

Cell3™ Direct

Rhesus D Fetal Blood Group Genotyping

Analysis Guidelines v1.3



Updates from Version 1.2

1. In the [Data Analysis Using Semi-Automated Excel Worksheet](#) section, an upper Ct threshold of 40 cycles has been added as a recommendation for analysing samples where extracted cfDNA is used as input material.
2. Further recommendations on how to handle samples which yield inconclusive calls has been included in the [Calling RhD Positive and Negative Outcomes](#) section.
3. The section [Calling RhD Exon 5 Variants](#) has been added.



Intended Use

The analysis process described in this document is intended to be used as a guideline to interpret quantitative/Real-Time PCR (qPCR) data generated by the Cell3™ Direct: Rhesus D Fetal Blood Group Genotyping kit. These guidelines are not intended to substitute standard operating procedures and we recommend that validation of the kit be conducted on a set number of samples with known outcomes by the end user, in order to assess the best analysis process for data interpretation.

Premise

The following guidelines describe how to analyse qPCR data obtained by following the setup process outlined in the Cell3™ Direct: Rhesus D Fetal Blood Group Genotyping, Protocol Guide; and in conjunction with the Cell3 Direct - Fetal RhD analysis template excel worksheet. Therefore, it is assumed that 6 replicates of each tested sample were loaded onto the qPCR plate (5 replicates for the EX assay and 1 for the C5 assay).

Setting Analysis Parameters on the qPCR Instrument and Data Export

Depending on the qPCR instrument used, the associated software interface can differ considerably. However, each software should allow the user to set the analysis settings of their preference. The following settings should be inputted for all targets/dyes:

- Baseline set to automatic*
- Ct threshold: 0.02 for instruments showing the ΔRn on the Y axis of the amplification plot**; 200 for instruments showing the raw fluorescent units (RFU) on the Y axis

However, we strongly advise that users find the optimal Ct threshold to use based on their experience with the qPCR instrument in question and the data they obtain.



As a way of roughly determining whether the Ct threshold is in the right range, aim to observe the following Ct values in the positive control:

- RhD exon 5 should show a Ct value between 32 and 35
- RhD exon 7-10 multiplex should show a Ct value between 31 and 34
- CCR5 should show a Ct value between 28 and 31

Once the baseline and Ct threshold are set, export all the data in a .CSV or .xls format that can be opened with Microsoft Excel.

***NOTE:** occasionally, certain targets may show artefactual/unspecific amplification curves in female/negative samples. In these cases, manually setting the baseline “start cycle” to 5 and the “end cycle” to 25 for these targets may remove these unspecific curves and render the data cleaner.

****NOTE:** in older instruments, the 0.02 and 0.04 Ct thresholds may result too low and should be adjusted to 0.2 and 0.4.

Data Analysis Using Semi-Automated Excel Worksheet

Open the Cell3™ Direct - Fetal RhD analysis template Excel worksheet and select the “Raw Data Analysis” tab. The top of the sheet can be filled in with experiment details. From the results.csv (or .xls) file containing the data exported from the qPCR instrument, copy over the information contained in the columns of cells titled “Well”, “Sample Name”, “Type” (optional), “Target”, “Reporter Dye” (optional) and “Ct”.

Sort the data so that all the Ct values for the RhD exon 5 target are contained between row 14 and row 109 (starting from row 14). Do the same for Ct values for the RhD exon 7-10 target so that they are contained between row 110 and 189 (starting from row 110). Finally, repeat the process for Ct values for the CCR5 target so that they are contained between row 190 and row 205 (starting at row 190).

Sort the data again by sample name for each one of the three targets. This way, the “Analysed results” boxes on the right hand side of the screen will display the mean Ct value and standard error for each sample, as well as the number of positive and negative replicates identified. This identification is calculated in column I, where Ct values are copied



over only if they are lower than 42 and higher than 24. Any Ct values higher than 42 are considered as unspecific amplification; while Ct values lower than 24 are considered artefacts.

NOTE: the 24 and 42 Ct thresholds for discriminating a positive from a negative replicate were obtained empirically on samples tested using plasma as input material. For samples tested using extracted cfDNA as input material, an upper Ct threshold of 40 is recommended. However, it is ultimately the decision of the user on whether to use these thresholds as advised in these guidelines or change them.

Calling RhD Positive and Negative Outcomes

The analysed data from the “Raw data analysis” tab is automatically copied over to the “Outcomes and graphs” tab in a summarised form. The amount of positive replicates identified for the RhD exon 5 target (6 replicates in total) and the RhD exons 7-10 multiplexed target (5 replicates in total) is represented in percentages in the “Replicates used (%)” box. Based on these values, a calling algorithm will determine whether the sample is:

- RhD positive (Pos): 3/11 replicates or more are identified as positive;
- RhD negative (Neg): 0/11 replicates or less are identified as positive;
- Inconclusive (Inc)*: between 1/11 and 2/11 replicates are identified as positive.

This algorithm has been developed empirically using samples tested direct from plasma. However, it is not meant to replace existing classification approaches of individual users and it is ultimately up to the user to determine the thresholds to use for data interpretation.

A histogram of the summarised data containing mean Ct values and calculated standard error bars for all samples tested is also included for the user’s convenience.

* NOTE: inconclusive calls are usually a result of:



- a low level contamination in a RhD negative sample resulting in 1 or 2 replicates showing a positive signal;
- a low fetal fraction or low cell free DNA concentration in a RhD positive sample.

For samples tested direct from plasma and showing an inconclusive call, it is recommended to re-test direct from plasma and/or from extracted cell free DNA to reach a conclusive call.

Calling RhD Exon 5 Variants

The RhD exon 5 assay is designed to discriminate between the wild type sequence and the PSI variant, which is predominantly found in populations of African origin. It is possible that the RhD exon 5 assay is capable of discriminating other rarer variants of the exon 5 sequence, although this has not been internally validated.

Due to the lower efficiency of PCR amplification when testing direct from plasma, the RhD exon 5 assay should be used to discriminate RhD exon 5 variants only when using extracted cell free DNA as input material. In the presence of a RhD exon 5 variant which can be discriminated by the RhD exon 5 assay, the qPCR data should show 0/6 positive replicates for the RhD exon 5 assay and 3/5 or more positive replicates for the RhD exon 7-10 assay. It is the user's decision how to handle outcomes which suggest the presence of a RhD exon 5 variant.

Internal Control: CCR5

CCR5 is a housekeeping gene commonly used as an internal quality control target in qPCR assays. We have included 1 replicate for CCR5 in the Cell3™ Direct: Rhesus D Fetal Blood Group Genotyping kit. As this is a general target, it will amplify both fetal and maternal DNA. Therefore, in most cases it will produce a lower Ct value than the RhD specific targets. The intended use of this target is to determine the overall quantity of cell free DNA present in the sample and to ensure that the qPCR reaction is working direct from plasma. Samples showing a Ct value higher than 42 for CCR5 should be handled with caution, as



this could mean that the qPCR reaction has performed poorly or that there is not enough cell free DNA concentration within the sample. In these cases, cell free DNA extraction from 1 ml of plasma is advised. The Cell3™ Direct: Rhesus D Fetal Blood Group Genotyping kit can then be used on extracted cell free DNA following the cell free DNA protocol to determine the fetal RhD status.

Artefactual and Unspecific Amplification Curves

Due to the diversity in molecular make up of plasma samples, artefactual or unspecific amplification curves may occasionally be observed. These curves can be easily identified in the amplification plot graph, as they tend to start considerably earlier than genuine amplification curves and proceed in a non-logarithmic and more linear fashion. This type of unspecific amplification usually occurs in female/negative samples. Setting the baseline start and end cycles manually as indicated previously should remove most artefactual amplification curves. However, in the event that one or more of these curves is still present, make a note of the well in which it occurs and the target, and delete the resulting Ct value from the analysis.

Practical Notes - Excel Analysis Worksheet

The Cell3 Direct - Fetal RhD analysis template excel file is a semi-automated worksheet intended to make data analysis and interpretation easier and faster for the user. The cells where analysed data appears contain formulas and algorithms which apply to set cell positions where the raw data is copied over by the user from the exported results.csv (or .xls) file. Therefore, said data should be inputted only in the appropriate cells as explained in this guide. It is important that cell content is moved by copying, pasting and deleting cells. If cells are cut and pasted or moved by dragging the cells in different positions in the worksheet, this will alter the cell position to which the formulas and algorithms apply to, resulting in incorrect calculations. It is advised that a new copy of the Cell3 Direct - Fetal RhD analysis template excel file be saved prior to starting data analysis, so that the template file remains unaltered and can be used to repeat the analysis when needed.

