

Original article

Noninvasive fetal *RHD* genotyping from maternal plasma Use of a new developed Free DNA Fetal Kit RhD[®]

Détermination non invasive du génotype foetal Rhésus D Utilisation d'une nouvelle trousse de génotypage *Free DNA Fetal Kit RhD*[®]

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Abstract

Fetal *RHD* genotyping from maternal plasma was performed by real-time PCR amplification of exons 7 and 10 of the *RHD* gene and the amplified products were detected either with SYBR Green I dye according to our previously published method [Mol Diagn 8 (2004) 23–31] or with hydrolysis probes in a new Free DNA Fetal Kit RhD[®]. Plasma specimen from 300 RhD-negative pregnant women (between 10 to 34 weeks of gestation) were analysed and validation of the results was ascertained either by *RHD* genotyping on amniotic cells or by blood typing of the neonate at birth. We found 100% concordant results when comparing the two methods. Two false-positive but no false-negative results were found. Thus, the sensitivity of the assay was 100% and the specificity superior than 99%. These data confirm the accuracy of fetal *RHD* genotyping on maternal plasma using the Free DNA Fetal Kit RhD[®], thus allowing to propose non invasive PCR-based fetal *RHD* genotyping for all RhD-negative pregnant women and to restrict the use of anti-D immunoglobulins only to those bearing an RhD-positive fetus.

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Résumé

Le génotypage *RHD* foetal à partir du plasma maternel a été effectué par amplification PCR en temps réel des exons 7 et 10 du gène *RHD* et détection des produits d'amplification soit par incorporation du colorant SYBR Green I selon une méthode publiée auparavant [Mol Diagn 8 (2004) 23–31], soit à l'aide d'une nouvelle trousse diagnostic *Free DNA Fetal Kit RhD*[®]. Une cohorte de 300 femmes de phénotype RhD-négatif enceintes de dix à 34 semaines de gestation a été analysée et les résultats ont été validés soit par génotypage sur des cellules amniotiques, soit par le phénotype Rh de l'enfant à la naissance. Nous avons observé une concordance totale entre les deux méthodes de génotypage. Deux faux-positifs, mais aucun faux-négatif n'ayant été identifiés ; la sensibilité du test est de 100 % et la spécificité supérieure à 99 %. Ces résultats confirment la précision du génotypage *RHD* foetal à partir du plasma maternel basé sur la trousse *Free DNA Fetal Kit RhD*[®]. Un génotypage foetal *RHD* non invasif peut donc être proposé à toutes les femmes enceintes de phénotype RhD-négatif afin de restreindre l'injection d'Ig anti-D uniquement à celles portant un foetus RhD-positif.

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Keywords: Rhesus (Rh); *RHD* genotyping; Fetal DNA; Maternal plasma; Hemolytic disease of the newborn; Anti-D prophylaxis

Mots clés : Rhésus (Rh) ; Génotypage *RHD* ; ADN foetal ; Plasma maternel ; Maladie hémolytique du nouveau-né ; Prophylaxie anti-D

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1. Background

The discovery of the Rhesus (Rh) blood group system is closely associated with the first description of the hemolytic disease of the newborn (HDN), a severe affection most often caused upon fetal RhD-positive red blood cells (RBC) destruction by placenta transfer of anti-D antibodies present in the blood of an immunized RhD-negative pregnant mother. In France, the incidence is about 1/1000 birth and may lead, in the absence of treatment, to stillbirth in 15% of cases. Since the 1960s, the introduction of an immunoprophylaxis by passive anti-D administration to RhD-negative mothers within 72 h following delivery of a RhD-positive child, as well as curative improvements (phototherapy, fetal transfusion), contributed to reduce the mortality by 90%, at least in countries where national programs to control the disease have been implemented [1]. Currently, about 160,000 women in France benefited this immunoprophylaxis each year, including those RhD-negative that delivered a RhD-positive child (70,000), spontaneous abortions (20,000), elective termination of pregnancy (40,000), amniocentesis for chromosomal anomaly detection (10,000) and every cause of fetomaternal microtransfusion (20,000). Importantly, a systematic antenatal immunoprophylaxis, particularly when the *RHD* status of the fetus is unknown, has the drawback of useless administration of Ig anti-D (with associated potential risks) in the 40% of cases where the fetus is RhD-negative. A targeted Rh immunoprophylaxis to non immunized RhD-negative women carrying a RhD-positive fetus is therefore highly desirable [2].

In the 1990s, the molecular basis of the RH blood group system and RhD+/RhD- polymorphism [3,4] was elucidated allowing the first prenatal determination of fetal *RHD* genotype early during pregnancy, by PCR amplification of DNA from amniotic cells or chorionic villus samples [5,6]. Indeed, the *RH* locus is composed of two homologous genes *RHD* and *RHCE* (96% homology) closely linked on chromosome 1p34–p36, and the *RHD*-pos/*RHD*-neg polymorphism is associated, at least in Caucasians where it is more frequent than in other populations, to the presence or absence of the *RHD* gene, respectively [4]. Accordingly, the *RHD*-positive haplotype is composed of the two genes, whereas the *RHD*-negative haplotype only carries the *RHCE* gene (Fig. 1A). Therefore, the first generation of PCR tests was based on a single *RHD* gene region amplification (exon 10). Subsequent studies revealed an additional level of complexity showing evidence for a number of *RHD* variants [7,8] and occurrence of RhD-negative individuals (rare) harboring a copy of a *RHCE* gene and of a silent *RHD* gene [9]. Various mechanisms may explain these unusual phenotypes, including gene conversion events between homologous *RHD* and *RHCE* producing hybrid genes, as well as nonsense mutations, and deletions or insertions interrupting the reading frame of the messenger RNA [for a compilation of Rh variants see <http://www.uni-ulm.de/~wflegel/RH/> and http://www.ncbi.nlm.nih.gov/projects/mhc/xslcgi.fcgi?cmd=bgmut/systems_info&system=rh]. In the black population of African origin, the most common RhD-negative phenotype is caused by the

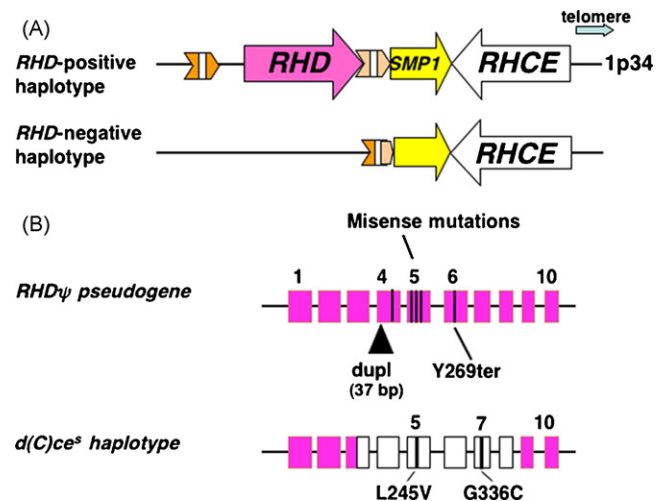


Fig. 1. Structure of the RH locus and some well characterized variants. A: in RhD-positive individuals, the *RH* locus is composed of two homologous genes, *RHD* and *RHCE* (96% homology). In almost all RhD-negative individuals of Caucasian origin, the Rh locus consists of a single gene *RHCE*, most likely following *RHD* gene deletion by unequal crossing-over. *RHD* (pink) and *RHCE* (white) genes are tandemly organized on chromosome 1p34–p36, but in opposite orientation. The two genes are each composed of 10 exons and are separated by about 30 kb DNA including the *SMP1* gene (yellow) of unknown function. The *RHD* gene is flanked by two “rhesus boxes” of 9 kb sharing 98% sequence identity, which are involved in the unequal crossing-over generating the *RHD*-negative haplotype. *RHD* gene deletion occurs in about 15% Caucasians but is rare in non Caucasians – being about 3–5% in Blacks and less than 1% in Asians; B: *RHD* gene fragments can be detected in some individuals phenotyped as RhD-negative, which could be the source of false-positive results in *RHD* genotyping. Typical examples are *RHDψ* pseudogene and *d(C)ce^S* gene [9]. *RHDψ* carries several mutations including a 37 bp duplication in exon 4, several missense mutations in exon 5 and a stop codon in exon 6 (Y269ter). The *d(C)ce^S* haplotype include a *RHCE* gene (not shown here) and a hybrid *RHD-CE(3-8)-D* gene in which the region of the *RHD* gene encompassing the exon 3 (in part) to exon 8 has been exchanged by equivalent *RHCE* gene sequences and which carries, in addition, a L245V and a G336C mutations in peptide regions encoded by exons 5 and 7, respectively. Of note, many other *RHD-CE-D* hybrid genes as well as several nonsense mutations or small deletions within the *RHD* gene have been described and may be responsible for false-positive results in *RHD* genotyping by PCR. The frequency of such phenotypes varies according to the ethnical origin of the samples under study. Among RhD-negative individuals of Black African origin, 18% carries a *RHD* gene deletion, 66% a *RHDψ* (only 24% in Black Americans and 17% in South Africans), 15% an *RHD-CE-D* hybrid gene and 2% a *RHD* mutation. In RhD-negative Asians, 70–80% have a *RHD* deletion, 2–11% a hybrid gene and 5–28% a *RHD* gene mutation (most of the Del type).

presence of a *RHDψ* pseudogene [5] carrying several mutations and another frequent haplotype in this population is *d(C)ce^S* (Fig. 1B). As these phenotypes could generate “false-positive” results in *RHD* genotyping assays, sometimes “false-negative” (for instance when a primer should hybridize to a given exon lacking in a particular variant), second generation PCR tests were designed in different laboratories, essentially based on the use of several primer pairs to amplify various regions (exons and introns) of the *RHD* gene [5,10], four regions in our modified test [11]. These studies also pointed to the crucial importance of ethnical origin of the samples in which unusual variants could be present.

2. Noninvasive fetal *RHD* genotyping from maternal plasma

The finding that maternal blood contained cell-free fetal DNA, the level of which rises during pregnancy and quickly disappears after delivery, was pivotal to settle a noninvasive test of third generation [12]. A prediagnosis study in our laboratory performed on 851 plasma samples from RhD-negative pregnant women showed that the fetal *RHD* gene could be detected by conventional or real-time PCR (with two primer pairs in exon 7 and exon 10), with a sensitivity which was very good beyond eight weeks of gestation, excellent beyond 13 weeks and a genotype/phenotype concordance of 99.5% [13]. Every fetal *RHD-negative* genotype is confirmed on a second sample collected few weeks later. Currently, more than 2500 samples have been genotyped by this technology, confirming previous results, with no false-negative but about 0.2% false-positive results (Rh variants, including *RHDψ* inherited from the father). Similar tests on large series of samples have been performed in other laboratories [14–16].

2.1. Rationale for a *RHD* diagnostic kit

Several arguments suggest that the development of a standard protocol might be useful to warrant reproducibility, sensitivity and specificity between laboratories: (i) there are notable differences between techniques used (DNA extraction, primer pairs, PCR conditions, detection of amplified products, etc); (ii) in a multicentric study on the detection of male fetal DNA (SRY sequences) in maternal blood, large variations were noticed between laboratories, particularly with regard to sensitivity (31 to 97%) [17]; (iii) in France, since 2005, the National College of Gynecologists and Obstetricians ([18], and <http://www.cngof.asso.fr>) recommend to perform a prophylaxis by anti-D administration at the third trimester of pregnancy (28 ± 1 weeks with a single 300 µg injection or two 100 µg injections at 28 and 34 weeks in addition to the doses given after potentially sensitizing events that is, antenatally, after amniocentesis, external version of the fetus, or within 72 h after delivery), a disposition already adopted in other countries, and thus knowledge of the fetal *RHD* status would be desirable before Ig anti-D administration; (iv) fetal *RHD* genotyping on maternal plasma is patented (Oxford University, Isis-Innovation).

Accordingly, a kit for fetal *RHD* genotyping on maternal plasma (*Free DNA Fetal Kit RhD*[®], Institut de Biotechnologies Jacques-Boy, Reims, France) based on our previous results by real-time PCR has been developed with some modifications, such as the use of hydrolysis probes.

2.2. *Free DNA Fetal Kit RhD*[®]

2.2.1. Kit content

RHD-positive and *RHD*-negative plasmas were included in the kit as positive and negative controls, respectively. Exogenous DNA (maize) was also provided as extraction/amplification control since no universal internal control for fetal DNA is yet available. Finally, three sets of primers and

hydrolysis probes (5' nuclease assay [20]) specific for exons 7 and 10 of the *RHD* gene and for the exogenous DNA (maize) are also included in the kit. The *Free DNA Fetal Kit RhD*[®] neither contains DNA extraction system nor real-time PCR consumables and reagents.

2.2.2. Methodology

RhD-negative pregnant women were recruited either one or two weeks before undergoing amniocentesis for karyotype or during a prenatal visit for anti-D immunization. 5–10 ml of blood samples were collected on EDTA and ship to the laboratory within 48 h at room temperature. Blood samples were centrifuged at 1730 g for 10 min at room temperature and the plasma was carefully removed, then aliquoted by 500 µl into polypropylene cryogenic vials and stored at -20°C until further processing.

DNA was extracted from 500 µl of plasma sample containing 5 µl of diluted maize DNA (1/100 v/v) with a QIAamp[®] MinElute[®] Virus Vacuum Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. DNA was eluted using 30 µl of the elution buffer provided with the kit. For each series of extraction, three controls were added: RhD-positive and RhD-negative plasma controls provided with the kit and a blank control for which 500 µl of water was used instead of plasma.

Because variants *RHD* genes may occur, two *RHD* specific exons (7 and 10) were amplified by real-time PCR [13]. Importantly, the size of the two amplicons is 133 and 131 bp for exon 7 and 10, respectively, which is below the mean size of circulating cell-free fetal DNA in maternal mother (range 145–201 bp) [19]. For each patient, PCR amplifications of the exons 7 and 10 of the *RHD* gene were performed as well as of the maize to check the absence of a PCR inhibitor in the sample and the quality of the DNA extraction. Real-time PCR analysis were performed using a LightCycler[®] 1.5 or 2.0 apparatus (Roche Molecular Biochemicals, Meylan, France). PCR reactions were performed in the LightCycler[®] glass capillaries using the LightCycler[®] Taqman[®] Master (Roche Molecular Biochemicals, Meylan, France) in a final volume of 20 µl containing 5 µl of plasma DNA, 1 µl of the mix of primers and probe provided in the kit and 4 µl of the LightCycler[®] Taqman Master mix (Roche Molecular Biochemicals, Meylan, France). Amplification conditions were initial denaturation at 95°C for 10 min, followed by 50 cycles with denaturation at 95°C for 10 s, annealing and extension at 59°C for 45 s, and extension and fluorescence measurement at 72°C for 1 s.

2.2.3. Presentation and interpretation of results

Preliminary studies of fetal *RHD* genotyping with the *Free DNA Fetal Kit RhD*[®] were performed with known maternal plasmas collected from previous studies [13]. Representative real-time PCR amplification plots are shown in Fig. 2. For each group of samples, Cp values (crossing-point, point/number of cycles at which the fluorescence of a sample rises above the background fluorescence, that is, the beginning of the exponential phase) for exons 7 and 10 assays were determined. Plasma samples from pregnant women carrying a RhD-positive

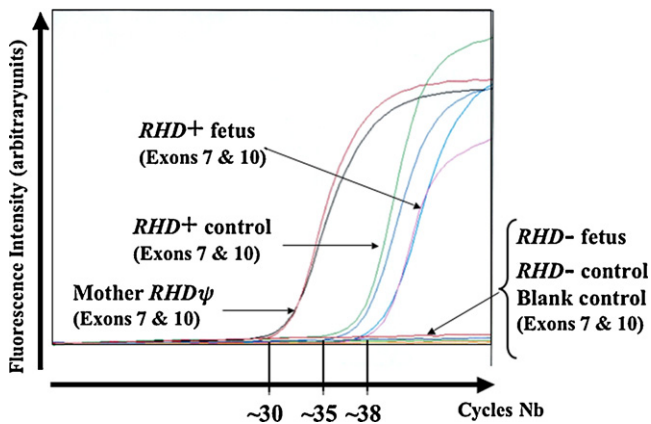


Fig. 2. Real-time PCR: representative amplification plots. Representative amplification curves showing real-time monitoring of fluorescence intensity (arbitrary units) for PCR *RHD* exon 7 and *RHD* exon 10 as a function of the number of amplification cycles. Cp values (crossing-point or cycle threshold): number of cycles to reach a threshold value of fluorescence. RHD+ control: RHD+ plasma control provided with the kit; RHD+ fetusplasma sample from pregnant woman carrying an RhD-positive fetus; RHD- control: plasma control from an RhD-negative individual provided with the kit; RHD- fetus: plasma sample from a pregnant woman carrying an RhD-negative fetus; mother *RHDψ*: plasma sample (RHD+ fetus) from a mother carrying a *RHD* pseudogene.

fetus (RHD+ fetus) gave Cp values in the range 35–40 (Fig. 2) according to the gestational age of pregnancy, whereas no Cp values were observed when the fetus was RhD-negative (RHD-fetus). The fetal *RHD* genotype was invalidated when Cp values in the range of 26–30 cycles, since it highlighted the presence of a silent variant *RHD* gene such as a *RHDψ* pseudogene in the maternal genome. This was confirmed by *RHD* genotyping of genomic DNA extracted from mother leukocytes. The full protocol is summarized on Fig. 3.

Current interpretations are summarized in Table 1. Importantly, results can be safely considered only if (i) no amplification curve is observed for the negative and blank controls; (ii) the Cp values for exons 7 and 10 for the positive controls are in the range 35.66 ± 1.55 and 35.9 ± 1.45 ; and (iii) the exogenous DNA (maize) is correctly amplified (Cp value: 34.33 ± 0.6) during the assay. Patterns 1 and 2 correspond to

RHD-negative and *RHD*-positive genotypes, respectively. However, since no internal control for the presence of fetal DNA in each sample is yet available in routine (see below), a *RHD*-negative results should be confirmed on a second plasma sample collected few weeks later; this may also avoid a false-negative result caused by a low level of fetal DNA at time of blood sampling. Patterns 3 and 4 show discrepant results between exons 7 and exon 10. Pattern 3 (exon 7+; exon 10-) may occur as exon 7 PCR is more sensitive than exon 10 PCR [11,13] and is usually indicative of a sample collected early during pregnancy (less than 10 weeks of gestation) when the level of fetal DNA in mother plasma is low. Accordingly, pattern 3 result is *RHD*-positive. Pattern 4 (exon 7-; exon 10+) is suggestive of a *RH* variant that could be either *RHD*-negative such as *d(C)ce^s*, or *RHD*-positive (D category: D^{IV} or DBT) for which exon 7 of the *RHD* gene is lacking but exon 10 of *RHD* is present. Accordingly, pattern 4 will be considered as a *RHD*-positive result. Patterns 5 and 6 (exon 10+, Cp < 35) are characteristic of a *d(C)ce^s* haplotype present in the maternal genome. As a consequence, only the exon 7 PCR results can be useful for the determination of the fetal *RHD* genotype. If no exon 7 PCR amplification occurs (pattern 5), the fetal *RHD* genotype is undetermined, as explained for pattern 4. On the other hand, *RHD* exon 7 amplification featured a fetal *RHD*-positive genotype (pattern 6). Pattern 7 in which Cp values are below 35 for both exons highlights the presence of a silent *RHD* gene in the maternal genome such as a *RHDψ* pseudogene and invalidate completely the fetal *RHD* genotype. In this case, the fetus has also to be considered as *RHD*-positive.

2.2.4. Kit evaluation for CE marking

Following the preliminary studies reported above, the performances of the kit was evaluated for non-invasive fetal *RHD* genotyping on maternal plasma from 300 RhD-negative pregnant women (10 to 34 g.w.). The samples were collected and processed as described above. Fetal *RHD* genotypes from maternal plasmas were confirmed either by determination of the *RHD* fetal genotype on amniotic cells and/or RhD blood typing of the infant at birth. Accordingly, 229 samples were correctly genotyped as *RHD*-positive and 79 as *RHD*-negative,

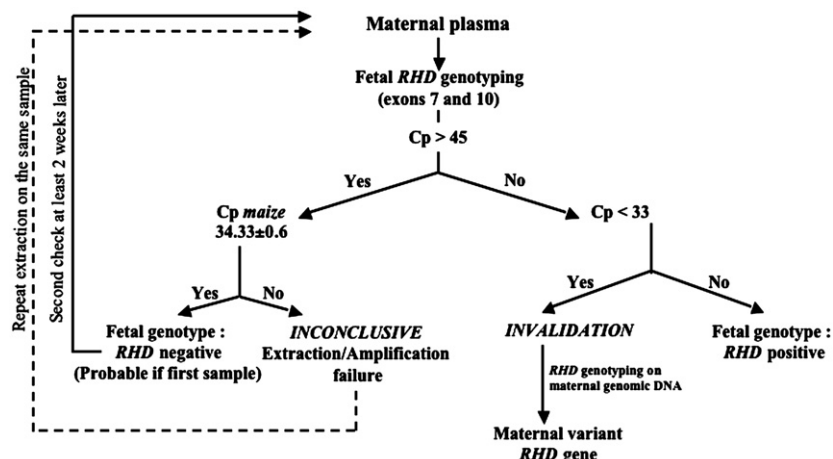


Fig. 3. Chart flow for fetal *RHD* genotyping from maternal plasma. See text for details.

Table 1
RHD genotyping: typical patterns of PCR results

Pattern	Exon 7	Exon 10	Maize ^a	Maternal <i>RHD</i> gene ^b	Conclusion Fetal <i>RHD</i> genotype
1	Neg	Neg	Ok	No	<i>RHD</i> -negative (if validated by second sample)
2	Pos (Cp \geq 35)	Pos (Cp \geq 35)	Ok	No	<i>RHD</i> -positive
3	Pos (Cp \geq 35)	Neg	Ok	No	<i>RHD</i> -positive (PCR exon 7 more sensitive; early gestation age)
4	Neg	Pos (Cp \geq 35)	Ok	No	To be considered as <i>RHD</i> -positive (<i>RHD</i> variant, <i>d(C)cc^s</i> , D ^{IV} , DBT)
5	Neg	Pos (Cp $<$ 35)	Ok	Yes	Inconclusive, but will be considered as <i>RHD</i> -positive
6	Pos (Cp \geq 35)	Pos (Cp $<$ 35)	Ok	Yes	<i>RHD</i> -positive
7	Pos (Cp $<$ 35)	Pos (Cp $<$ 35)	Ok	Yes	Inconclusive, but will be considered as <i>RHD</i> -positive

Patterns of PCR results for *RHD* exons 7 and 10, maize DNA and mother genomic DNA. Neg: negative (no amplification after 45 cycles); Pos: positive.

^a For exogenous PCR (maize), OK means correct extraction procedure with Cp values of 34.33 ± 0.6 .

^b *RHD* genomic analysis of mother leukocytes from buffy-coat.

with 100% concordant results when comparing the SYBR green I method [8] and the hydrolysis probe assay with the *Free DNA Fetal Kit RhD*[®]. Two false-positive results were found with all Cp for exons 7 and 10 that were > 39 cycles, which was not concordant with the gestational age of these pregnancies (16 and 20 g.w.). When a second extraction was performed, no amplification was observed, suggesting a contamination during the extraction process for these two samples. Most importantly, no false-negative result was observed in this study. Thus, the sensitivity of the assay was 100% and the specificity $> 99\%$.

The kit received the CE marking (as to the European Directive 98/79/CE) and owns the exclusive license for the fetal *RHD* genotyping from maternal plasma in Europe. A medicoeconomical evaluation for systematic application of the noninvasive determination of fetal *RHD* genotyping using this kit to the management of 4000 RhD-negative pregnant women is in progress in five centers in France (Paris, Marseille, Lille, Nantes, Poissy).

2.3. Future improvements of non invasive fetal *RHD* genotyping

As discussed above, the first generation kit is not suitable for correct genotyping of fetuses from women carrying a *RHD* ψ pseudogene in their genome. In order to overcome this problem, a study is in progress in our laboratory to assess a recently published procedure [21], consisting to perform a *RHD* exon 5 PCR designed to amplify a 82 bp fragment of *RHD* but not *RHD* ψ [22].

More importantly, one drawback of the current fetal genotyping assay on maternal plasma is the occurrence of a potentially harmful “false-negative” result, due either to the low level (absence?) of fetal DNA in the maternal blood at early gestational week or to a genotyping method not sensitive enough to detect low amount of fetal DNA. However, a reliable universal positive control proving the presence of fetal DNA in the sample, particularly when the genotyping result is “*RHD*-negative” is missing. This is why we do recommend that a fetal “*RHD*-negative” genotype has to be confirmed on a second plasma sample collected at least two weeks later, specially when the first sample was obtained before 13 weeks of gestation.

Several strategies have been proposed to confirm the presence of fetal DNA in the maternal plasma. One is based on the specific detection of *SRY* sequences, but is obviously not applicable to the 50% of pregnancies involving a female fetus [22,23]. Other approaches rely on the detection of repeated sequences or bi-allelic polymorphisms, but the sensitivity and informativity of these systems are limited [24,25]. A completely different approach to the development of a universal fetal DNA marker consists of exploiting epigenetic differences between fetal and maternal DNA molecules present in maternal plasma. One of the best studied epigenetic marker is DNA methylation [26]. Along this line, it has been shown that the DNA methylation profile of CpG islands in the tumor suppressor gene mammary serine protease inhibitor (*maspin*, SERPIN B5) is hypomethylated in placenta cells but hypermethylated in maternal blood cells, and on this basis a methylation-specific PCR amplification was developed [27]. However, a bisulfite treatment is required to detect unmethylated sequences (fetal origin) in an ocean of hypermethylated sequences of maternal origin, and it has been shown that DNA degradation is an undesired side-effect of bisulfite treatment [28], which has an impact on the detection limit of the method. More recently, the promoter region of the tumor suppressor gene Ras association domain family 1A gene (*RASSF1A*) was selected since its methylation pattern is opposite to that of *maspin*: *RASSF1A* is hypermethylated in placenta cells, but hypomethylated in maternal blood cells [29]. In this case, methylation analysis, is performed using methylation-sensitive endonucleases, thus cutting hypomethylated *RASSF1A* sequences from maternal origin but leaving hypermethylated placental *RASSF1A* sequences intact for amplification. Thus, *RASSF1A* might represent a universal fetal DNA marker in maternal blood and its potential use is in progress in our laboratory in a large series of maternal plasmas.

3. Conclusion

Our current findings confirm the accuracy of fetal *RHD* genotyping on maternal plasma using a *Free DNA Fetal Kit RhD*[®]. Based on an accuracy of the test $> 99.3\%$ (two false positive/300 samples, no false negative), fetal *RHD* genotyping from maternal plasma should be applicable in clinical routine practice. Non invasive fetal *RHD* genotyping not only

allows to target immunoprophylaxis only to non immunized RhD-negative pregnant women carrying a RhD-positive fetus (60% of them), thus avoiding unnecessary administration of Ig anti-D (which is in short supply as monoclonal anti-D preparations are not yet in clinical use) to those carrying a RhD-negative fetus (40% of them), but is also an important tool for antenatal diagnosis of fetomaternal incompatibility and for a better follow-up of anti-D immunized women. Iterative irregular antibodies screening should be avoided in non-immunized RhD-negative pregnant women carrying an RhD-negative fetus. Finally, at least in France, systematic or targeted prevention based on fetal *RHD* genotyping from maternal plasma should be evaluated to define the impact on health care costs.

References

- [1] Bowman JM. Rh-immunoglobulin: Rh prophylaxis. *Best Pract Res Clin Haematol* 2006;19:27–34.
- [2] Brossard Y, Sender A, Cartron JP, Huchet J, Pinon F, Blot P, et al. Intérêt du génotype foetal *RHD* dans la prévention de l'immunisation anti-D. *Bull Acad Natl Med* 2001;185:329–36.
- [3] Le Van Kim C, Colin Y, Cartron JP. Rh proteins: key structural and functional components of the red cell membrane. *Blood Rev* 2006;20:93–110.
- [4] Colin Y, Cherif-Zahar B, Le Van Kim C, Raynal V, Van Huffel V, Cartron JP. Genetic basis of the RhD-positive and RhD-negative blood group polymorphism as determined by Southern analysis. *Blood* 1991;78:2747–52.
- [5] Bennett PR, Le Van Kim C, Colin Y, Warwick RM, Cherif-Zahar B, Fisk NM, et al. Prenatal determination of fetal RhD type by DNA amplification. *N Engl J Med* 1993;329:607–10.
- [6] Lo YM, Howell PJ, Selinger M, Mackenzie IZ, Chamberlain P, Gillmer MD, et al. Prenatal determination of fetal RhD type by DNA amplification. *Lancet* 1993;341:1147–8.
- [7] Avent ND, Reid ME. The Rh blood group system: a review. *Blood* 2000;95:375–87.
- [8] Wagner FF, Flegel WA. Review: the molecular basis of the Rh blood group phenotypes. *Immunohematol* 2004;20:23–36.
- [9] Singleton BK, Green CA, Avent ND, Martin PG, Smart E, Daka A, et al. The presence of an *RHD* pseudogene containing a 37 base pair duplication and a nonsense mutation in africans with the Rh D-negative blood group phenotype. *Blood* 2000;95:12–8.
- [10] Maaskant-van Wijk PA, Faas BH, de Ruijter JA, Overbeeke MA, von dem Borne AE, van Rhenen DJ, et al. Genotyping of *RHD* by multiplex polymerase chain reaction analysis of six *RHD*-specific exons. *Transfusion* 1998;38:1015–21.
- [11] Aubin JT, Le Van Kim C, Mouro I, Colin Y, Bignozzi C, Brossard Y, et al. Specificity and sensitivity of *RHD* genotyping methods by PCR-based DNA amplification. *Br J Haematol* 1997;98:356–64.
- [12] Lo YM, Hjelm NM, Fidler C, Sargent IL, Murphy MF, Chamberlain PF, et al. Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. *N Engl J Med* 1998;339:1734–8.
- [13] Rouillac-Le Sciellour C, Puillandre P, Gillot R, Baulard C, Métral S, Le Van Kim C, et al. Large scale prediagnosis study of fetal RhD genotyping by PCR on plasma DNA from Rh-negative pregnant women. *Mol Diagn* 2004;8:23–31.
- [14] Finning K, Martin P, Daniels G. A clinical service in the UK to predict fetal Rh (rhesus) D blood group using free fetal DNA in maternal plasma. *Ann N Y Acad Sci* 2004;1022:119–23.
- [15] Gautier E, Benachi A, Giovangrandi Y, Ernault P, Olivi M, Gaillon T, et al. Fetal RhD genotyping by maternal serum analysis: a two-year experience. *Am J Obstet Gynecol* 2005;192:666–9.
- [16] Van der Schoot CE, Soussan AA, Koelewijn J, Bonsel G, Paget-Christiaens LGC, de Haas M. Non-invasive antenatal *RHD* typing. *Transfus Clin Biol* 2006;13:53–7.
- [17] Johnson KL, Dukes KA, Vidaver J, LeShane ES, Ramirez I, Weber WD, et al. *Clin Chem* 2004;50:516–21.
- [18] Recommandation du Collège national des obstétriciens gynécologues français. Prévention de l'allo-immunisation rhésus fœtomaternelle. *J Gynecol Obstet Biol Reprod* 2006; 35 (Suppl. au no 1): 1S81–1S13.
- [19] Chan KC, Zhang J, Hui AB, Wong N, Lau TK, Leung TN, et al. Size distribution of maternal and fetal DNA in maternal plasma. *Clin Chim* 2004;50:88–92.
- [20] Holland PM, Abramson RD, Watson R, Gelfand DH. Detection of specific polymerase chain reaction products by utilizing the 5'->3' exonuclease activity of *thermus aquaticus* DNA polymerase. *Proc Natl Acad Sci USA* 1991;88:7276–80.
- [21] Grootkerk-Tax MGHM, Soussan AA, de Haas M, Maaskant-van Wijk PA, van der Schoot CE. Evaluation of prenatal *RHD* typing strategies on cell-free fetal DNA from maternal plasma. *Transfusion* 2006;46:2142–8.
- [22] Finning KM, Martin PG, Soothill PW, Avent ND. Prediction of fetal D status from maternal plasma: introduction of a new noninvasive fetal *RHD* genotyping service. *Transfusion* 2002;42:1079–85.
- [23] Lo YM, Tein MS, Lau TK, et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. *Am J Hum Genet* 1998;62:768–75.
- [24] Pertl B, Sekizawa A, Samura O, Oresvic I, Rahaim PT, Bianchi DW. Detection of male and female fetal DNA in maternal plasma by multiplex fluorescent polymerase chain reaction amplification of short tandem repeats. *Hum Genet* 2000;106:45–9.
- [25] Page-Christiaens GC, Bossers B, Van der Schoot CE, de Haas M. Use of bi-allelic insertion/deletion polymorphisms as a positive control for fetal genotyping in maternal blood: first clinical experience. *Ann N Y Acad Sci* 2006;1075:123–9.
- [26] Poon LLM, Leung TN, Lau TK, Chow KC, Lo YMD. Differential DNA methylation between fetus and mother as a strategy for detecting fetal DNA in maternal plasma. *Clin Chem* 2002;48:35–41.
- [27] Chim SSC, Tong YK, Chiu RWK, Lau TK, Leung TN, Chan LY, et al. Detection of the placental epigenetic signature of the *maspin* gene in maternal plasma. *Proc Natl Acad Sci USA* 2005;102:14753–8.
- [28] Grunau C, Clark SJ, Rosenthal A. Bisulfite genomic sequencing: systematic investigation of critical experimental parameters. *Nucleic Acids Res* 2001;29:e65.
- [29] Chan KCA, Ding C, Gerovassili A, Yeung SW, Chiu RWK, Leung TN, et al. Hypermethylated *RASSF1A* in maternal plasma: a universal fetal DNA marker that improves the reliability of noninvasive prenatal diagnosis. *Clin Chem* 2006;52:2211–8.