Additional step in detection of cell-free fetal DNA from maternal plasma to improve non-invasive prenatal diagnostic

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Introduction

Rhesus D (RhD) blood group incompatibility between RhD-negative mother and RhD-positive fetus can occasionally result in maternal alloimmunization where the resultant anti-D antibodies can cross the placenta and attack the fetal red cells, which in worse case scenarios can cause fetal anemia and ultimately death. Determination of fetal blood group RhD in immunized pregnant women is necessary for the prediction of the haemolytic disease of the newborn (HDN) due to anti-D antibodies (1, 2).

Noninvasive fetal genotyping became feasible after cell-free fetal DNA was isolated from the maternal plasma. In the last few years very sensitive methods for the detection of cell-free fetal DNA from maternal plasma have been developed. The method also gives us a chance to limit the prenatal prophylaxis with anti-RhD IgG only with RhD-negative women bearing RhD positive fetus (3, 4, 5, 6). The isolation and detection of free-fetal DNA is critical because of low yield of isolated free fetal-DNA.

In our study the automatic isolation of free-DNA was performed and a new preanalytical step was included for improvement in test robustness for reliable results in early pregnancy.

Material and Methods

Ten different blood samples of RhD-negative pregnant woman were collected. The automatic isolation by EZ1 instrument (Qiagen) using EZ1 Virus Mini Kit v2.0 (Qiagen) was used for the isolation of free-DNA. The 400 µL of plasma was used as a starting material for the isolation. The eluted volume of free-nucleic acid was 60 µL.

The 5µL of isolated free-DNA was denaturated for three minutes at 95 ºC and then chilled in an ice-water bath. The samples were then preamplified using PreAmp Master Mix (Applied Biosystems) according to the manufacturer's instructions (Applied Biosystems). The 180 nM concentration of each primer for amplification and 40 nM concentration of each probe (Applied Biosystems) gene *RHD* exon 7 and exon 10, gene *SRY* and gene *albumin* were used. Preamplified reactions were finally diluted 5x.

All isolated cell-free DNA samples and all preamplified 5X diluted samples were analyzed by real-time TaqMan PCR for gene *albumin*, gene *RHD* exon 7 & exon 10 and gene *SRY*. The reactions were performed according to manufacturer's instructions in 10 µL reaction volume on ABI PRISM 7900HT (Applied Biosystems). Standard curves were performed for all tested genes using human RhD-positive male genomic DNA standard (Promega).

References

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Results

Results are shown in Table I. The linear view of amplification plots for sample S1 are shown in Figure I, Figure II, Figure III and Figure IV, respectively. The examples of standard curves performed using human RhD-positive male genomic DNA standard (unpreamplified and preamplified 5X diluted) for *albumin* and *RHD* exon 7 are shown in Figure V.

SAMPLE	TARGET GENES							
	ALBUMIN		*RhD Exon 10*		*RhD Exon 7*		*SRY*	
	2µL isolated free-DNA	2µL 5x diluted preamplified free-DNA	2µL isolated free-DNA	2µL 5x diluted preamplified free-DNA	2µL isolated free-DNA	2µL 5x diluted preamplified free-DNA	2µL isolated free-DNA	2µL 5x diluted preamplified free-DNA
	C_t ± SD	C_t ± SD	C_t ± SD	C_t ± SD	C_t ± SD	C_t ± SD	C_t ± SD	C_t ± SD
S 1	34,57±0,81	24,08±0,39	34,66±0,11	25,67±0,15	37,60±1,55	29,32±0,13	37,33±0,57	27,44±0,21
S 2	34,50±0,84	23,40±0,31	38,85±0,07	27,22±0,22	0	28,67±0,02	35,76±0,37	26,84±0,05
S 3	35,44±0,03	24,18±0,51	36,75±0,06	28,65±0,03	36,97±0,60	29,72±0,57	35,85±0,04	26,36±0,06
S 4	35,63±0,19	23,75±0,12	0		0		0	0
S 5	34,03±0,31	22,71±0,09	38,14±0,19	28,72±0,17	38,86±0,35	26,83±0,16	35,34±0,19	26,13±0,01
S 6	33,91±0,90	22,63±0,08	34,98±0,88	28,16±0,81	38,93±0,16	29,14±0,10	0	0
S 7	33,98±0,31	23,09±0,14	0	0	0	0	0	0
S 8	35,35±0,68	24,46±0,79	0	28,64±0,23	39,42±0,36	29,76±0,23	0	26,27±0,07
S 9	35,68±0,87	23,97±0,22	0	27,60±0,24	34,94±0,56	30,51±0,21	0	26,40±0,05
S 10	35,21±0,88	24,77±0,41	0	0	37,87±0,70	31,20±0,65	0	0

Table I: The analysis of different samples of isolated free fetal-DNA from the maternal plasma for gene *albumin* (as endogene control), gene *RHD* (exon 7 & exon 10) and gene *SRY*.

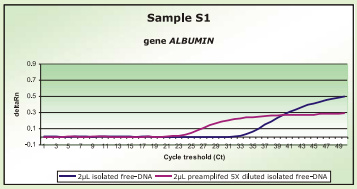

Figure I: Real-Time PCR amplification plots for gene *albumin* unamplified DNA vs. preamplified DNA for sample S1.

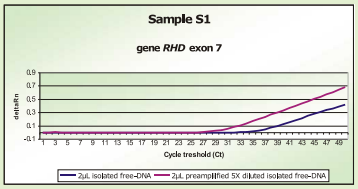

Figure II: Real-Time PCR amplification plots for gene *RHD* exon 7 unamplified DNA vs. preamplified DNA for sample S1.

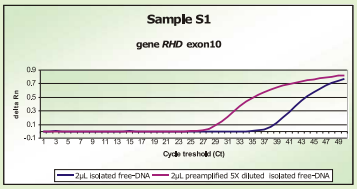

Figure III: Real-Time PCR amplification plots for gene *RHD* exon 10 unamplified DNA vs. preamplified DNA for sample S1.

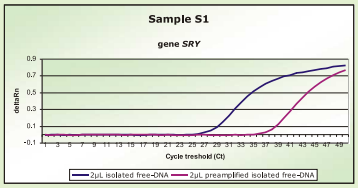

Figure IV: Real-Time PCR amplification plots for gene *SRY* unamplified DNA vs. preamplified DNA for sample S1.

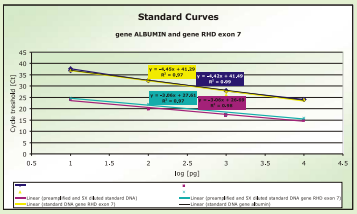

Figure V: Standard curves for amplification of gene *RHD* exon 7 and gene *albumin* for unamplified standard DNA and preamplified 5x diluted standard DNA.

Conclusions

The advantage of fetal *RHD* determination from maternal plasma compared to classical sampling is enormous since there is no risk for the mother or the fetus.

Advantages of our new preanalytical step are:

- Lower concentration of fetal free-DNA can be detected using additional step of preamplification
- The standard deviation of Cts between unpreamplified and premplified dilutions of samples were lower

This study presents a suitable method for non-invasive fetal *RHD* genotyping, especially for determination of fetal Rhesus D status in early pregnancy, when the amount of cell-free fetal DNA is low.