Protocol guide v1.2

Cell3™ Direct

Rhesus D Fetal Blood Group Genotyping (tube format)

Updates from version 1.09

1. New protocol for kit provided with primers and probes in tubes.

Intended use

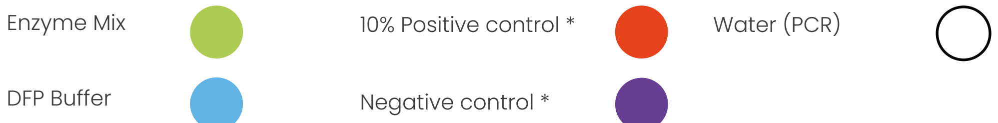
The intended use of the Cell3™ Direct Rhesus D Fetal Blood Group Genotyping kit is for the detection of Rhesus D (RhD) gene-specific sequences in plasma obtained from RhD negative pregnant women at 11 or more weeks of gestation. Cell free DNA is present in the blood of every individual and in pregnant women around 2-20% of cell free DNA is of fetal origin. Women who are RhD negative are at risk of developing Hemolytic Disease of the Fetus and Newborn (HDFN) if carrying an RhD positive child. A test to determine the RhD genotype of the fetus is often conducted to determine whether anti-D prophylaxis administration is necessary to avoid anti-D immunization.

The Cell3™ Direct Rhesus D Fetal Blood Group Genotyping kit provides all necessary reagents to conduct Rhesus D fetal blood group genotyping by real-time quantitative PCR using plasma or extracted cfDNA as the input material. The assay provides high sensitivity and specificity by targeting multiple RhD gene-specific sequences. Positive and negative controls are also included in the kit for quality control purposes.

Kit contents

EX assay = primers / probes targeting exon 5 (FAM labelled) and exons 7 and 10 (HEX labelled) of the RhD gene

C5 assay = primers / probes targeting exon 5 of the RhD gene (FAM labelled) and the CCR5 gene (HEX labelled)



*** Positive and negative genomic DNA controls are at a final concentration of 1 ng per replicate or 300 genomic equivalents. Therefore, within the 10% positive control 30 genomic equivalents are of RhD (+) origin and 270 of RhD (-) origin.**

Required equipment

Centrifuge equipped with swinging bucket rotor capable of accommodating 96 well plates

Pipette p10 / p100 / p200 / p1000

Class II Biosafety Cabinet or similar

Microcentrifuge (1.5 – 2.0 ml)

Vortexer

96 well plate holder

Real Time (qPCR) machine with minimum of 2 channels (FAM/HEX) or (FAM/VIC)

Additional user supplied consumables

96 well qPCR plate (standard or low profile) with white wells that fit the qPCR instrument of choice

0.5 – 1.5 – 2 ml PCR clean / DNA free micro centrifuge tubes

Pipette filter tips for p10 / p100 / p200 / p1000

Disposable sterile laboratory gloves

Storage and handling

All components are shipped at room temperature and are stable in these conditions for transportation. Upon arrival briefly centrifuge all kit components to ensure that content is collected at the bottom of the tube/wells. Store the DFP Buffer solution at +4°C and all other kit components at -20°C. The EX assay and C5 assay tubes containing primers and probes and the Enzyme Mix are light sensitive and should be kept in the dark. Before use please ensure all reagents are fully thawed, thoroughly mixed and spun down. The Enzyme Mix is ready to use and contains a concentration of ROX compatible with all qPCR machines. Avoid repeated freezing and thawing.

Plasma isolation

Please note that blood samples received in EDTA tubes (purple lid) should be stored at +4°C and processed within 24 hours of blood draw. Samples received in Cell-Free DNA BCT Streck tubes should be stored at room temperature and processed within 7 days of blood draw. The sample should not be processed if received in any other tube or if clotted.

All work should be carried out in a Class II biosafety cabinet using standard aseptic techniques to maintain sample sterility.

1. Centrifuge the blood sample at 2,000 g for 10 minutes using a swinging bucket rotor.

2. Collect the separated plasma using a 1,000 µl pipette ensuring that the buffy coat remains undisturbed.

3. Aliquot into 1.5 – 2 ml sterile 'PCR Clean' microcentrifuge tubes.

4. Centrifuge at maximum speed (minimum 10,000 g) for 10 minutes in a microcentrifuge.

NOTE: the second centrifugation step ensures that isolated plasma is free of cell debris, which can reduce qPCR efficiency and result in higher Ct values.

5. Taking care not to disturb the pellet retrieve the supernatant using a 1,000 µl pipette and aliquot into a fresh sterile 'PCR Clean' microcentrifuge 1.5 – 2 ml tube.

6. Plasma can be stored at -20°C for long term storage. The sample is now ready for direct from plasma testing (Direct from Plasma qPCR) or alternatively the sample can be processed following cell free DNA extraction (follow optional qPCR on extracted cell free DNA protocol below).

NOTE: frozen plasma can be thawed at room temperature or in a heat block/bath at 37°C and used for direct from plasma testing or cfDNA extraction. When used for direct from plasma testing, vortex briefly once thawed and spin down at maximum speed in a microcentrifuge for 2 minutes. Direct from plasma testing has been conducted on plasma samples subjected to up to 4 freeze/thaw cycles with no observed negative effect on test performance.

Direct from plasma qPCR

Table 1 outlines the required volumes of sample and reagents needed to set up the direct from plasma qPCR reaction for a total of six replicates per sample (5x EX and 1x C5). Refer to Table 2 to prepare the recommended positive, negative and no template controls. All procedures should be carried out in a sterile environment, ideally a Class II biosafety cabinet.

Table 1. Plasma preparation for 1 sample (includes 7% excess)

Plasma samples	Plasma (μl)	DFP buffer (μl)	Enzyme mix (μl)	Final volume (μl)
x1 sample -6 replicates	80	80	160	320

Table 2. Control preparation for 1 sample (includes 7% excess)

Controls	Control (μl)	DFP buffer (μl)	Water (μl)	Enzyme mix (μl)	Final volume (μl)
x1 sample -6 replicates	6.4	80.0	73.6	160	320

1. Add plasma to DFP Buffer (using the volumes indicated in Table 1) in individual sterile 'PCR Clean' 0.5 – 1.5 ml microcentrifuge tube.
2. Vortex for 10 seconds, spin down and incubate at room temperature for 2 minutes.
3. Add control to PCR grade water and DFP Buffer (using the volumes indicated in Table 2) in individual sterile 'PCR Clean' 0.5 – 1.5 ml microcentrifuge tube.
4. Vortex for 5 seconds and spin down.
5. Add the enzyme mix to the tubes containing plasma or controls to prepare the final reaction mix.
6. Vortex for 5 seconds and spin down.
7. Split each sample / control into two tubes containing the following:
    - Tube 1: containing 266 μl of the final reaction mix and 10.7 μl of the EX assay primer/probe mix.
    - Tube 2: containing 54 μl of the final reaction mix and 2.1 μl of the C5 assay primer/probe mix.
8. Vortex each tube for 5 seconds and spin down.
9. For each sample aliquot 52 μl of the final reaction mix containing the EX assay primer/probe mix (tube-1) to 5 wells of a 96 well qPCR plate; and 52 μl of the final reaction mix containing the C5 assay primer/probe mix (tube-2) to 1 well of the same qPCR plate.

NOTE: to ensure the sensitivity of the assay, it is highly recommended that 96 well qPCR plates with white wells are used.

10. Carefully seal plate using an optically clear qPCR seal or optically clear 8 well cap strips.

11. Spin down the plate in a 96 well plate centrifuge at 1,000 g for 2 minutes.

qPCR instrument setup and cycling conditions direct from plasma

Set up the qPCR instrument using manufacturers guidelines. Where possible, choose the Quantitation by Comparative Ct (ΔΔCt) method with TaqMan or "Other" reagents (do not add a melt curve option). For qPCR instruments with "FAST" blocks, select the Standard ramp speed. Select the FAM filter for the exon 5 target; and the HEX or VIC filter for exons 7 and 10 and CCR5 targets. Where possible, select NFQ-MGB as quencher; or alternatively leave this field empty. Do not select a dye as quencher (such as TAMRA). Select Program cycling conditions as shown in table below. Set reaction volume to 5o μl and select the white well plate setting (when possible).

Cycle step	Temperature	Time	Cycles
Initial activation	98°C	10 min	1
Denaturation	95°C	10 sec	50
Annealing/ Elongation	62°C	30 sec	

Optional qPCR setup for extracted cell free DNA

Cell free DNA should be extracted from plasma using a recognised and validated kit specific for cell free DNA extraction. Table 3 outlines the required volumes of sample and reagents needed to set up the qPCR reaction for extracted cell free DNA for a total of six replicates per sample (5x EX and 1x C5). Refer to Table 4 to prepare the recommended positive, negative and no template controls. All procedures should be carried out in a sterile environment, ideally a Class II biosafety cabinet.

Table 3. cfDNA preparation for 1 sample (includes 7% excess)

cfDNA samples	cfDNA (µl)	Water (µl)	Enzyme mix (µl)	Final volume (µl)
x1 sample -6 replicates	32*	128	160	320

Table 4. Control preparation for 1 sample (includes 7% excess)

Controls	Control (µl)	Water (µl)	Enzyme mix (µl)	Final volume (µl)
x1 sample -6 replicates	6.4	153.6	160	320

1. Add cell free DNA and PCR grade water to the Enzyme Mix (using the volumes in the table above) in individual sterile 'PCR Clean' 0.5 – 1.5 ml microcentrifuge tube to prepare the final reaction mix.

2. Vortex for 5 seconds and spin down.

3. Add control and PCR grade water to the Enzyme Mix (using the volumes indicated in Table 4) in individual sterile 'PCR Clean' 0.5 – 1.5 ml microcentrifuge tube.

4. Vortex for 5 seconds and spin down.

5. Split each sample / control into two tubes containing the following:

- Tube 1: containing 266 µl of the final reaction mix and 10.7 µl of the EX assay primer/probe mix.
- Tube 2: containing 54 µl of the final reaction mix and 2.1 µl of the C5 assay primer/probe mix.

6. Vortex each tube for 5 seconds and spin down.

7. For each sample aliquot 52 µl of the final reaction mix containing the EX assay primer/probe mix (tube-1) to 5 wells of a 96 well qPCR plate; and 52 µl of the final reaction mix containing the C5 assay primer/probe mix (tube-2) to 1 well of the same qPCR plate.

NOTE: to ensure the sensitivity of the assay, it is highly recommended that 96 well qPCR plates with white wells are used.

8. Carefully seal plate using an optically clear qPCR seal or optically clear 8 well cap strips.

9. Spin down the plate in a 96 well plate centrifuge at 1,000 g for 2 minutes.

qPCR instrument setup and cycling conditions for extracted cell free DNA

Set up the qPCR instrument using manufacturers guidelines and as indicated in the qPCR instrument setup and cycling conditions for direct from plasma protocol above. Program cycling conditions as shown in table below.

Cycle step	Temperature	Time	Cycles
Initial activation	98°C	10 min	1

qPCR data analysis

Please note data analysis may vary between qPCR machines and thresholds must be determined empirically by the end user or laboratory. This assay has been designed specifically to be highly sensitive to the presence of RhD (+) DNA against a background of RhD (-) DNA. Therefore, the end user must ensure there is no possibility of RhD (+) DNA contamination.

Typically, a threshold of 42 cycles is used to determine whether the result is positive or negative for the presence of RhD (+) DNA when using plasma as input material; and a threshold of 40 cycles is used when using extracted cfDNA as input material. CCR5 specific targets are used to determine if cell free DNA is present in the sample or not. We recommend using x5 replicates for the EX multiplex and x1 replicate for the C5 multiplex, as this gives a total of 11 replicates of RhD specific targets from which to interpret.

For further guidelines on how to analyse and interpret qPCR data generated by the Cell3™ Direct Rhesus D Fetal Blood Group Genotyping kit, please download the Analysis Guidelines document from our website. For further information on kit performance, please refer to our Cell3™ Direct Rhesus D Fetal Blood Group Genotyping datasheet.

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