

Determination of RhD Zygosity: Comparison of a Double Amplification Refractory Mutation System Approach and a Multiplex Real-Time Quantitative PCR Approach

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Background: Rh isoimmunization and hemolytic disease of the newborn still occur despite the availability of Rh immunoglobulin. For the prenatal investigation of sensitized RhD-negative pregnant women, determination of the zygosity of the RhD-positive father has important implications. The currently available molecular methods for RhD zygosity assessment, in general, are technically demanding and labor-intensive. Therefore, at present, rhesus genotype assessment is most commonly inferred from results of serological tests. The recent elucidation of the genetic structure of the prevalent *RHD* deletion in Caucasians, as well as the development of real-time PCR, allowed us to explore two new approaches for the molecular determination of RhD zygosity.

Methods: Two methods for RhD zygosity determination were developed. The first was based on the double Amplification Refractory Mutation System (double ARMS). The second was based on multiplex real-time quantitative PCR. For the double ARMS assay, allele-specific primers were designed to directly amplify the most prevalent *RHD* deletion found in RhD-negative individuals in the Caucasian population. The multiplex real-time quantitative PCR assay, on the other hand, involved coamplification and quantification of *RHD*-

specific sequences in relation to a reference gene, *albumin*, in a single PCR reaction. A ratio, ΔC_t , based on the threshold cycle, was then determined and reflects the *RHD* gene dosage.

Results: The allele-specific primers of the double ARMS assay reliably amplified the *RHD*-deleted allele and therefore accurately distinguished homozygous from heterozygous RhD-positive samples. The results were in complete concordance with serological testing. For the multiplex real-time quantitative PCR assay, the ΔC_t values clearly segregated into two distinct populations according to the *RHD* gene dosage, with mean values of 1.70 (SD, 0.17) and 2.62 (SD, 0.29) for the homozygous and heterozygous samples, respectively ($P < 0.001$, t -test). The results were in complete concordance with the results of serological testing as well as with the double ARMS assay.

Conclusion: Double ARMS and real-time quantitative PCR are alternative robust assays for the determination of RhD zygosity.

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The Rh blood group system is involved in hemolytic disease of the newborn (HDN),⁵ transfusion reactions, and autoimmune hemolytic anemia (1). Despite the widespread use of prophylactic Rh immunoglobulin, Rh isoimmunization during pregnancy still occurs and is a significant cause of neonatal morbidity and mortality (2). Fifty-six percent of RhD-positive individuals in Caucasian populations are heterozygous at the *RHD* locus (3). De-

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⁵Nonstandard abbreviations: HDN, hemolytic disease of the newborn; ARMS, Amplification Refractory Mutation System; JOE, 2,7-dimethoxy-4,5-dichloro-6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; FAM, 6-carboxy-fluorescein; and C_t , threshold cycle.

termination of the zygosity of the RhD-positive father can aid in the assessment of a couple's risk of carrying a RhD-positive offspring, particularly in the case of preconception counseling of RhD-negative mothers immunized from a previous pregnancy. For example, heterozygous males have a 50% chance of producing a RhD-negative offspring, in which case the pregnancy will not be at risk for HDN. On the other hand, homozygous RhD-positive males can produce only RhD-positive offspring, and therefore, close monitoring of the pregnancy is needed.

At present, determination of RhD zygosity in RhD-positive individuals is commonly inferred from results of serological testing, firstly, because the currently available molecular methods, such as single-sperm analysis, microsatellite analysis, and other gel-based electrophoretic techniques (4–7), are either too labor-intensive or technically demanding for routine use, and secondly, because the genetic structure of the prevalent *RHD* deletion in Caucasians had not been elucidated until recently (8), further assay development for zygosity determination has been hampered by the lack of reference assays that could reliably and accurately determine *RHD* genotype. Consequently, reliable and robust methods for RhD zygosity determination have been lacking.

Recently, Wagner and Flegel (8) elucidated the genetic structure of the *RH* locus and characterized the location of the *RHD* deletion that is responsible for the majority of RhD-negative phenotypes in Caucasians. Such sequence information allows development of assays for the specific detection of the *RHD* deletion, which previously was not possible. We have developed two approaches for the determination of RhD zygosity. The first assay, which is based on the work of Wagner and Flegel (8), relies on specific detection of the *RHD* deletion through amplification of a 1507-bp fragment by a double Amplification Refractory Mutation System (double ARMS) approach (9). In addition, in view of the need for a rapid, robust, and automatable assay adaptable for routine laboratory testing, we have developed a second assay for determination of RhD zygosity. This assay is based on a multiplex real-time quantitative PCR approach through amplification and quantification of *RHD* sequences in relation to a reference gene, *albumin*. The reliability of this assay has been assessed by comparison with the double ARMS assay.

Materials and Methods

SUBJECTS

Blood (5 mL) was collected from 46 blood donors into EDTA tubes. Ethics approval was obtained from the Central Oxford Research Ethics Committee. The blood samples were subjected to full Rh phenotyping for D, C, c, E, and e antigens, performed by the National Blood Service, Oxford, United Kingdom. Ten of the subjects were RhD-negative, and the remaining subjects were RhD-positive; 18 of the RhD-positive subjects tested homozygous and the others heterozygous for RhD. The

method of Rh phenotyping consisted of serological methods with calculation of the most probable Rh genotype from known frequencies of Rh genotypes in an English population (10).

After collection, the blood samples were centrifuged at 1600g for 10 min, and the plasma was removed. The buffy coat was stored at –20 °C until further processing. DNA was extracted from the buffy coat (200 µL) with the Nucleon reagent set (Amersham Life Science) according to the “blood and cell culture 2 (BACC2)” protocol recommended by the manufacturer.

All of the primers used in this study were synthesized by Life Technologies.

DOUBLE ARMS APPROACH

Allele-specific primers were designed to detect the *RHD*-deleted allele by a double ARMS approach (9). The double ARMS approach involves the use of two allele-specific primers simultaneously during PCR when there is a need to distinguish a sequence of interest from two or more closely related sequences. According to Wagner and Flegel (8), 5' and 3' to the *RHD* gene there are two highly homologous DNA segments, designated “Rhesus boxes”, which are ~9000 bp in length (Fig. 1A). The two Rhesus boxes share 98.6% homology within which there is a segment of 1463 bp, termed the “identity region”, where the sequence is completely identical. For the most prevalent RhD-negative haplotype in Caucasians, the breakpoints of the *RHD* deletion are located within the identity region of the Rhesus boxes. Consequently, in the RhD-negative haplotype, only one Rhesus box was found, which is of a hybrid nature, comprising the 5' end of the Rhesus box upstream to the *RHD* gene and the 3' end of the downstream Rhesus box.

We exploited the differences in the DNA sequences of the upstream, downstream, and hybrid Rhesus boxes and designed double ARMS primers that specifically amplify hybrid one, and therefore detect the *RHD*-deleted allele (Fig. 1B). The forward and reverse primers were designed to anneal to the 5' and 3' ends of the 1463-bp identity region. At the 5' end of the identity region, the downstream Rhesus box differs by 1 bp in sequence from the upstream and hybrid Rhesus boxes. The last base pair of the forward primer, HYB2-U, matches the sequence of the upstream and hybrid Rhesus boxes, but not the downstream one. The reverse primer, HYB2-L, was designed to anneal to the 3' end of the identity region, where there is a 1-bp insertion in the upstream Rhesus box compared with the downstream and hybrid Rhesus boxes. The reverse primer sequence matches that of the hybrid and downstream Rhesus boxes, but not the upstream one. Consequently, the primers selectively amplify a 1507-bp segment of the hybrid Rhesus box. To enhance the specificity of the primers, an additional mismatch was introduced into the third base pair from the 3' end of both primers (Fig. 1B).

PCR amplification reactions were set up according to

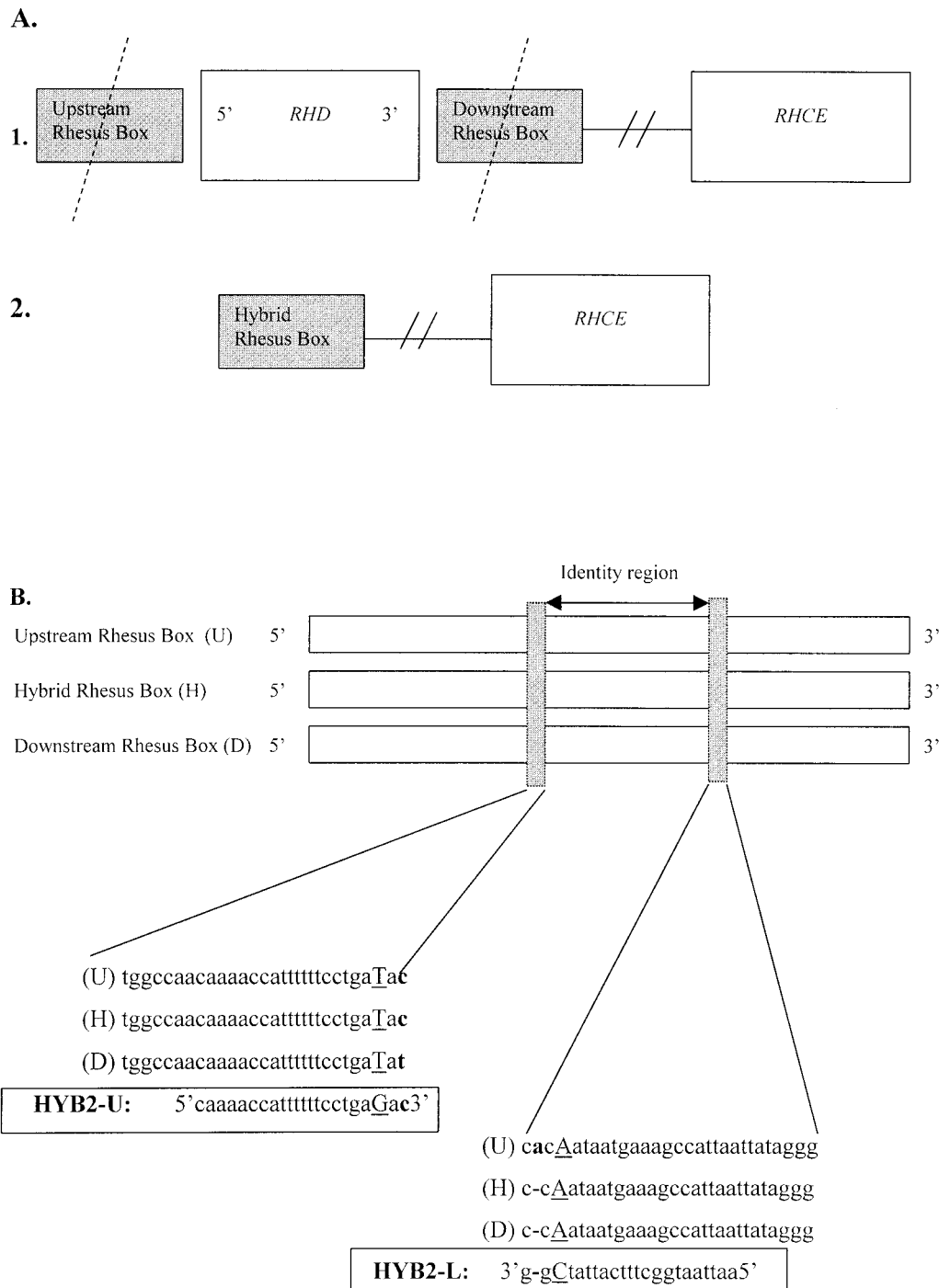


Fig. 1. Schematic diagrams of the genetic structure at the *RH* locus (A) and orientation and sequences of the HYB2-U and HYB2-L primers (B). (A), panel 1, structure of gene organization of the RhD-positive haplotype. There are two highly homologous DNA segments, termed Rhesus boxes, which are located 5' and 3' to the *RHD* gene. Dashed lines represent the breakpoints in the Rhesus boxes that produce the prevalent *RHD* deletion in Caucasians. Panel 2, structure of gene organization of the RhD-negative haplotype. The deletion of the *RHD* gene produces a hybrid Rhesus box, which comprises the 5' end of the upstream Rhesus box and the 3' end of the downstream one. (B), the upstream, hybrid, and downstream Rhesus boxes are designated U, H, and D, respectively. HYB2-U and HYB2-L amplify the identity region of the hybrid Rhesus box specifically. The primers anneal to the regions adjacent to the identity region (shaded area), and the corresponding sequences of the upstream, hybrid, and downstream Rhesus boxes are shown in detail. Sequences of HYB2-U and HYB2-L are boxed. Allele specificity is conferred by the bases identified in bold, as well as an additional mismatch (underlined and capitalized) introduced at the third base from the 3' end of each primer.

the manufacturer's (Applied Biosystems) instructions in a reaction volume of 50 μ L. DNA (100 ng) was added to each reaction mixture, which consisted of 5 μ L of 10×

buffer II; 300 nM each amplification primer; 200 nM each of dATP, dCTP, dGTP, dTTP; 4 mM $MgCl_2$, and 2.5 U of AmpliTaq Gold. PCR was carried out in a MJ Research

PTC-200 thermal cycler (MJ Research) with a thermal profile that started with a first denaturation step of 2 min at 95 °C, followed by 40 cycles of 95 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 5 min, with a final step of 72 °C for 10 min. The PCR products were visualized on 1% agarose gels with ethidium bromide staining.

MULTIPLEX REAL-TIME QUANTITATIVE PCR

A multiplex real-time quantitative PCR-based assay has been developed for the homogeneous detection of RhD zygosity. This assay is based on the amplification and quantification of *RHD* sequences in relation to a reference gene, *albumin*, in a single PCR. The *RHD* TaqMan system consists of the amplification primers RD-A and RD-B, as well as the dual-labeled fluorescent probe RD-T, as described previously (11). Similarly, the *albumin* system consists of the primers Alb-up (5'-GCTGTCATCTCT-TGTGGGCTGT-3'), Alb-low (5'-ACTCATGGGAGCTG-CTGGTTC-3'), and the Alb-probe [5'(JOE)-CCTGTCA-TGCCACACAAATCTCTCC-(TAMRA)3'], where JOE is 2,7-dimethoxy-4,5-dichloro-6-carboxyfluorescein, and TAMRA is 6-carboxytetramethylrhodamine. The primers and probe were synthesized according to Laurendeau et al. (12), although the probe was synthesized in the reverse sense. The dual-labeled fluorescent probes were synthesized by Applied Biosystems, and each contained a 5' reporter dye, 6-carboxy-fluorescein (FAM) and JOE, respectively, and a 3' quencher, TAMRA. The different spectral characteristics of the reporter dyes, FAM and JOE, enable each amplification system to be monitored independently.

Multiplex amplification reaction mixtures were set up in a reaction volume of 50 µL. All components other than the primers and probes were supplied in a TaqMan PCR Core reagent set (Applied Biosystems). DNA (100 ng) was added to each reaction mixture, which consisted of 5 µL of 10× buffer A; 300 nM each amplification primer (*RHD* and *albumin*); 62.5 nM RD-T probe; 100 nM Alb-probe; 3 mM MgCl₂; 200 nM each of dATP, dCTP, dGTP; 400 nM dUTP; 1.25 U of AmpliTaq Gold; and 0.5 U of AmpErase uracil N-glycosylase. The following thermal profile was used: 2 min incubation at 50 °C, followed by a first denaturation step of 10 min at 95 °C, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Real-time quantitative PCR was carried out in an Applied Biosystems 7700 Sequence Detector. The theoretical and practical aspects of this technology have been described in detail elsewhere (13). The amplification and product-reporting systems used are based on the 5' nuclease assay, in which the liberation of a fluorescent reporter, tagged onto a sequence-specific probe, is coupled to the amplification reaction (14). The sequence detector monitors the increase in fluorescent signal of each reaction well and determines the number of amplification cycles required to reach a fixed threshold signal intensity, termed the threshold cycle (Ct), which is re-

corded by the 7700 Sequence Detection Software (Ver. 1.6.3). The Ct is inversely proportional to the logarithmic scale of the starting quantity of template DNA. Consequently, the gene dosage at a particular gene locus, e.g., *RHD* in this study, can be deduced by calculating the difference in Ct from the Ct of a reference gene, e.g., *albumin*, termed ΔCt (15). Thus:

$$\Delta Ct = [Ct(RHD) - Ct(albumin)]$$

where Ct(*RHD*) denotes the Ct value of the *RHD* PCR, and Ct(*albumin*) denotes the Ct value of the *albumin* PCR.

The average analysis time, including blood component separation and DNA extraction, was 3 h.

STATISTICAL ANALYSIS

Descriptive statistics were carried out using SigmaStat 2.0 software. To determine the discriminating power of the multiplex real-time PCR assay for RhD-positive homozygotes from heterozygotes, the mean ΔCt and its standard deviation for the homozygotes and heterozygotes were calculated. The 99% confidence interval for the difference between the ΔCt values of the heterozygous and homozygous cases is given by the following equation:

$$(\overline{\Delta Ct}_{hetero} - \overline{\Delta Ct}_{homo}) \pm 2.58 \sqrt{\frac{\sigma_{hetero}^2 + \sigma_{homo}^2}{n_{hetero} + n_{homo} - 2}}$$

where,

$\overline{\Delta Ct}_{hetero}$	denotes the mean ΔCt value of the heterozygous cases
$\overline{\Delta Ct}_{homo}$	denotes the mean ΔCt value of the homozygous cases
σ_{hetero}	denotes the standard deviation of the ΔCt values of the heterozygous cases
σ_{homo}	denotes the standard deviation of the ΔCt values of the homozygous cases
n_{hetero}	denotes the number of heterozygous cases tested (18 in the current study)
n_{homo}	denotes the number of homozygous cases tested (18 in the current study)

$$\sqrt{\frac{\sigma_{hetero}^2 + \sigma_{homo}^2}{n_{hetero} + n_{homo} - 2}} \quad \text{denotes the pooled standard deviation}$$

Results

DOUBLE ARMS PCR ASSAY

In the double ARMS approach, RhD-negative samples were analyzed as controls to indicate positive amplification of the *RHD*-deleted allele. Among the RhD-positive samples, the hybrid Rhesus box should be amplifiable only in the heterozygous state because these individuals have inherited one allele containing the *RHD* deletion. The RhD-positive samples were analyzed, and the results were in complete concordance with serological typing. As expected, PCR products were observed only in RhD-negative and heterozygous RhD-positive samples, but not in the homozygous RhD-positive samples.

MULTIPLEX REAL-TIME QUANTITATIVE PCR ASSAY

For the real-time quantitative PCR approach, *RHD*-specific sequences were amplifiable only from RhD-positive samples and not from the RhD-negative samples. ΔC_t values were calculated for the RhD-positive samples. The ΔC_t values obtained clearly segregated into two distinct populations (Fig. 2), with mean ΔC_t values of 1.70 (SD, 0.17) and 2.62 (SD, 0.29) for the homozygous and heterozygous samples, respectively ($P < 0.001$, t -test). The distinction between the homozygous and heterozygous RhD-positive samples was in complete concordance with typing by both the serological and double ARMS assays. The larger numerical values obtained for the heterozygous samples were as expected and reflected the smaller *RHD* gene dosage in these samples, producing a larger difference between the $C_t(RHD)$ and the $C_t(albumin)$.

The mean difference in the ΔC_t values of the heterozygous and homozygous samples was $2.62 - 1.70 = 0.92$. The pooled SD was 0.057. The 99% confidence interval for the mean difference in the ΔC_t values of the heterozygous and homozygous samples was 0.77–1.07, which did not straddle the value of 0. In other words, the real-time PCR assay had at least a 99% chance of distinguishing a heterozygous from a homozygous RhD-positive individual.

Discussion

Despite modern obstetrical practices and the availability of Rh immunoglobulin, Rh incompatibility remains the leading cause of HDN. HDN occurs when a previously sensitized RhD-negative pregnant woman conceives a RhD-positive fetus. A noninvasive prenatal test is now available for assessment of the fetal RhD status based on the detection of fetal-derived *RHD* sequences in maternal plasma (11). However, the sensitivity of this assay is reduced during the first trimester because the concentration of fetal DNA in maternal plasma is relatively low during this period (11, 16). Consequently, RhD zygosity determination of the RhD-positive father provides useful information for risk estimation during preconception counseling or early pregnancy.

Wagner and Flegel (8) have elucidated the site of the most prevalent *RHD* deletion in Caucasians and developed two PCR-based assays for the specific detection of the *RHD* deletion. The assays described include a long-range PCR assay, which relies on the amplification of a 9416-bp fragment that is amplifiable only in the *RHD*-deleted allele, and a PCR-restriction fragment length polymorphism assay for amplification of a 3100-bp fragment with subsequent *Pst*I digestion. However, both assays have several shortcomings. The long-range PCR requires relatively difficult optimization, whereas the PCR-restriction fragment length polymorphism assay relies on post-PCR enzyme digestion and interpretation of the restriction pattern. Conversely, the amplification of a 1507-bp segment of the hybrid Rhesus box using the double ARMS approach described in this study can robustly and accurately distinguish heterozygous from homozygous RhD-positive genotypes.

In Caucasians, a complete deletion of *RHD* is the most common cause for lack of expression of the D antigen, whereas the remaining cases are the result of structural abnormalities in the *RHD* gene, where the gene is often amplifiable. Consequently, such *RHD*-positive D-antigen-negative alleles cannot be accurately determined by tests for RhD genotyping based on direct detection of the *RHD* deletion. However, a recent study has shown that such mutations occur very rarely (17). In Caucasians, ~6% of RhD-negative but C- or E-positive individuals, and 0.03% of individuals with cde haplotypes are found to have amplifiable *RHD*. Consequently, when such values are combined with the frequency of occurrence of the various RhD-negative haplotypes (10), it can be estimated that among Caucasians, *RHD* is amplifiable in <0.4% of RhD-negative alleles. In other words, 99.3% of RhD-negative alleles can be correctly typed by direct detection of the *RHD* deletion.

In addition to the direct detection of the *RHD* deletion as described above, many alternative approaches have been used for the determination of RhD zygosity, including single-sperm analysis, microsatellite analysis, and

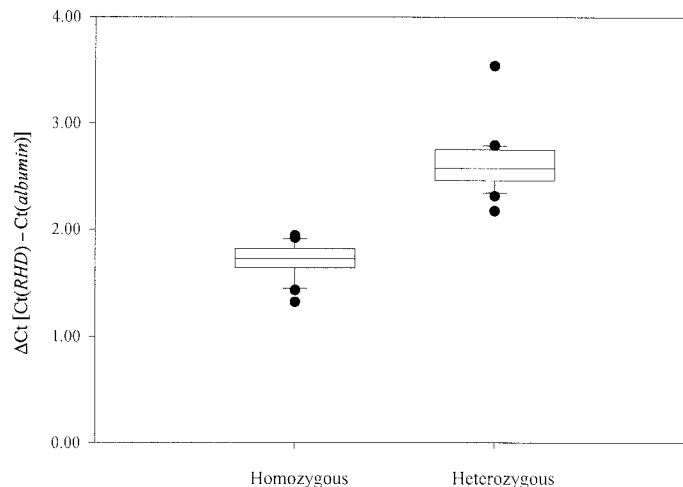


Fig. 2. Box-plot showing distribution of ΔC_t values for RhD-positive homozygous and heterozygous samples, respectively.

The upper and lower limits of the boxes represent the 75th and 25th percentiles, respectively; the lines inside the boxes indicate the median. The upper and lower horizontal bars indicate the 90th and 10th percentiles, respectively. Outliers are illustrated as ●.

other gel-based electrophoretic techniques (4–7). Compared with these techniques, the real-time quantitative PCR approach has much reduced risk of contamination because it is a homogeneous assay, requiring no post-PCR processing. In addition, it is less labor-intensive, more robust, and automatable. The results are clearly presented in a numerical format that can be easily interpreted. Coupled with a short turnaround time, the approach can potentially be used by any routine laboratory for high-throughput analysis. An advantage of this multiplex approach is that the *albumin* system also acts as an internal amplification control in addition to being the reference gene for gene dosage calculation. This design also allows for the simultaneous determination of RhD-negative, RhD-positive homozygous and heterozygous genotypes in a single assay.

In conclusion, we have developed two assays for determination of RhD zygosity. The double ARMS approach is based on direct detection of the *RHD* deletion, whereas the multiplex real-time quantitative PCR assay is based on *RHD* gene dosage comparison with the reference gene, *albumin*. The results of both assays are concordant with each other as well as with the serology-based method. The real-time quantitative PCR can be adapted for high-throughput determination of RhD zygosity in a routine setting. It is hoped that the availability of tests for assessing RhD zygosity will lead to better management of sensitized RhD-negative pregnant women.

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References

1. Avent ND, Reid ME. The Rh blood group system: a review [Review]. *Blood* 2000;95:375–87.
2. Chavez GF, Mulinare J, Edmonds LD. Epidemiology of Rh hemolytic disease of the newborn in the United States. *JAMA* 1991;265:3270–4.
3. Race RR, Sanger R. Blood groups in man, 6th ed. Oxford: Blackwell Scientific Publications, 1975:659pp.
4. Wolter LC, Hyland CA, Saul A. Rhesus D genotyping using polymerase chain reaction [Letter]. *Blood* 1993;82:1682–3.
5. Cossu G, Angius A, Gelfi C, Righetti PG. Rh D/d genotyping by quantitative polymerase chain reaction and capillary zone electrophoresis. *Electrophoresis* 1996;17:1911–5.
6. Reubini BE, Avner R, Rojansky N, Manny N, Friedmann A, Laufer N, et al. RhD genotype determination by single sperm cell analysis. *Am J Obstet Gynecol* 1996;174:1300–5.
7. Kemp TJ, Poulter M, Carritt B. Microsatellite variation within the human RHCE gene. *Vox Sang* 1999;77:159–63.
8. Wagner FF, Flegel WA. RHD gene deletion occurred in the Rhesus box. *Blood* 2000;95:3662–8.
9. Lo YMD, Patel P, Newton CR, Markham AF, Fleming KA, Wainscoat JS. Direct haplotype determination by double ARMS: specificity, sensitivity and genetic applications. *Nucleic Acids Res* 1991;19:3561–7.
10. Mollison PL, Contreras M, Engelfriet CP. Blood transfusion in clinical medicine, 10th ed. Oxford: Blackwell Science, 1997:754pp.
11. Lo YMD, 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.
12. Laurendeau I, Bahuau M, Vodovar N, Larramendy C, Olivi M, Bieche I, et al. TaqMan PCR-based gene dosage assay for predictive testing in individuals from a cancer family with INK4 locus haploinsufficiency. *Clin Chem* 1999;45:982–6.
13. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome Res* 1996;6:986–94.
14. Holland PM, Abramson RD, Watson R, Gelfand DH. Detection of specific polymerase chain reaction product by utilizing the 5'-3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc Natl Acad Sci U S A* 1991;88:7276–80.
15. Boulay JL, Reuter J, Ritschard R, Terracciano L, Herrmann R, Rochlitz C. Gene dosage by quantitative real-time PCR. *Biotechniques* 1999;27:228–32.
16. Lo YMD, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM, 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.
17. Wagner FF, Frohmayer A, Flegel WA. RHD positive alleles in D negative whites [Abstract]. *Transfusion* 2000;40(Suppl 10S):7S.