A Direct from Plasma Approach to Non-Invasive Prenatal Fetal Sex Determination and RhesusD Genotyping by Quantitative PCR

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Introduction

In the field of non-invasive prenatal diagnosis (NIPD), cell free fetal DNA (cffDNA) analysis by quantitative PCR (qPCR) is commonly used to determine fetal sex at early gestation in pregnancy[1] ; and fetal RhesusD (RhD) genotype in pregnant women at risk of developing Hemolytic Disease of the Fetus and Newborn (HDFN)[2] . Non-invasive screening of fetal sex is warranted where the fetus is at risk of inheriting a sex-linked disorder or congenital adrenal hyperplasia. This reduces by 50% the need for further diagnostic testing by invasive techniques, such as amniocentesis and chorionic villus sampling, which carry a 0.5-1% risk of miscarriage[3] . Women who have a negative RhD genotype are at risk of developing HDFN in the event of exposure to the RhD antigen when bearing an RhD positive child. In these cases, anti-D immunoglobulin is administered to avoid anti-D immunization in the mother. Unfortunately, this results in a significant percentage (38%) of RhD negative mothers receiving the anti-D human blood products unnecessarily[4] . In the UK, recent NICE recommendations [5] and other publications [6] demonstrate the diagnostic and health economic benefits of performing RhD fetal testing using NIPD as opposed to the current practice of providing all RhD negative pregnancies with a prophylactic treatment.

Aims

In current practice, NIPD of fetal sex and RhD genotype is conducted by extracting cell free DNA (cfDNA) from plasma isolated from peripheral blood and conducting qPCR to determine the presence/absence of the Y-chromosome or RhD gene in cffDNA. In the current study, we aim to showcase the faster and more streamlined approach of using the Cell3™Direct technology (Nonacus, UK), which enables qPCR testing to be conducted directly from plasma, thus avoiding the time consuming and costly necessity of cfDNA extraction.

Methods

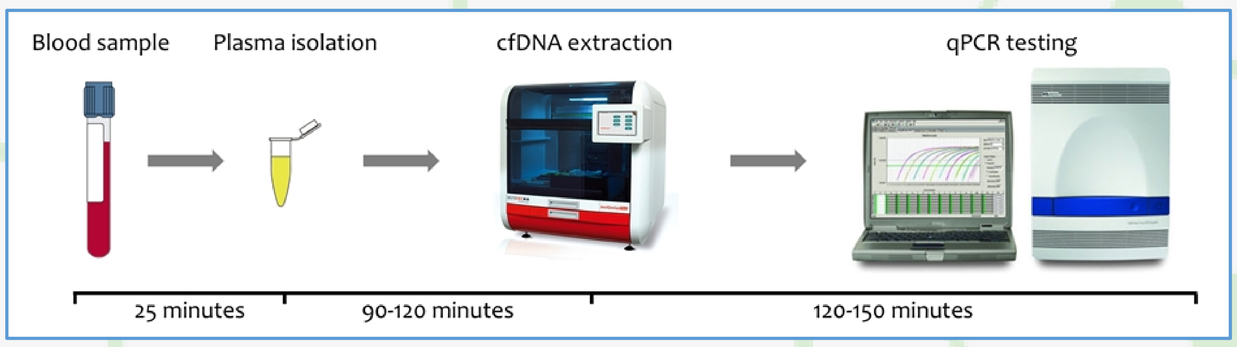
Samples: samples containing RhD positive genomic DNA (gDNA) spiked in RhD negative gDNA; and male gDNA spiked in female gDNA at different ratios was used for initial assay evaluation. 100 plasma samples from RhD negative pregnant women (24-26 weeks gestation) and 50 samples from healthy pregnancies (10-27 weeks of gestation) were used to assess the sensitivity and specificity of this technology.

qPCR: the Cell3™Direct: Rhesus D Fetal Blood Group Genotyping kit was used to investigate the fetal RhD genotype in the 100 plasma samples from RhD negative pregnant women. The kit targets sequences specific for exons 5, 7 and 10 of the RhD gene and therefore can distinguish between fetal RhD positive, negative and RhD PSI genotypes in a background of RhD negative maternal DNA. A target for the CCR5 control gene is also included to confirm adequate cfDNA quantity within the sample. The Cell3™Direct: Fetal Sex Determination kit was used to determine the fetal sex in the 50 plasma samples from healthy pregnancies. The kit detects the Y-chromosome specific genes SRY, TSPY and DAZ; as well as the CCR5 control gene.

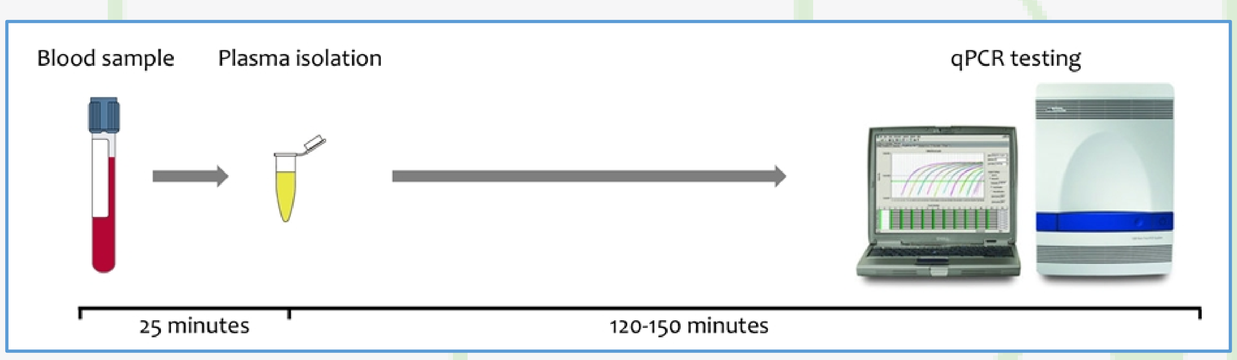
Data Analysis: the samples were blinded and analyzed independently using parameters comparable to those used routinely in a clinical setting.

Workflow: advantages of testing direct from plasma

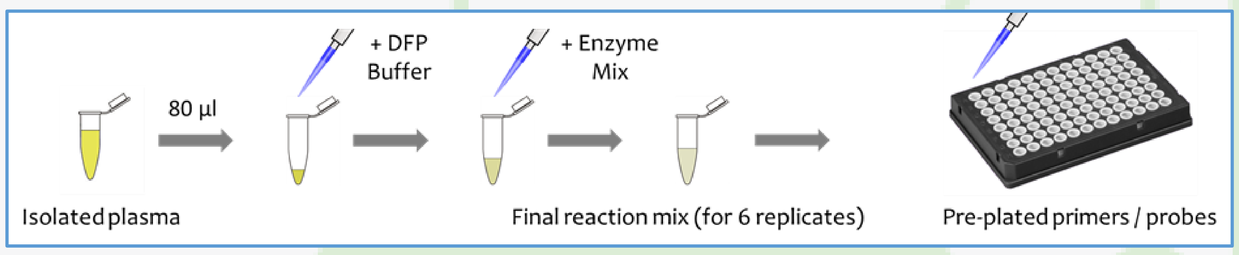
The routine workflow used in current practice for NIPD of fetal RhD genotype and fetal sex determination requires cfDNA extraction, resulting in a sample to result turn-around-time of 4-5 hours.



By using the direct from plasma approach, cfDNA extraction is no longer required, which results in a faster and less expensive workflow with a sample to result turn-around-time of 2.5-3 hours.

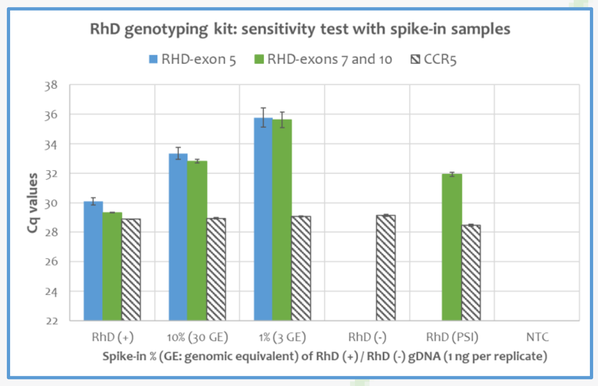


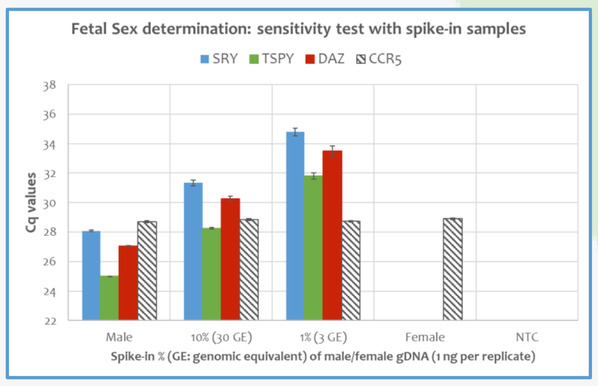
With the streamlined workflow of the Cell3™Direct: Rhesus D Fetal Blood Group Genotyping kit, sample setup requires only three simple steps.



Technical evaluation

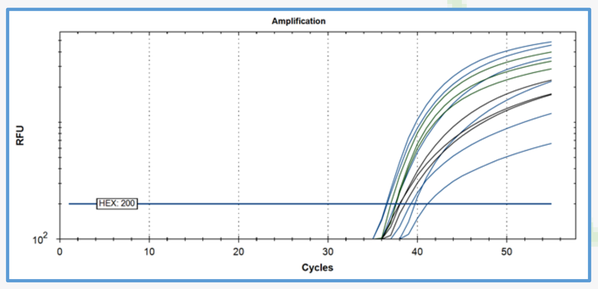
The accuracy and sensitivity of the Cell3™Direct: Rhesus D Fetal Blood Group Genotyping kit was evaluated on artificially prepared samples containing RhD positive gDNA spiked into RhD negative gDNA at 10% and 1% ratios. A 10% Rh D PSI variant spike-in sample was also tested to prove that the exon 5 target could discriminate it from the RhD wild type sequence. Similarly, the Cell3™Direct: Fetal Sex Determination kit was evaluated on samples containing male gDNA spiked into female gDNA at the same ratios. Mean Cq values were calculated from 3 replicates per target and 1 ng of total DNA was used per replicate.



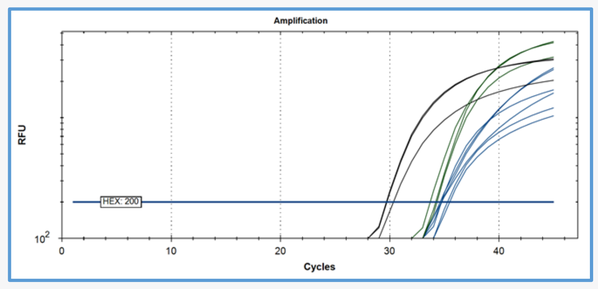


The Cell3™Direct technology showed robust target amplification direct from plasma when compared to the data obtained from the corresponding extracted cfDNA from the same sample, as seen in the below qPCR amplification plots.

RhD genotyping kit

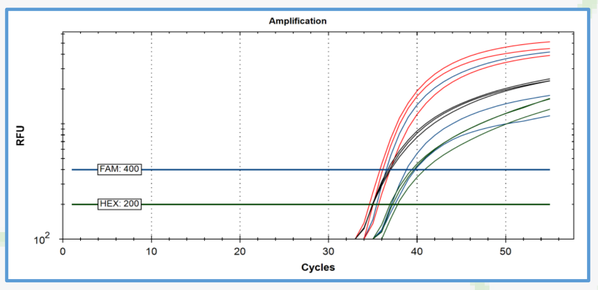


Direct from plasma amplification plot

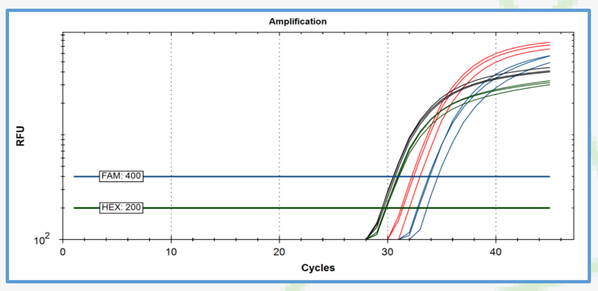


cfDNA amplification plot

Fetal Sex determination kit



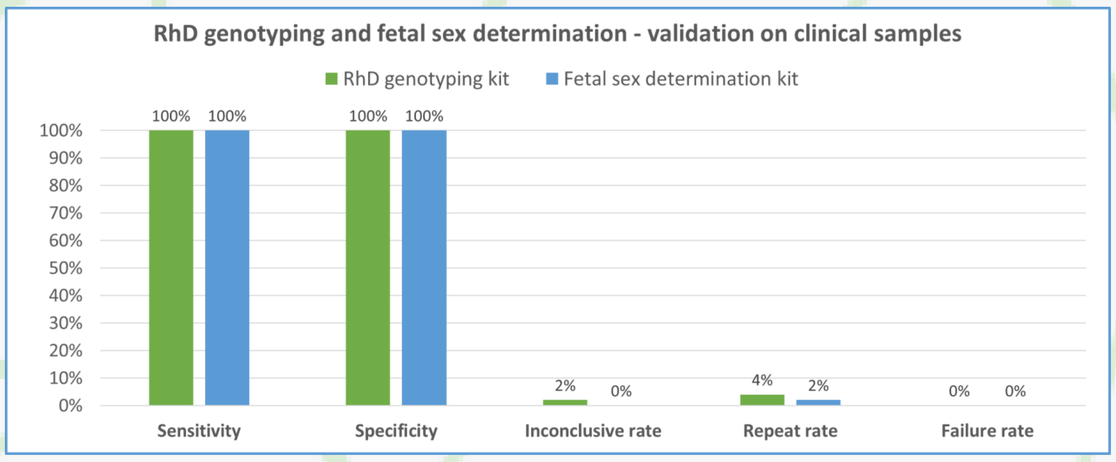
Direct from plasma amplification plot



cfDNA amplification plot

Validation on clinical samples

Testing on the 100 plasma samples obtained from RhD negative pregnant women at 24-26 weeks of gestation was conducted using the Cell3™Direct: Rhesus D Fetal Blood Group Genotyping kit. Fetal RhD genotype was known for the entire cohort, with 68 RhD positive and 32 RhD negative outcomes. Results showed sensitivity and specificity rates of 100%, with a 2% inconclusive rate; a 4% repeat rate and a 0% failure rate. Testing on 50 plasma samples from healthy pregnancies at 10-27 weeks of gestation (average gestation of 15.3 weeks) was conducted using the Cell3™Direct: Fetal Sex Determination kit. Fetal sex was known for all samples, with 25 male and 25 female outcomes. Results revealed sensitivity and specificity rates of 100%, with a 2% repeat rate and a 0% inconclusive and failure rates. Test setup and analysis was conducted blinded for all samples.



Conclusions

The Cell3™Direct technology is the first commercially available solution that enables Non-Invasive Prenatal Testing of fetal Rhesus D genotype and fetal sex determination direct from plasma. These kits set a new benchmark in simplicity of setup with results generated within 3 hours. The protocol offers significantly reduced hands-on time due to no sample extraction requirement and flexible pre-plated assays. Additionally, the total plasma quantity required is 80 µl per sample, therefore allowing for a smaller volume of blood to be drawn and plenty of sample remaining for further tests to be undertaken.

References

1. Diagnostic accuracy of routine antenatal determination of fetal RHD status across gestation: population based cohort study: BMJ 2014;349:g5243 doi: 10.1136/bmj.g5243.
2. NICE recommends test to identify fetal rhesus D status: BMJ 2016;355:i6106 BMJ 2016;354:i3944.
3. Sensitivity of fetal RHD screening for safe guidance of targeted anti-D immunoglobulin prophylaxis: prospective cohort study of a nationwide program in the Netherlands: BMJ 2016;355:i5789.
4. Diagnostic accuracy of routine antenatal determination of fetal RHD status across gestation: population based cohort study. BMJ 2014;349:g5243 doi: 10.1136/bmj.g5243.
5. NICE recommends test to identify fetal rhesus D status. BMJ 2016;355:i6106 BMJ 2016;354:i3944.
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