. *RASSF1A* is hypermethylated in the placenta but hypomethylated in maternal blood cells (12). In

Correspondence to: Aggeliki Kolialexi, MD, PhD, Department of Medical Genetics, Athens University School of Medicine, Aghia Sofia Children's Hospital, Thivon & Levadias, 115 27, Athens, Greece. Tel: +30 2107467462, Fax: +30 2107795553, e-mail: akolial@med.uoa.gr

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this case, methylation analysis can be performed using methylation-sensitive endonuclases, which cut maternal hypomethylated *RASSF1A* sequences, leaving intact for amplification those of placental origin.

The aim of the present study was to design and optimize a rapid and easily applicable two-step protocol for the determination of fetal RhD status in RhD-negative pregnant women. The protocol involves enzyme digestion of maternal plasma cell-free DNA (cfDNA) using a methylation-specific endonuclease, followed by a multiplex polymerase chain reaction (PCR) that allows simultaneous amplification of exons 7 and 10 of the *RHD* gene, *SRY, RASSF1A* and *ACTB* sequences. Amplification of these loci provides an indication of the fetal RhD status, confirming, at the same time, the presence of cffDNA and allowing assessment of digestion efficiency. The protocol was validated for clinical use in 84 RhD-negative pregnant women.

Materials and Methods

Samples. Genomic DNA from ten blood donors (five RhD-positive males and five RhD-negative non-pregnant females), plasma cfDNA from four pregnant and four non-pregnant RhD-negative women and DNA from five CVS were used to standardize the multiplex PCR assay and to evaluate enzyme digestion efficiency for unmethylated *RASSF1A* and *ACTB* sequences.

A total of 120 single lymphocytes, obtained from RhD-positive males (n=60) and RhD-negative females (n=60) were used to test the sensitivity and accuracy of the designed multiplex-PCR protocol. Single cells were collected by micromanipulation and placed in 10 μl double-distilled water in 0.2 ml DNAse-free, RNAse-free Eppendorf tubes (1 cell/tube) prior to further processing (19).

Following standardization, the protocol was validated for RhD NIPD using blood samples collected from 84 RhD-negative pregnant women (eight immunized and 76 previously non-immunized) at 7-24 weeks of gestation, during a routine prenatal visit or before an invasive procedure for prenatal karyotyping. Samples (3-6 ml) of maternal blood were collected from each woman in ethylenediaminetetraacetic acid (EDTA). In all cases the fetal *RHD* genotype was confirmed either by testing genomic DNA obtained from amniotic fluid cells or serologically at birth.

The study was approved by the Ethics Committee of Athens University and informed consent was obtained from all donors and pregnant women.

Template preparation. Genomic DNA was extracted from 200 μl EDTA whole-blood or 0.2 *g* CVS, using QIAamp DNA Blood Minikit (Qiagen Inc., Hilden, Germany), following the manufacturer's instructions. Single-cell DNA was released as previously described (19).

Isolation of cfDNA. Plasma was separated within 48 h after blood collection by two serial centrifugations, at 2,200 ×*g* for 15 min and 14000 ×*g* for 10 min. Two aliquots of the sample, approximately 800 μl each, were stored at –80˚C in polypropylene cryogenic vials until further processing. CfDNA was isolated from 500-μl thawed maternal plasma using the QIAamp DSP Virus Kit (Qiagen Inc.), according to the manufacturer's instructions. Each series included five samples collected from RhD-negative pregnant women and one negative control from a non-pregnant RhD-negative female, to test for contamination during cfDNA isolation. cfDNA was eluted in 50 μl of double-distilled water, placed in two separate siliconized Eppendorf tubes (25 μl/tube) (Axygen Scientific Inc, Union City, CA, USA) and either processed immediately or stored at –80˚C.

Multiplex PCR. A multiplex PCR was designed to allow for the simultaneous amplification of specific *RHD* sequences (exons 7 and 10), *SRY, RASSF1A* and *ACTB*, with Cy5.5 fluorescently-labeled forward primers (Table I). PCR was initially optimized using as template 20-50 pg of genomic DNA (approximately 4-10 g.e.) from blood donors, by testing different annealing temperatures ranging from 53 to 62˚C, primer concentrations from 0.4 to 2 μM and the addition of betaine (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and/or DMSO (Sigma-Aldrich Chemie GmbH). The multiplex PCR reaction was finally set up in a volume of 50 μl which included 25 μl of premixed buffer/dNTPs/$MgCl_2$/Taq polymerase (HotStarTaq; Qiagen Inc.), primers for all five sequences to be amplified (Table I), betaine and DMSO at a final concentration of 8% and 4%, respectively. The first denaturation step at 95˚C for 15 min was followed by 40 cycles of 95˚C for 30 s, 55˚C for 45 s, 72˚C for 45 s and a final extension step at 72˚C for 10 min. The fluorescently (Cy5.5)-tagged PCR-generated products of the five regions were sized and analyzed on a VisibleGenetics OpenGenet System automatic DNA sequencer with Gene Objects software (Visible Genetics, High Wycombe, UK). The expected length of each amplified sequence is noted in Table I. A no-template control, containing DNA-free water, was also included, in order to test for contamination during the PCR set up.

The efficiency of the optimized multiplex PCR protocol was evaluated at the one genome-equivalent DNA level using as template 15 μl of each lysed single lymphocyte. Only lymphocytes where at least one locus was amplified were considered for evaluation.

Enzyme digestion for unmethylated RASSF1A and ACTB. *Aci* I (New England Biolabs, Inc., Ipswich, MA, USA) was selected as an appropriate methylation-sensitive restriction enzyme for cfDNA digestion, using the RestrictionMapper database (http://www.restrictionmapper.org). Selection was based on the ability of *Aci* I to selectively digest only the unmethylated *RASSF1A* and *ACTB* sequences, while the *RHD* and *SRY* sequences remain intact.

Digestion efficiency and expected methylation pattern were tested using genomic DNA from RhD-positive male donors, CVS, as well as plasma DNA from RhD-negative pregnant and non-pregnant females. Samples of genomic DNA (20-50 ng) and plasma DNA (18 μl), along with a blank, in a final reaction volume of 25 μl, were digested using 10 U of *Aci*I and 1× NEBuffer 3 (New England Biolabs, Inc.) at 37˚C. Various incubation periods ranging from 10 min to 16 h were tested, followed by enzyme inactivation at 65˚C for 20 min in a thermoblock. Samples of digested genomic DNA (10 μl) and digested plasma DNA (16 μl) were used as a template for the optimized multiplex PCR immediately after enzyme digestion.

Clinical validation. In the clinical validation study no more than five cfDNA samples were simultaneously processed. In the same reaction, plasma DNA obtained from an RhD-negative non-pregnant female, a genomic DNA sample obtained from a RhD-positive male blood donor, CVS DNA and a no-template sample were digested and used as controls to verify the efficiency and specificity of enzyme digestion for unmethylated *RASSF1A* and *ACTB* sequences and the absence of contamination.

Table I. *Sequences and characteristics of primers used in the multiplex PCR assay.*

Target	Name	Sequence	Product size (bp)	PCR Concentration (μM)	Reference
RHD exon 7	RHD-ex7 f[a]	5'-GGG TGT TGT AAC CGA GTG CTG-3'	125	0.4	(31)
RHD exon 7	RHD-ex7 r	5'-CCG GCT CCG ACG GTA TC-3'		0.4	
RHD exon 10	RHD-ex10 f[a]	5'-CTC TCA CTG TTG CCT GCA T-3'[b]	134	0.4	
RHD exon 10	RHD-ex10 r	5'-ATG GTG AGA TTC TCC TCA AAG AGT-3'[b]		0.4	
SRY	SRY f[a]	5'-GGC AAC GTC GTC CAG GAT AGA GTG A-3'	115	0.4	(32)
SRY	SRY r	5'-TGC TGA TCT CTG AGT TTC GCA TT-3'		0.4	
RASSF1A	RSF f[a]	5'-AGC CTG AGC TCA TTG AGC TG-3'	130	1.2	(12)
RASSF1A	RSF r	5'-ACC AGC TGC CGT GTG G-3'		1.2	
β-Actin	ACTB f[a]	5'-GCG CCG TTC CGA AAG TT-3'	150	1.2	(12)
β-Actin	ACTB r	5'-GGG TGT GGA CGG GCG-3'[b]		1.2	

[a]5' Fluorescenly labeled with Cy5.5; [b]PCR primers designed with the aid of the computer software Amplify; f, forward primer; r, reverse primer.

Table II. *Criteria used for the interpretation of protocol results.*

Pattern	Replicate 1		Replicate 2		Concluded fetal RhD status	No of cases
	Exon 7	Exon 10	Exon 7	Exon 10		
1	-	-	-	-	RhD-negative	26
2	+	+	+	+	RhD-positive	48
3	+	+	+	-	RhD-positive	3
4	+	+	-	+	RhD-positive	0
5	+	+	-	-	RhD-positive	1
6	+	-	-	+	RhD-positive	0
7	+	-	+	-	RhD-positive[a]	0
8	-	+	-	+	To be considered RhD-positive[b]	1
9	+	-	-	-	Inconclusive result, test should be repeated by a second DNA extraction from the same sample	0
10	-	+	-	-	Inconclusive result, test should be repeated by a second DNA extraction from the same sample	0
						Total=79

[a]Analysis of a second sample is recommended later during pregnancy; [b]parental DNA should be tested for *RHD* variant alleles.

Two lysed single lymphocytes (one from an RhD-negative female and one from an RhD-positive male) and a DNA-free water sample were amplified, along with all digested samples in each set of reactions, to prove successful amplification at the level of one genome-equivalent and the absence of contamination during the PCR set up, respectively.

Interpretation criteria. Results were evaluated only if: (i) no amplification was observed in the DNA-free water samples and (ii) the specificity and efficiency of the *Aci*I enzyme digestion were confirmed in CVS and RhD-positive male genomic DNA controls. Fetuses were characterized as RhD-negative following the patterns described in Table II.

Results

Single lymphocyte analysis. Eight out of the 120 single lymphocytes initially isolated were excluded from the analysis since no amplification was obtained in any locus, possibly due

Table III. *Results obtained from multiplex PCR application on single cells.*

Donor	PCR amplification				
	RHD exon7 No. cells (%)	*RHD* exon10 No. cells (%)	*SRY* No. cells (%)	*RASSF1A* No. cells (%)	*ACTB* No. cells (%)
Male RhD-positive (n=55)	55 (100)	52 (94.54)	53 (96.36)	55 (100)	51 (92.73)
Female RhD-negative (n=57)	0 (0)	0 (0)	0 (0)	56 (98.25)	57 (100)
Total (n=112)	-	-	-	111 (99.1)	108 (96.4)

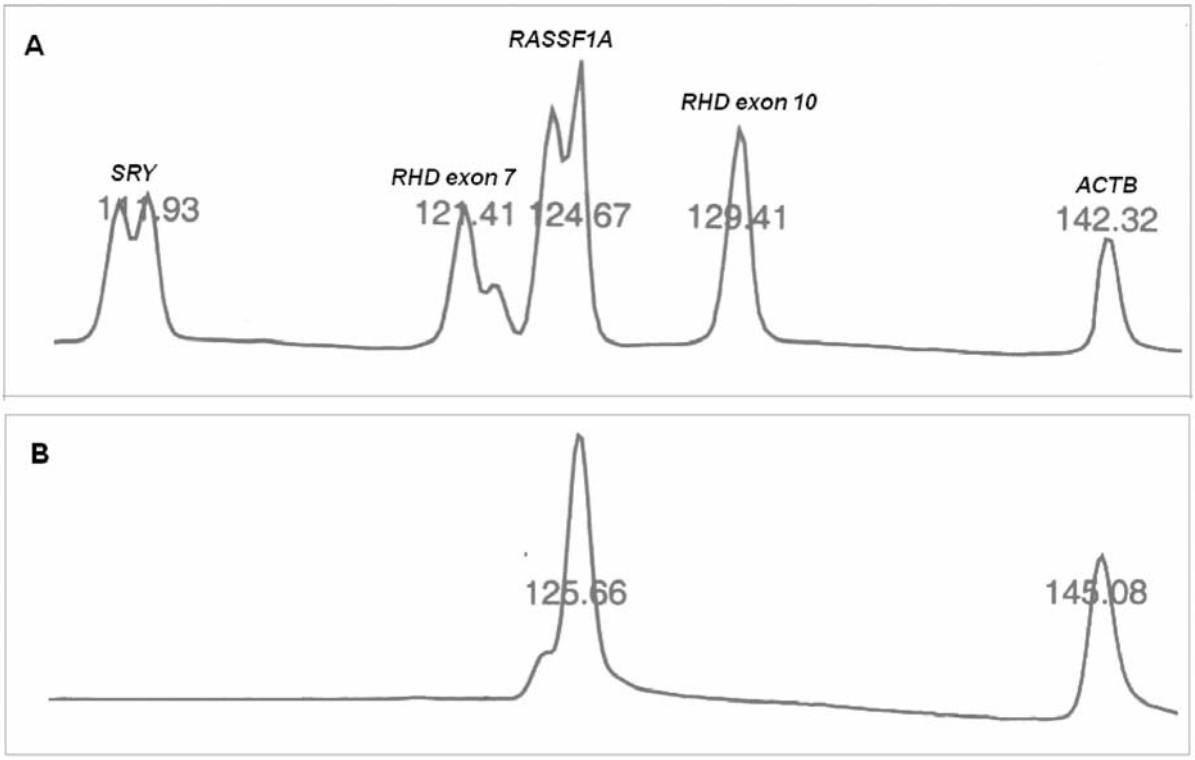

Figure 1. *PCR amplification of the five tested loci in a single lymphocyte obtained from A: RhD-positive male and B: RhD-negative female. Numbers are provided by the automatic DNA sequencer and correspond to the size of the fragments (bp) (Table I).*

to unsuccessful cell isolation. Evaluation of PCR success was, therefore, performed for each locus in a total of 112 single lymphocytes based on the detection of a PCR product of the expected amplicon (Figure 1). In single cells obtained from 55 RhD-positive males, *RHD* exons 7 and 10 and *SRY* sequences were successfully amplified in 55, 52 and 53 single cells, respectively (Table III). No amplification was noted in any of the 57 single cells from RhD-negative females. Thus PCR efficiency, on the one genome-equivalent level, for each of the three sequences tested was ≥94.5%. *RASFF1A* and *ACTB* sequences were amplified in 111/112 and 108/112 cells analysed, respectively, resulting in PCR efficiency ≥96.4%.

Enzyme digestion specificity for unmethylated RASSF1A and ACTB. Following enzyme digestion, *RASSF1A* sequences were amplified only in CVS and plasma DNA samples obtained from pregnant women and were completely absent from genomic DNA and plasma cfDNA coming from non-pregnant females. *ACTB* sequences were absent from all samples analysed, indicating successful enzyme digestion. In addition, *RHD* and *SRY* sequences were normally amplified, confirming that the selected enzyme has no restriction sites on these sequences. Complete digestion was achieved at all incubation times tested and therefore a 10 min digestion period was selected.

Clinical validation. Conclusive results from the first set of reactions were obtained in 79 cases (94%). In total, 53 RhD-positive and 26 RhD-negative fetuses were identified (Table II and Figure 2). In 78/79 cases, non-invasive prenatal *RHD* genotyping was concordant with that reported from the analysis of amniotic fluid cells or serologically determined at birth. In one sample, a serologically RhD-negative male fetus was incorrectly predicted as being RhD-positive. In another case, only exon 10 was amplified in both plasma and amniotic fluid cells (Table II, pattern 8). In this case, the presence of an *RHD* variant allele was suspected, but parental DNA samples were not available for further investigation. No false-negative results were observed in this study.

In 26 samples with no *RHD* amplification, the presence of cffDNA was confirmed by the amplification of *RASSF1A* and/or *SRY* sequences. Specifically, SRY sequences were detected in 12 samples (46%) and in 14 cases (54%) only amplification of enzyme digestion–resistant *RASSF1A* sequences was noted, indicating the existence of fetal material in the sample.

Five cases (6%) were considered inconclusive. In three of them, no amplification was obtained in any of the five loci and in the remaining two amplification of *RASSF1A* along with *ACTB* sequences was observed, indicating incomplete enzyme

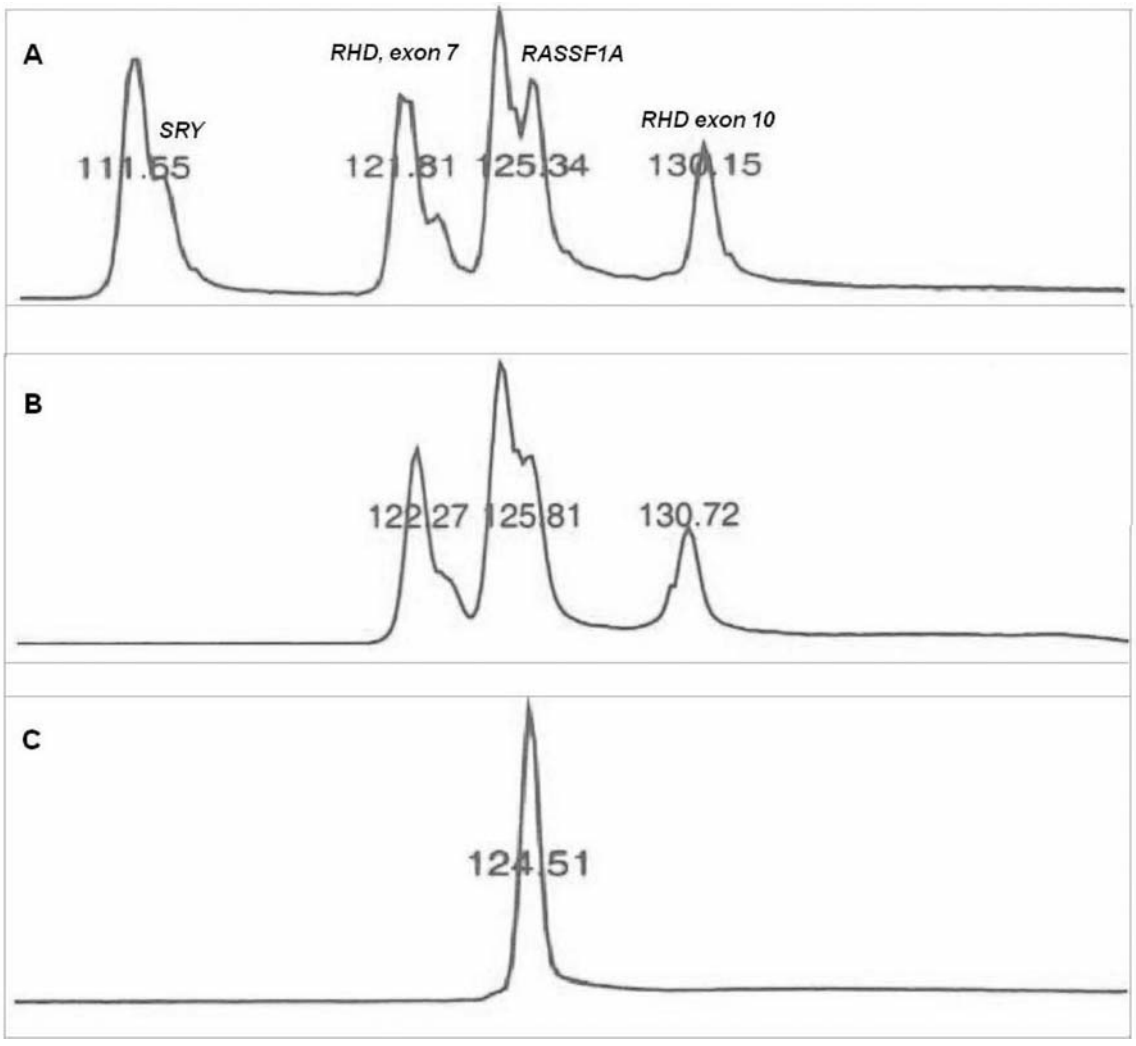

Figure 2. *Application of the multiplex PCR protocol in maternal plasma cffDNA, following enzyme digestion. A: RhD-positive male fetus, B: RhD positive female fetus and C: RhD-negative female fetus. Numbers are provided by the automatic DNA sequencer and correspond to the size of the fragments (bp) Table I).*

digestion. The analysis was repeated using cfDNA isolated from stored plasma and all five cases were correctly diagnosed.

Overall, after exclusion of the case with the suspected *RHD* variant allele, accurate diagnosis was achieved in 98.8% of cases, with 3.3% false-positive rate, resulting in 96.7% specificity and 100% sensitivity.

Discussion

This study reported, for the first time, the application of a multiplex PCR-based protocol for the simultaneous detection of four fetal loci in maternal plasma. This assay provided, in one step, confirmation of the presence of cffDNA and of the fetal *RHD* status and precluded further post-PCR handling, in cases of RhD-negative fetuses (12, 20). Contrary to methods currently used which detect Y-chromosome sequences and genetic variations between the fetus and the mother, in order to prove the presence of cffDNA in maternal plasma, the proposed method is independent of fetal gender and parental polymorphisms. (7, 8, 12, 21). In 14 maternal blood samples analysed, where neither *RHD* nor *SRY* amplification were detected, the presence of fetal DNA was ascertained through the amplification of *RASFF1A* sequences, confirming an RhD-negative female fetus and avoiding the need for a second sample testing.

Although the *RASSF1A/ACTB* system has already been used by several groups in order to confirm the presence of cffDNA, this was the first time that this fetal-specific marker was incorporated in a multiplex PCR assay for routine NIPD testing in clinical practice (12, 20, 22-24). However, since the protocol

involves the promoter region of *RASSF1A*, which is a tumor suppressor gene, it may be of little value when testing pregnant women with a medical history of cancer (12, 25).

Aci I was selected as the appropriate methylation-specific restriction enzyme. During enzyme digestion optimization, *Aci*I completely digested template DNA in 10 min. Using the optimized protocol, complete digestion of the internal control *ACTB* sequences was achieved in 82/84 samples tested. Similar results were obtained in studies when two enzymes were simultaneously used for longer incubation times (24). Although it is known that there are three *Aci*I restriction sites within the *RASSF1A* amplicon and five *Aci*I restriction sites within the *ACTB*, both sequences were sufficiently degraded, as indicated by the simultaneous digestion of genomic and cfDNA (not containing fetal sequences) which were used as external controls in each set of reactions.

Prior to its clinical validation, the efficiency of the multiplex PCR was evaluated in single lymphocytes. Successful amplification ≥94.5% for each of the five loci analyzed, at this low DNA concentration, indicated high efficiency of the method, granting it suitable for NIPD. There is general agreement that more than one region of the *RHD* gene should be tested for *RHD* typing, since the high complexity of the Rh system may lead to false results (26). In the present study, amplification of exon 7 was shown to be more sensitive for *RHD* genotyping (Table III), but as is known, exon 7 primers do not detect hybrids *RHD*-CE(3-7)-D or *RHD*-CE(2-9)-D present in some RhD-negative individuals of African descent, giving false-negative results in some D variants (26). Rouillac-Le Sciellour *et al*. analyzed 893 RhD-negative pregnant women of various ethnic backgrounds using exons 7 and 10 primers and identified 42 samples exhibiting a non-functional or rearranged *RHD* gene (9). In the present study of Caucasian women, this discrepancy was observed in one case, when only exon 10 was amplified in both maternal plasma and amniotic fluid cells. Parents were invited for further analysis but they did not respond. The PCR set up, however, does not cover all genetic *RHD* variants and *RHD* genes rare in the European population, such as *RHDψ*, and carriers of the pseudogene will be detected as RhD-positive. Primers for the detection of *RHDψ* were not incorporated in the multiplex PCR since the variant is not frequent in the local population and no samples were available for validation of the assay. A single PCR for the detection of *RHDψ*, however, was optimized, using dilutions of genomic DNA (kindly provided by Professor Tobias Legler, Department of Transfusion Medicine at the University Hospital Gottingen, Germany) and is ready for use in cases where patients of African origin request the test.

The application of the proposed technique gave conclusive results appropriate for clinical management in 82 out of 83 cases (after exclusion of the suspected *RHD* variant case). The one discordant result, obtained at the beginning of the study, was possibly due to contamination during cfDNA isolation, since the possibilities of an anembryonic pregnancy or a vanishing twin were excluded (14, 27). To minimize sample contamination, precautions should be most stringent and even the cfDNA isolation kit reagents should be aliquoted and used once by properly trained personnel.

During protocol validation, only two PCR replicates from a single cfDNA isolation of each sample were performed. Fetal RhD status was correctly reported, after one trial, in 77/83 cases. In five cases, inconclusive results were obtained. Although this may be due to low levels of cffDNA in early gestation (28), in this study, there was no correlation with gestational age, since the particular samples came from pregnancies that were more than 10 weeks old. Inconclusive results, therefore, were attributed to failure to isolate or amplify cffDNA sequences.

When the proposed protocol is applied in clinical practice, it is recommended that cfDNA is isolated from two separate plasma aliquots and each one is analysed twice so that a total of four PCR replicates is available for reliable fetal RhD determination. When the results are unclear or inconclusive, analysis may be repeated increasing the number of tested replicates to eight. Preliminary application of the protocol in ten cases for clinical management confirmed the reliability of this approach (data not shown).

The protocol has been designed primarily to accurately report the fetal *RHD* genotype, but it may also be useful for fetal gender determination. The assay accurately identified fetal gender in 100% of cases tested, but is not recommended for clinical use as such, since other male-specific sequences should be added (14, 15, 29, 30). In addition, the sensitivity of the test for fetal gender determination at <10 weeks of gestation should be tested in order to evaluate its accuracy in cases of gender-linked and endocrine disorders.

In conclusion, the proposed protocol is rapid, robust and applicable in most molecular diagnostic laboratories and provides a good basis for non-invasive routine examination of fetal RhD status of immunized RhD-negative women as well as for screening for fetal *RHD* typing in non-immunized, RhD-negative pregnant women.

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