Catlab Informa

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FETAL RhD GENOTYPING: FROM THE GENETIC BASIS TO MOLECULAR DETERMINATION

Introduction

The Rh system is one of the most clinically important red blood cell blood group systems. Within this system, the D antigen is particularly relevant because of its high immunogenicity. In simplified terms, the presence or absence of this antigen determines whether an individual is RhD positive or RhD negative.

During pregnancy, RhD incompatibility between the mother and fetus may have clinical consequences when an RhD-negative pregnant woman comes into contact with fetal red blood cells expressing the D antigen. This exposure may lead to maternal alloimmunization, with the formation of anti-D antibodies that can cross the placenta and react against fetal erythrocytes.

The ability to determine fetal RhD status during pregnancy from a maternal blood sample has represented a major advance in this field. This determination is possible because cell-free fetal DNA (cffDNA) is present in maternal plasma, allowing certain fetal genetic characteristics to be studied without obtaining a fetal sample by an invasive procedure.

Genetic basis of the RhD system

The main genes responsible for Rh system antigens are located on the short arm of chromosome 1 and are *RHD* and *RHCE*. The two genes are located very close to each other and show a high degree of sequence homology, as they arose from duplication of a common ancestral gene.

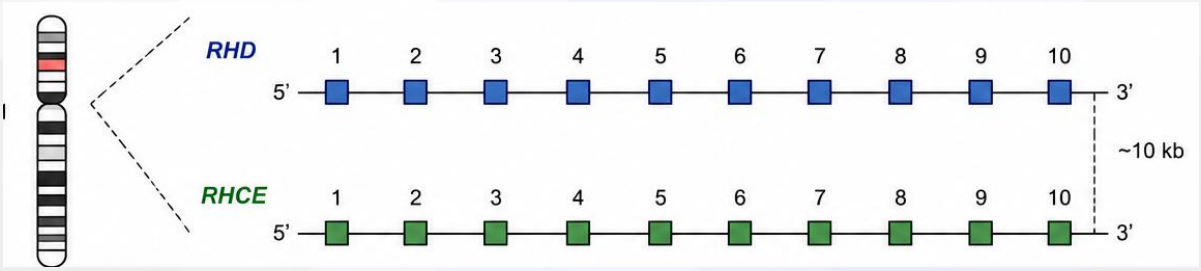

Figure 1. Location of the RHD and RHCE genes on the short arm of chromosome 1 (1p36.11) and organization into 10 exons. Source: original figure based on NCBI. The Rh blood group. Blood Groups and Red Cell Antigens. NCBI Bookshelf.

The *RHD* gene encodes the RhD protein, a 417-amino-acid transmembrane protein that crosses the erythrocyte membrane in 12 transmembrane segments. RhD is part of the Rh complex in the erythrocyte membrane and is the protein carrying the D antigen, one of the most immunogenic blood group antigens. Its structure includes multiple transmembrane segments and extracellular loops that help define the regions exposed at the cell surface and contribute to the different epitopes of the D antigen.

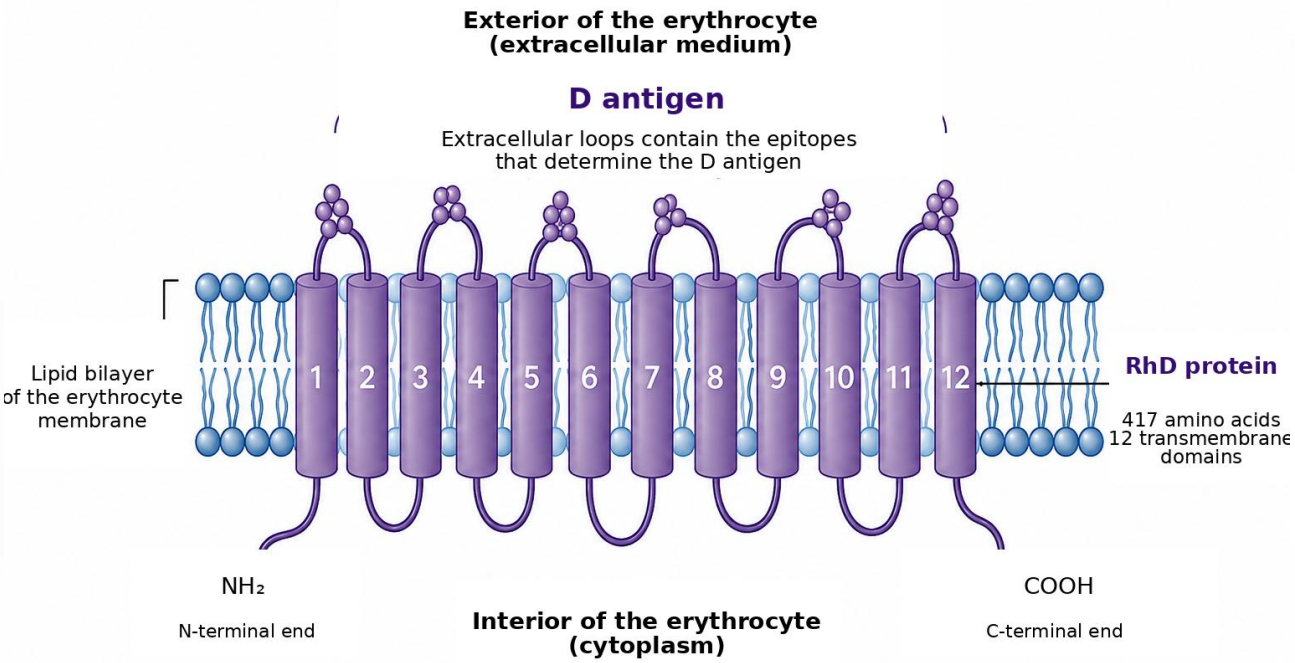

Figure 2. RhD protein in the erythrocyte membrane.
Source: original figure based on Avent ND, Reid ME. The Rh blood group system: a review. Blood. 2000;95(2):375–387.

The close homology between *RhD* and *RhCE* is particularly relevant at the molecular level, because recombination and gene-conversion events between *RHD* and *RHCE* can generate hybrid genes and D-antigen variants, including weak D and partial D phenotypes.

RhD positive and RhD negative

In the European population, the most frequent molecular cause of the RhD-negative phenotype is complete deletion of the *RHD* gene. This deletion is associated with homologous recombination between highly similar regions flanking the gene, known as Rhesus boxes.

However, not all RhD-negative phenotypes result from complete RHD deletion. Numerous gene variants can alter D-antigen expression, including weak D, partial D, DEL variants and

RHD-RHCE hybrid genes. This genetic diversity is one of the main challenges in molecular RhD genotyping.

Cell-free fetal DNA

During pregnancy, a small proportion of the cell-free DNA circulating in maternal plasma is of fetal origin. This cell-free fetal DNA originates mainly from the placenta and is mixed with a much larger amount of maternal cell-free DNA.

This makes it possible to exploit a key genetic feature: if the mother is RhD negative and does not carry a functional RHD gene, detection of RHD-specific sequences in maternal plasma may indicate the presence of fetal DNA from a fetus carrying RHD.

In this way, a maternal blood sample can be used to analyze the fetal *RHD* gene indirectly during pregnancy. This approach is one of the most established clinical applications of non-invasive prenatal diagnosis based on cell-free DNA.

Fetal RhD genotyping at Catlab

From maternal blood to fetal DNA

Fetal RhD genotyping is performed in RhD-negative pregnant women using a maternal blood sample collected in a 10 mL EDTA tube. Plasma is obtained from this sample and contains cell-free fetal DNA (cffDNA), which is the genetic material analyzed.

At Catlab, plasma is separated by centrifugation and stored at approximately −80 °C until extraction. DNA is subsequently extracted semi-automatically using the QIAcube system and the QIAamp MinElute ccfDNA Mini Kit.

Molecular detection of the fetal RhD genotype

The extracted cffDNA is analyzed using the FastQ® RHD fetal kit. The methodology is based on allele-specific PCR (PCR-SSP), with amplification detected by real-time PCR.

The reaction analyzes three specific regions of the *RHD* gene: exons 5, 7 and 10. Each sample is processed in triplicate, yielding up to nine specific detections per sample.

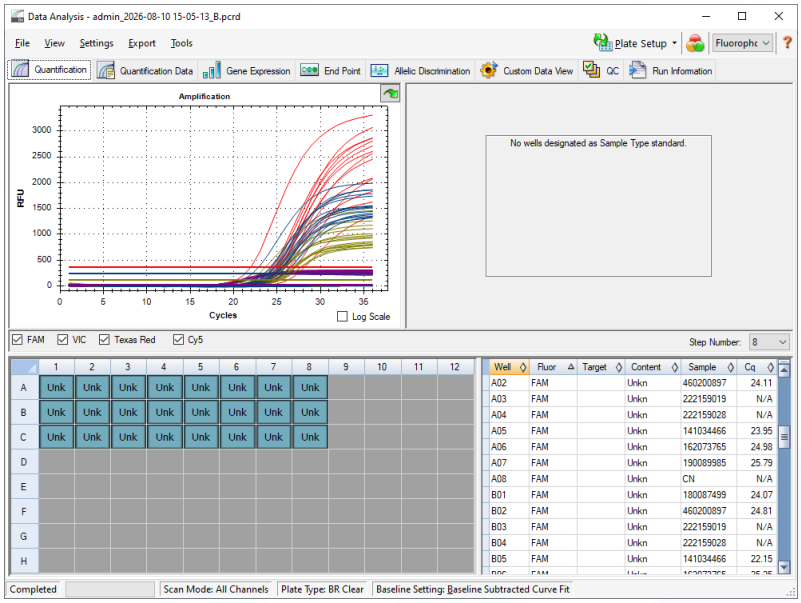

Figure 3. Example of amplification curves obtained with the CFX96 system during fetal RhD genotyping.

Each molecular target is detected using a specific fluorophore: exon 5 in the FAM channel, exon 7 in VIC and exon 10 in Texas Red. The reaction also includes an internal amplification control (IAC), associated with the human HCG gene, detected in the Cy5 channel.

For result interpretation, specific amplifications are considered positive when the Cq value is ≤30. The internal control confirms that the reaction contains amplifiable human DNA.

Specificity	Fluorophore	Criterion
Internal amplification control (IAC)	Cy5	Cq ≤ 30
Exó 5	FAM	Cq ≤ 30
Exó 7	VIC	Cq ≤ 30
Exó 10	Texas Red	Cq ≤ 30

Each analytical run includes a negative control (NTC) containing distilled water to monitor possible contamination. Under appropriate conditions, the negative control should not show a fluorescence signal within the established Cq range.

Interpretation also takes into account possible PCR artifacts, particularly in the Cy5 channel of negative controls, which may appear as late signals and require assessment together with the other run results.

Interpretation of the fetal RhD genotype

The determination is based on the number of positive specific detections obtained among the nine possible measurements (three exons analyzed in triplicate).

Interpretation is not based solely on the total number of positive signals. The distribution of signals among exons 5, 7 and 10 provides additional information. Discordant detection patterns among these exons may be related to *RHD* variants, such as *RHD-RHCE* hybrid genes, weak D or partial D variants, and may result in an inconclusive result.

Detection pattern	Interpretation	Action / result
≥4 of the 9 specific detections positive	Fetal RHD genotype positive	Positive result.
1–2 of the 9 specific detections positive	Fetal RHD genotype negative	If gestational age is ≤20 weeks, the study should be repeated from week 20 onwards.
3 positive detections or a discordant pattern among exons 5, 7 and 10	Inconclusive result	Repeat the study and perform a specific assessment of the pattern.
No specific exon detected	Fetal RHD genotype negative	Negative result, provided that the controls are valid.
Internal control (Cy5) absent / inhibited reaction	Invalid result	Uninterpretable reaction; the study must be repeated.

All inconclusive results are repeated. If the result remains inconclusive after repeat testing, the pregnancy is managed as a possible RhD-positive fetus as an obstetric precaution.

Fetal RhD genotyping in RhD-negative pregnant women using cell-free fetal DNA makes it possible to determine whether the fetus is RhD positive or negative from a simple maternal blood sample, without invasive procedures. This determination provides relevant information for pregnancy management and allows anti-D prophylaxis to be tailored to each pregnant woman's needs, contributing to more precise, safe and personalized care.

References

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