DEVYSER
RESULTS FOR LIFE

Devyser RHD

Art. No.: 8-A406

For Research Use Only

Handbook

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1. INTRODUCTION

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1.1 Intended use

The Devyser RHD kit enables detection of fetal RHD DNA from maternal plasma.

The Devyser RHD kit is for research use only, not for use in diagnostic procedures. The test kit is not intended for blood grouping.

1.2 Background

The discovery that a detectable fraction of cell-free DNA in the plasma of pregnant women is of fetal origin[1] has enabled several important applications in prenatal diagnostics. Among the applications it has been demonstrated that fetal *RHD* genes could be detected in RhD-negative women[2].

Immunization against the RhD antigen is the most common cause of hemolytic anemia in the fetus and the newborn. The introduction of postnatal anti-D prophylaxis has significantly reduced RhD immunization events[3,4,5]. At present, routine antenatal anti-D prophylaxis is offered to RhD-negative women in several countries, although approximately 40% of these women carry a RhD-negative fetus and are thus not at risk of immunization. Since anti-D immunoglobulin is derived from pooled human plasma, an effort should be made to avoid unnecessarily exposing the mother and the fetus to infectious agents[6].

1.3 Assay principle and design

Devyser RHD is based on real-time PCR enabling detection of PCR-amplified DNA fragment (amplicon) accumulation by means of an increase in fluorescence. Data collected in the exponential phase of the reaction provides quantitative information on the starting quantity of the amplified target. Fluorescent reporters used in this real-time product design are dye molecules attached to probes that hybridize with amplicons during thermocycling. The change in fluorescence over the course of the reaction is measured by a real-time PCR instrument, combining thermal cycling with fluorescence detection. By plotting fluorescence against a cycle number, an amplification plot is generated that represents the accumulation of the amplicons over the duration of the PCR reaction.

As post-amplification procedures are not required, analysis can be completed rapidly, allowing a high sample throughput. In addition, there is less risk of amplicon carry-over and contamination as reaction tubes remain closed after PCR.

For detection of fetal DNA, the Devyser RHD assay is designed for PCR amplification in *RHD* exon 4. The single exon amplicon design, without competing parallel PCR reactions, enables detection of cell-free fetal *RHD* DNA from maternal plasma with high sensitivity and robustness[7].

The single exon strategy has demonstrated similar or even better performance than multiple exon designs with respect to false negative and false positive results[8]. In addition, the single exon amplification approach enables high throughput testing and data analysis.

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The inclusion of *GAPDH* as an endogenous control enables estimation of the total DNA amount in a sample by comparing the obtained cycle threshold (Ct) value for GAPDH in the sample to the cycle threshold value for GAPDH obtained in the positive control with a known DNA concentration.

Inclusion of the passive reference dye ROX allows normalization of the fluorescent reporter signals between different samples within one run.

The fluorescent reporter dyes used in the design are FAM for RHD and VIC for GAPDH.

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2. MATERIALS AND EQUIPMENT

2.1 Kit configuration

The Devyser RHD kit is available in a 78-test format that is sufficient for the simultaneous analysis of up to 25 samples tested in triplicate and the kit-internal controls, two replicates of the RHD Pos and one replicate of the NTC. The kit contains two tubes each of RHD Mix and RHD Enzyme. Activation of a single RHD Mix tube provides activated RHD Mix sufficient for 12 samples tested in triplicates, two replicates of RHD Pos and one replicate of NTC in each run.

Table 1. Devyser RHD kit configuration (8-A406)

Component	Art. No.	Number/kit	Cap color	Storage condition
RHD Mix	4-A317	2	White	Below −18°C
RHD Enzyme	4-A318	2	Orange	Below −18°C
RHD Pos*	4-A290	1	Yellow	Below −18°C
NTC**	4-A292	1	Blue	Below −18°C

*genomic RHD-positive control DNA at a concentration of approximately 1 ng/µL
**non-template control (no DNA present)

2.2 Equipment and reagents required but not provided

2.2.1 General

- Micropipettes with aerosol barrier tips or dispenser with filter displacement tips dedicated for pre-PCR
- Disposable powder-free protective gloves
- Optical reaction tubes/plates and caps/sealers
- Vortex
- Table-top centrifuge for plates/strips

2.2.2 DNA extraction

- DNA extraction reagents according to manufacturer’s instructions for use
- QIAsymphony DSP Virus/Pathogen Midi Kit (Cat.No./ID: 937055) with the QIAsymphony SP instrument to extract circulating cell-free DNA from maternal plasma (Qiagen)

2.2.3 PCR reaction set-up

- Manually according to this handbook
- Consumables according to manufacturer's instruction for use for automated procedure using the QIAsymphony AS instrument (Qiagen)

2.2.4 Amplification and detection

- Real-time PCR consumables according to manufacturer's instructions for use
- Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher) standard 96-well format (7500 Fast system is not supported)
- QuantStudio 6/7 Flex Real-Time PCR System (Thermo Fisher) standard 96-well format (96-well Fast and 384-well formats are not supported)
- Spectral calibration of the Real-Time PCR Systems should be performed for the fluorescent dyes FAM, VIC and ROX according to the manufacturer's instruction for use

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2.2.5 Real-time instrument software

- According to the manufacturer's instruction for use regarding 7500 system SDS software v2.3 and QuantStudio Real-Time PCR Software v1.3

2.2.6 Downloads

Supplementary information and files can be downloaded from https://devyser.com/resources/ifu and certificates/ using the download code printed on the kit label. See Table 2 for details.

Table 2. Downloads

Download file name	Description
BRC Devyser RHD LOT yyMnnn	Batch Release Certificate with Lot-specific criteria. Access the BRC using the product Lot number
Devyser RHD RUO 7-A145-EN	Handbook
Devyser RHD template 7500 Devyser RHD template QS6 Devyser RHD template QS7	PCR run template for Applied Biosystems 7500, QuantStudio 6 and 7 systems

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3. STORAGE REQUIREMENTS

- Store the Devyser RHD kit or individual components below −18°C (−28°C to −18°C)
- Do not use components beyond the kit lot expiration date
- If handled and stored properly, kit components will be stable until the expiration date of the kit
- Frozen kit components should be thawed in a refrigerator or at room temperature before use
- Remaining activated RHD Mix can be stored for up to 14 days at below −18°C with a single freeze-thaw cycle. The test procedure included the removal of half the volume from one activated RHD mix tube, i.e. sufficient volume to test 6 samples in triplicate including controls
- Avoid repeated freeze-thaw cycles
- Do not aliquot activated RHD Mix for storage

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4. WARNINGS AND PRECAUTIONS

- Use of this product should be limited to personnel trained in PCR techniques
- The procedure should be performed according to this handbook
- Deviations from the handbook will compromise kit performance
- Wear powder-free disposable gloves, laboratory coat and eye protection when handling samples and kit reagents
- Do not pool reagents with different kit lot numbers, as this will compromise the kit performance
- Do not use damaged reagent vials
- Frozen components should be completely thawed in a refrigerator or at room temperature before use
- Use, storage and disposal of kit components and samples should be in accordance with the procedures defined by national bio-hazard safety guidelines and in accordance with country, federal, state and local regulations
- Use sterile disposable aerosol barrier pipette tips to avoid microbial contamination of reagents
- Supplies and equipment should be dedicated to each activity
- The positive control (RHD Pos) provided in the kit has been tested negative for the presence of Human Immunodeficiency Virus (HIV) 1 and 2, Hepatitis C Virus (HCV) and Hepatitis B Virus (HBV)
- All instrumentation and further equipment should be maintained and calibrated regularly to verify performance

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5. PROCEDURAL LIMITATIONS

- Sequence variants within the target regions of Devyser RHD could affect primer and/or probe binding resulting in failure to detect the presence of fetal *RHD* DNA
- The Devyser RHD kit does not allow discrimination between the *RHD* gene and the *RHD* pseudogene (RHDΨ)
- The Devyser RHD kit should only be used in accordance with the intended use and handling described in this handbook
- The Devyser RHD kit is intended for research use only with cell-free DNA extracted from maternal plasma using the methods stated in section 2.2.2. Performance with other sample materials and DNA extraction methods have not been tested
- The Devyser RHD kit has been tested for use with the real-time PCR systems listed in section 2.2.4. Performance with other real-time PCR systems has not been tested
- Inconclusive results can be obtained in the rare event of the presence of unknown and/or not yet reported *RHD* alleles
- Devyser RHD is not recommended for individuals with a history of solid organ or hematopoietic stem cell transplantation

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6. SAMPLE REQUIREMENTS

DNA concentration, integrity and purity are important parameters for successful testing using PCR technology. DNA should be free from contaminating proteins, salts and other PCR inhibitors, e.g. residual ethanol from DNA extraction procedures. Low quality DNA may result in amplification failure and/or increased background signals.

6.1 Sample material

The Devyser RHD kit can be used for sensitive detection of cell-free fetal DNA extracted from maternal plasma. The primary sample is whole blood from which plasma is separated according to standard procedures. DNA extraction from plasma is performed according to section 6.2.

6.2 Sample handling

- Blood samples should not be collected before pregnancy week 10
- Blood samples can be stored at room temperature for up to 6 days
- Tubes containing K2-EDTA should be used for blood sampling
- Plasma should be separated within 6 days after blood sampling
- Plasma samples can be stored at −28°C to −18°C for up to 2 weeks or at −80°C for up to 18 months before DNA extraction

6.3 DNA extraction from plasma

- Results are reproducibly obtained from cell-free DNA extracted from maternal plasma using QIAsymphony DSP Virus/Pathogen Midi Kit (Cat.No./ID: 937055) and the QIAsymphony SP instrument
- Follow the manufacturer's instruction for use starting with 1 000 µL maternal plasma. Elute in 85 µL

6.4 Internal system control

We recommend performing regular internal system controls of all equipment and software used to perform the procedure according to this handbook. Samples with pre-characterized *RHD* status (in-house or externally sourced) are suitable as system controls.

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7. INSTRUCTIONS FOR USE

7.1 Run sizes

Devyser RHD contains reagents for 78 (2×39) PCR reactions. Recommended run sizes are 39 or 78 PCR reactions.

7.2 Sample replicates

Each sample (cfDNA) should be tested in triplicate PCR reactions.

7.3 Number of samples

Run size 39 PCR reactions: a total of 12 samples in triplicates and three controls.
Run size 78 PCR reactions: a total of 25 samples in triplicates and three controls.

For usability and appropriate use of kit reagents, the recommended number of samples to be tested in each run is 24, which corresponds to the maximum number of samples that can be extracted in one run on the QIAsymphony SP instrument.

7.4 Controls

One replicate of NTC and two replicates of RHD Pos must be included in each run to allow data analysis.

7.5 DNA extraction

Follow the manufacturer's protocol and recommendations for DNA extraction, (see section 6.3).

NOTE	To reduce the risk of contamination, it is recommended that different operators handle the reagent preparation and the DNA extraction if both are performed the same day.

7.6 Workflow

7.6.1 Reagent preparation

Required kit components: **RHD Mix (4-A317)** and **RHD Enzyme (4-A318)**.

Determine the number of **RHD Mix** and **RHD Enzyme** tubes required. Each tube is sufficient for 39 reactions.

A. Ensure that the **RHD Mix** is completely thawed before use, then briefly vortex
B. Centrifuge the **RHD Mix** and the **RHD Enzyme** briefly
C. Add 1320 µL (2 x 660 µL) **RHD Mix** to one tube of **RHD Enzyme** to obtain activated **RHD Mix**

NOTE	Mix thoroughly by manually pipetting up and down for a minimum of 10 times using a volume of at least 660 µL. Avoid creation of bubbles.

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D. Centrifuge briefly to collect the content
E. Dispense 30 µL of the activated **RHD mix** into separate tubes/plate wells by pipetting to the bottom of the tubes/wells
F. Remaining activated **RHD Mix** can be stored for up to 14 days at −28 °C to −18 °C with a single freeze-thaw cycle

7.6.2 Addition of samples and controls

Samples and controls should be added in a dedicated area separated from the reagent preparation and amplification areas.

A. Add 20 µL of either control or sample to separate, dedicated tubes/wells prepared in section 7.6.1
B. Seal the tubes/plate and centrifuge briefly to collect the contents

7.7 Amplification and detection

7.7.1 Performing real-time PCR

A. Program the thermal cycler according to section 7.7.2 - 7.7.5
B. Start the amplification (duration approximately 2 hours)
C. Following amplification, remove the plate containing the completed PCR amplification reaction from the thermal cycler and discard the plate without removing the seal. Do not bring amplified material into pre-amplification areas

7.7.2 Experiment conditions

Table 3: Experiment conditions

Setting	Applied Biosystems 7500 (SDS software v2.3)	QuantStudio 6/7 Flex (Software v1.3)
Instrument	Applied Biosystems 7500 (96 Wells)	QuantStudio 6/7 Flex
Block	Not applicable	96-Well (0.2mL)
Experiment	Quantitation_Standard Curve	Standard Curve
Reagents	TaqMan® Reagents	
Ramp speed	Standard	Standard

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7.7.3 Define and assign targets

Table 4: Define and assign targets

Setting	Applied Biosystems 7500 (SDS software v2.3)	QuantStudio 6/7 Flex (Software v1.3)
Define - Targets	Target 1: Target Name: RHD, Reporter: FAM Target 2: Target Name: GAPDH, Reporter: VIC	
Define - Passive Reference	Not applicable (to be adjusted in next menu point)	ROX
Assign - Targets	Select all wells to be run and choose both targets. Passive reference dye: ROX	Select all wells to be run and choose both targets.

Alternatively, download appropriate pre-set Devyser RHD run template(s) for the respective instrument type to be used: See Table 2 in section 2.2.6 for download of templates.

7.7.4 Run method

The run method consists of the thermal profile shown below in Figure 1.

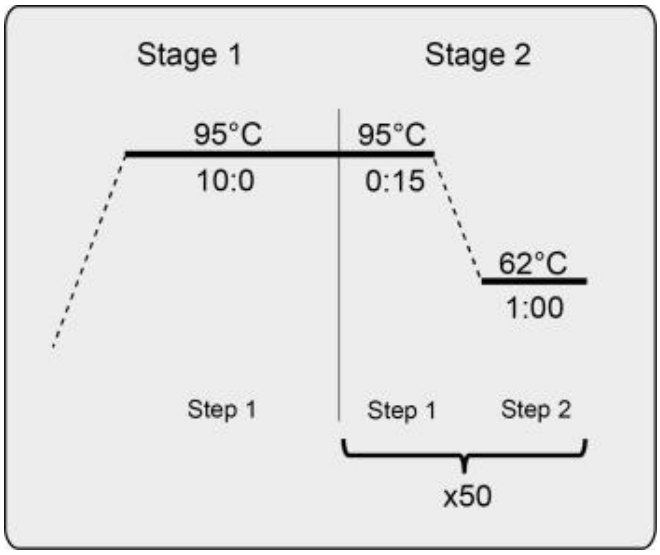

Figure 1. Thermal profile

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The following run method conditions need to be selected by the user:

A. Reaction volume: 50 µL
B. Data collection at stage 2, step 2
C. Number of Cycles: 50
D. Ramp speed (standard):
    - 7500 Real-Time PCR System / SDS v2.3: Heating/Holding at 50% and Cooling at 100%
    - QuantStudio 6/ -7 Flex Real-Time PCR System / v1.3: Heating/Holding at 0,8 ˚C/s and Cooling at 1,6 ˚C/s

7.7.5 Analysis

Under “Analysis Settings”, select the following:

A. Baseline Start Cycle: 3
B. Baseline End Cycle: 20

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8. REAL-TIME PCR NOMENCLATURE AND DEFINITIONS

The following general terms and definitions apply when analyzing real-time PCR data.

8.1 Baseline

The baseline refers to the signal level during the initial cycles of PCR in which there is little change in fluorescent signal. The low-level signal of the baseline can be equated to the background or the “noise” of the reaction.

8.2 Threshold

The threshold of the reaction is the level of signal that reflects a statistically significant increase over the calculated baseline signal. It is set to distinguish a relevant amplification signal from the background.

8.3 Threshold cycle (Ct)

The threshold cycle (Ct) value is the cycle number at which the fluorescence generated within a reaction crosses the threshold. A fluorescence signal crossing the threshold is defined as detected. Ct values are inversely proportional to the amount of target nucleic acid in the sample, i.e. the lower the Ct value the greater the amount of target nucleic acid in the sample.

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9. DATA ANALYSIS AND RESULTS INTERPRETATION

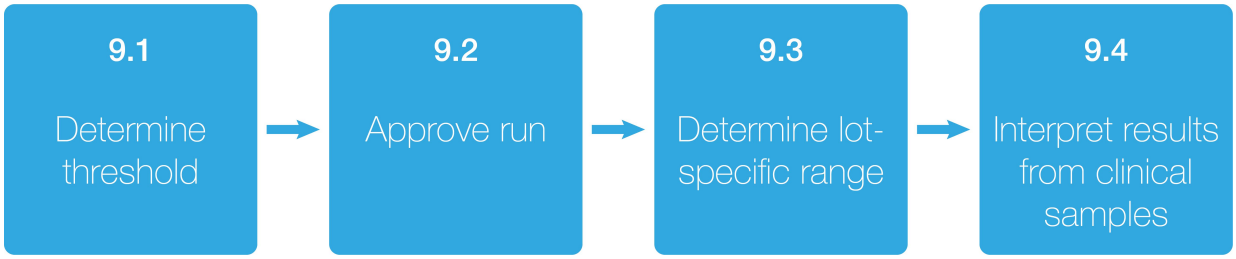

Figure 2. Data analysis and results interpretation overview

9.1 Determine threshold

- After run completion, adjust the threshold line manually to obtain a Ct^{RHD} of 28.5 (28.46 - 28.54) for the RHD Pos replicate with the higher cycle value (x-Axis), see Figure 3. The threshold should be identical in the FAM and VIC channel
- Export the data in Excel file format before proceeding to section 9.2

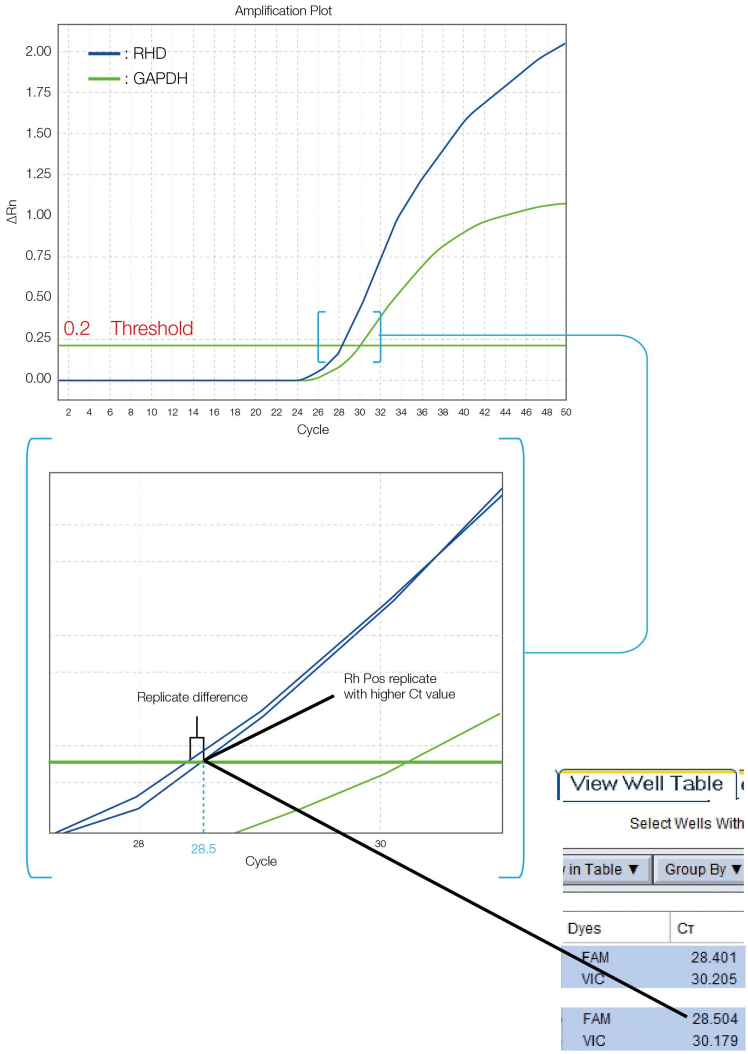

Figure 3. Determine threshold

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9.2 Approve run

NOTE	A signal is considered "detected" if it crosses the threshold and thus is assigned a Ct value. If a signal does not cross the threshold, Ct will be stated as "undetermined" in the exported Excel file.

1. Confirm that no RHD or GAPDH signals are detected in the NTC
2. Confirm that RHD and GAPDH signals are detected in both replicates of the RHD Pos
3. Confirm that the difference (ΔCt^{RHD}) between the Ct^{RHD} values for both RHD Pos replicates is less than or equal to 0.7
4. If one of the above-mentioned criteria is not met, consult section 10, Troubleshooting

9.3 Determine lot-specific range

For each run, calculate the average Ct^{GAPDH} ($\bar{x}$) of the two RHD Pos replicates which is required to calculate the lot-specific Ct^{GAPDH} range (Figure 4) valid for interpretation of samples:

1. Download the lot-specific BRC from https://devyser.com/resources/ifu and certificates/
2. Calculate the lot-specific Ct^{GAPDH} range as follows:

Lower range limit = $\bar{x}$ - ☐ (insert lower reference value from BRC)

Upper range limit = $\bar{x}$ + ☐ (insert upper reference value from BRC)

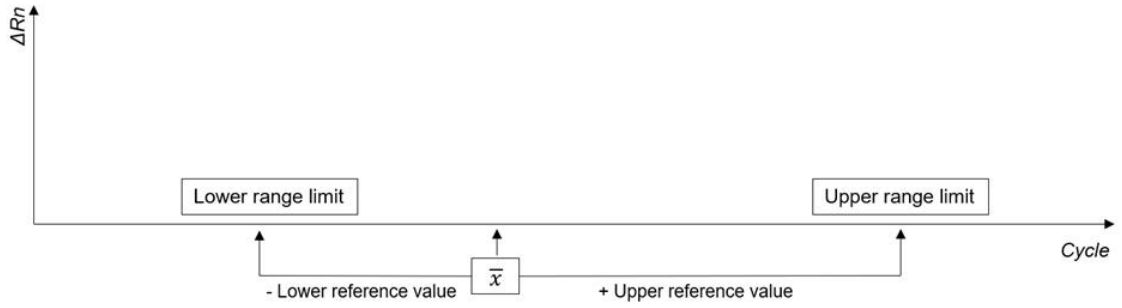

Figure 4. Determine lot-specific range

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9.4 Results interpretation

NOTE	A GAPDH signal must be detected in all replicates of a sample. If not, the sample must be re-tested. The lot-specific range for Ct^{GAPDH} applies only where indicated in Tables 5 and 6.

Table 5. Interpretation of typical results for samples

Detected replicates		Interpretation
RHD	**GAPDH**	
0 of 3	3 of 3 with Ct^{GAPDH} within lot-specific range	No fetal *RHD* DNA detected
2 of 3 with Ct^{RHD} later than Ct^{GAPDH}	3 of 3 (lot-specific range does not apply)	Fetal *RHD* DNA detected
3 of 3 with Ct^{RHD} later than Ct^{GAPDH}	3 of 3 (lot-specific range does not apply)	Fetal *RHD* DNA detected

Table 6. Results requiring further investigation

Detected replicates		Interpretation and recommendation
RHD	**GAPDH**	
0 of 3	1, 2 or 3 of 3 with Ct^{GAPDH} later than lot-specific range	Amount of fetal DNA too low. Re-testing recommended.†
0 of 3	1, 2 or 3 of 3 with Ct^{GAPDH} earlier than lot-specific range	Potential presence of maternal genomic DNA. Re-sampling recommended.
1 of 3 with Ct^{RHD} later than Ct^{GAPDH}	3 of 3 (lot-specific range does not apply)	Inconclusive. Re-testing recommended.†
1, 2 or 3 of 3 with Ct^{RHD} earlier than Ct^{GAPDH}	3 of 3 (lot-specific range does not apply)	Potential maternal *RHD* gene (pseudogene or other). Additional investigation recommended.

† If re-testing does not produce a conclusive result according to the criteria above, re-sampling at a later gestational week is recommended

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10. TROUBLESHOOTING

Possible results requiring troubleshooting are listed in Table 7:

Table 7. Troubleshooting

Observation	Possible cause	Interpretation and recommendation
RHD and/or GAPDH signal detected for NTC	Contamination during PCR set-up	The run should be assessed as failed and repeated
RHD and GAPDH signals not detected for both replicates of RHD Pos	Pipetting handling error	The run should be assessed as failed and repeated
The difference (ΔCt^{RHD}) between the Ct^{RHD} values for both RHD Pos replicates is larger than 0.7	Pipetting handling error and poor precision when adding the two replicates of RHD Pos	The run should be assessed as failed and repeated
RHD signals of sample(s) below the threshold	RHD signals below the threshold are probably unspecific	If the recommendations in this handbook are followed, a sample is interpreted as RHD negative despite weak RHD signals that are below the threshold
	If blood sampling is performed prior to pregnancy week 10, the amount of fetal cfDNA available may be below the detection limit of the assay and may therefore result in weak RHD signals that do not cross the threshold and should therefore not be evaluated nor interpreted	Re-sampling is recommended

Contact Devyser Technical support in the case of further questions, see section 13.

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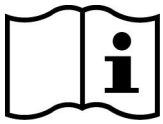
11. SYMBOLS USED ON LABELS

Lot or batch number

Expiry date

Number of tests

Store below temperature shown



Consult instructions for use

Catalogue number

Manufacturer

Research Use Only

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12. NOTICE TO PURCHASER

Results obtained using Devyser RHD should not be used for diagnostic purposes. Devyser AB will not accept responsibility for any decisions taken.

Purchase of this product does not provide a license to perform PCR under patents owned by any third party.

QuantStudio™, FAM™ and ROX™ are all trademarks of the Thermo Fischer Scientific Corporation.

QIAsymphony® is a registered trademark of Qiagen.

Applied Biosystems® and VIC® are registered trademarks of the Thermo Fischer Scientific Corporation.

Devyser® is a registered trademark of Devyser Diagnostics.

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13. CONTACT INFORMATION

13.1 Legal manufacturer

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Homepage: www.devyser.com

13.2 Technical support

E-mail: techsupport@devyser.com

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15. ABBREVIATIONS

Abbreviation	Explanation
BRC	batch release certificate
cfDNA	cell free DNA
Ct	cycle threshold
DNA	deoxy ribonucleic acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase gene
gDNA	genomic DNA
NTC	non-template control
RHD	rhesus D gene
RhD	rhesus antigen
PCR	polymerase chain reaction

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16. REVISION HISTORY

Version 2021-02-15
Editorial changes

2.1 Corrected number of total reactions per kit

6.2 Changed time interval for collection of blood samples

6.2 Added information regarding storage of blood samples

9.1 Included Ct range for adjustment of threshold and clarified that the threshold should be identical in the FAM and VIC channel

9.4 Clarified GAPDH column in rows 2 and 3 of Table 6

10 Clarified "weak" signals in Table 7

Version 2020-09-29
New

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