Designing a quantitative PCR assay for fetal RhD genotyping from maternal plasma derived cell-free fetal DNA (cffDNA)

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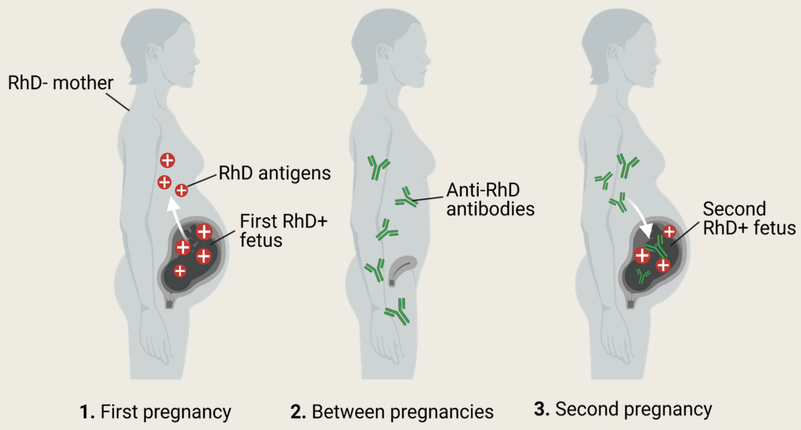
1 Introduction

Hemolytic disease of the fetus and newborn (HDFN) is typically caused by maternal alloimmunization against antigens of RhD positive red blood cells. During pregnancy, these anti-RhD antibodies can induce the destruction of fetal RhD positive red blood cells and lead to HDFN. Maternal alloimmunization can be prevented with antenatal and postnatal anti-D prophylaxis treatment, which is often routinely performed for all RhD negative mothers. However, fetal RhD genotyping allows anti-D prophylaxis to be targeted only for women carrying RhD positive fetus. Therefore, unnecessary anti-D prophylaxis treatments can be avoided.

The aim of the study was to develop a multiplexed, quantitative PCR (qPCR) assay for fetal RhD genotyping using maternal plasma derived cell-free fetal DNA (cffDNA) as sample material.

Key Features

- Multiplexed detection of *RHD* gene exon 5, 7 and 10 in addition to an internal control gene.
- Compatibility with PerkinElmer Vanadis Extract® Cell-Free DNA Extraction Unit.
- Dried qPCR chemistry and ready-to use qPCR plates enabling a simple and fast workflow.



The principle of maternal RhD alloimmunization.

2 Materials and methods

The multiplexed qPCR reaction was set up utilizing hydrolysis probes, including simultaneous detection of *RHD* gene exons 5, 7, 10, and human *RPP30* internal control gene. After optimizing assay conditions with synthetic plasmid samples, the assay was confirmed to be compatible with cffDNA samples extracted with PerkinElmer Vanadis Extract® system (Figure 1). Overall, 80 maternal plasma samples with unknown RhD phenotype were extracted with Vanadis Extract® and analysed with qPCR in addition to 6 control samples with known RhD phenotype. The presence of fetal DNA in the maternal samples was demonstrated by amplification of male-specific sex-determining region Y (*SRY*) gene. Assay performance was determined with both liquid and dried qPCR reagent mixes (Figure 2) and the specificity of the primers and probes was confirmed with qPCR amplification product analysis with PerkinElmer LabChip® capillary electrophoresis separation technology.

Figure 1. Vanadis Extract® Cell-Free DNA Extraction Unit was used for automatic cfDNA extraction from plasma samples.

Figure 2. qPCR workflow with dried reagents. Dried qPCR reagents enable qPCR reagent preparation by manufacturer and only sample addition is needed prior the qPCR run.

3 Results

Limit of detection (LoD 95 %), where lowest concentration at which 95% of positive samples are detected, was determined to be 30 copies per reaction with both dried and liquid qPCR reagents. Both dried and liquid qPCR reagents showed similar qPCR efficiency of *RHD* gene targets (95.8-107.3 %) (Table 1). The assay clearly distinguished RhD positive and negative cfDNA samples (Figure 3) and was specific to the *RHD* gene based on microfluidic electrophoresis determination. Male-specific sex-determining region Y (*SRY*) gene was detected from 10/20 randomly chosen maternal cfDNA samples, which demonstrated the compatibility of the selected cffDNA extraction method.

qPCR reaction mix	Dried qPCR Reagents			Liquid qPCR Reagents		
Target gene	*RHD* exon 5	*RHD* exon 7	*RHD* exon 10	*RHD* exon 5	*RHD* exon 7	*RHD* exon 10
qPCR Efficiency, %E	107.3 %	104.1 %	99 %	99.7 %	105.6 %	95.8 %
Linear correlation, R^2	0.99	0.99	0.99	0.99	0.98	0.99
LoD, 95 % (DNA copies/reaction)	30	30	30	30	30	30

Table 1. Comparison of limit of detection (LoD 95 %, n=20), qPCR reaction efficiency and linear correlations (R^2) between dried and liquid reaction mixes. Both reaction mixes showed similar performance and LoD, which was determined to be 30 DNA copies/reaction.

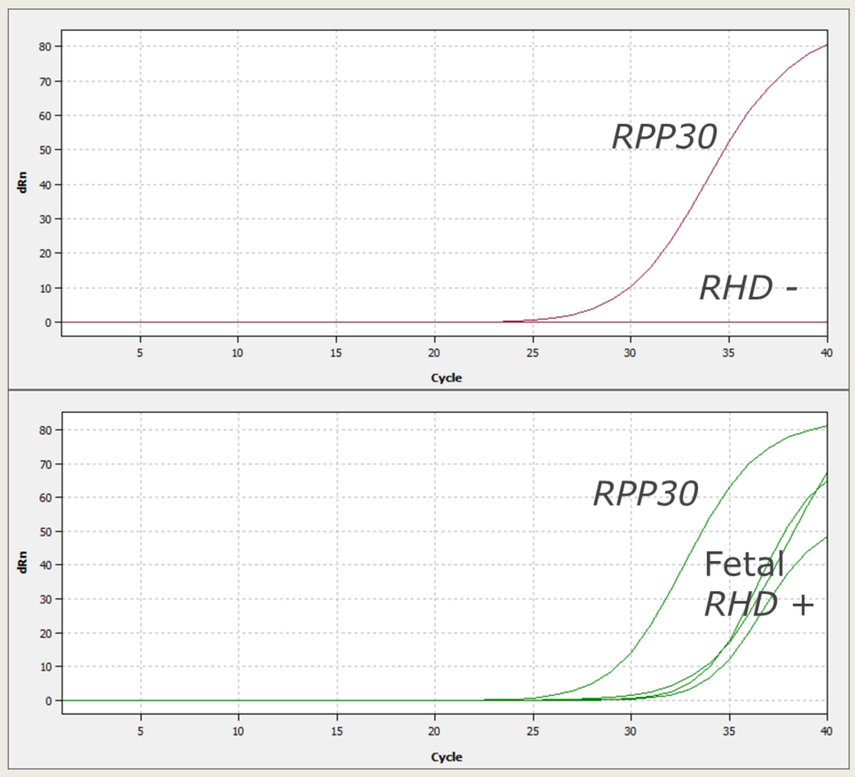


Figure 3. Amplification curves from RhD negative individual and RhD negative pregnant women with RhD positive fetus.

4 Conclusion

Preliminary results suggest, that the assay would be suitable for its intended use for qualitative detection of *RHD* gene from maternal cfDNA samples extracted with PerkinElmer Vanadis Extract® or equivalent cfDNA extraction system. Dried qPCR reagents showed similar performance compared to traditional liquid qPCR reagents. Usage of dried qPCR reagents enables faster and simpler workflow, as only sample addition is required. Thus, fetal RhD genotyping could be easily combined with other prenatal screening using cffDNA samples. To further evaluate clinical sensitivity and specificity of the assay, studies with a greater number of maternal cfDNA samples with known serological RhD phenotypes are needed.

5 Summary

This study demonstrated the possibility of using cfDNA samples extracted with Vanadis Extract® Cell-Free DNA Extraction Unit for additional qPCR-based assays. This RhD qPCR assay with dried reagents would offer simple and fast workflow for fetal RhD genotyping. Dried reagents enable shipping and storage at room temperature and removes the need for separate laboratory space for PCR reagent preparation. However, further clinical testing is required to determine the clinical sensitivity and specificity of the assay.

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