FetoGnost® Kit RHD

Kit Version 1.1

Instruction for use

CE

IVD **For *in vitro* diagnostic use**

REF **HUFG100** Σ **100 reactions (up to 30 plasma samples)**

REF **HUFG500** Σ **500 reactions (up to 150 plasma samples)**

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Explanation of Symbols

LOT	Batch code		Use-by date
REF	Catalogue number		Manufacturer
Σ	Contains sufficient for <n> tests		Temperature limit
CE	This product fulfills the requirements of the European Directive 98/79/EC on *in vitro* diagnostic medical devices	IVD	In vitro diagnostic *medical device*
	Consult instructions for use or consult electronic instructions for use	UDI	Unique device identifier
	Keep away from sunlight	QTY	Contents

1 Intended purpose

FetoGnost® Kit RHD is a non-automated IVD real-time PCR test for the qualitative detection of fetal *RHD* DNA from extracted human maternal plasma of non-immunized RhD negative pregnant women (non-invasive prenatal determination of fetal *RHD* status, NIPT-RHD). The test detects exons 5, 7 and 10 of the *RHD* gene.

This test is suitable for women of all ages from the 12th week of pregnancy (gestational age ≥ 11+0) with singleton or multiple pregnancies.
The test can be used both in a first pregnancy and in subsequent pregnancies.
It allows targeted anti-D prophylaxis in RhD-negative pregnant women without anti-D alloimmunization.

Contraindications:

- Pregnant women with anti-D alloimmunization. Thus, the immunization status of the pregnant woman should be known before starting the test.
- The test is not suitable for samples taken before gestational age 11+0. The limited performance of the test prior to the 12th week of pregnancy must be indicated on the report.
- The test is not intended for Rhesus D determination of transfusion recipients and blood donors.

The test is intended for professional use and is limited to qualified personnel instructed in the procedures of real-time PCR and *in vitro* diagnostic procedures.

2 Product description

FetoGnost® Kit RHD is a real-time PCR test und allows a rapid, sensitive, and non-invasive detection of the fetal rhesus factor D (*RHD*) gene in samples purified from maternal plasma of RhD-negative pregnant women. Probe-specific amplification-curves in VIC, FAM and NED channels indicate the amplification of exons 5, 7 and 10 of the *RHD* gene of RhD-positive fetuses, respectively. The internal DNA positive control (IPC) is detected in the fluorescence channel Cy5 and serves as a control for DNA extraction and possible real-time PCR inhibition. The target for the DNA IPC is an artificial short DNA fragment and is added during sample extraction.

The kit content of 100 reactions allows the analysis of 30 plasma samples including controls in triplicates on a fully loaded 96-well reaction plate.

The sensitive and robust multiplex test format of FetoGnost® Kit RHD for the detection of three exons of the *RHD* gene in triplicates minimizes false-negative results.

FetoGnost® Kit RHD has been validated with the ABI® 7500 Fast instrument and QuantStudio™ 7 Pro (Thermo Fisher Scientific) - fast cycle parameters are not supported by this test. It is, however, also compatible with other real-time PCR instruments which detect and differentiate fluorescence in FAM, VIC, NED, ROX and Cy5 channels.
When using PCR-platforms not tested by ingenetix, an evaluation of the multiplex-PCR shall be done. Please, keep in mind that some PCR-platforms first must be calibrated with the corresponding dye before performing multiplex-PCR.

Fragmented DNA (< 150 bp, Qiao et al, 2019) of fetal origin (cffDNA, cell-free fetal DNA) represents only a small fraction of the circulating cell-free DNA (ccfDNA) present in maternal plasma and increases during pregnancy. In plasma samples from the second trimester of pregnant women, the concentration of cffDNA can reach 2-151 genome equivalents of cffDNA/ml of blood (Birch et al., 2005).

Please also follow the official and country-specific recommendations or guidelines (e.g., recommendation by DGTI, Deutsche Gesellschaft für Transfusionsmedizin und Immunhämatologie) when implementing FetoGnost® Kit RHD in your lab.

3 Background

Rhesus factor D (RhD) or RhD antigen is the most common of the five main Rhesus antigens (C, c, D, E, and e) out of a variety of antigens on the surface of red blood cells. The dominantly inherited *RHD* gene determines whether a person is RhD-positive or -negative. Most of the population is RhD-positive. In a RhD-negative status, a complete deletion of the *RHD* gene is usually present. Less commonly, minor genetic alterations (e.g., point mutations, insertions, deletions, and gene rearrangements) result in non-functional or weak RhD antigen.

Prediction of the fetal *RHD* status is significant for the prevention of fetal haemolytic disease, where a RhD-negative mother becomes sensitized to an RhD-positive fetus causing a maternal immune response to produce IgG anti-D antibodies, which damage the fetus in a subsequent pregnancy.

4 Principle of real-time PCR

The test is based on multiplex real-time PCR by 5´-nuclease-assay technology. Specific DNA sequences are amplified, and the generated PCR-products are detected by oligonucleotide-probes labelled with fluorescent dyes. This allows a sequence-specific detection of PCR amplificates.

During PCR, primers are extended by *Taq* polymerase and probes hybridized to the target are cleaved by the 5'-exonuclease activity of *Taq* polymerase. According to the accumulation of PCR product, the fluorescence of the probe increases with each PCR cycle. The change in fluorescence of the different dyes is recorded cycle by cycle in the real-time PCR instrument in the closed reaction tube at different fluorescence wavelengths.
The Cq value (Cq = Quantification cycle, Ct = Cycle threshold, Cp = Crossing point) describes the cycle at which the fluorescence first rises significantly above the background fluorescence.

5 Contents of the kit, stability, and storage

Table 1

Labelling	Content	HUFG100	HUFG500	Storage
FetoGnost® Kit RHD Assay Mix (green cap)	Primer and probes for detection of RHD exon 5 (VIC), 7 (FAM), 10 (NED) and IPC (Cy5)	1 x 500 µl	5 x 500 µl	-25 to -15 °C
FetoGnost® Kit IPC Target (orange cap)	Target for IPC (internal DNA Positive Control, 100,000 target copies/µl)	1 x 300 µl	5 x 300 µl	-25 to -15 °C
FetoGnost® Kit RHD Positive Control (red cap)	DNA Positive Control (mixture of three DNA target sequences for exon 5, 7, and 10, approx. 1,000 target copies/µl each)	1 x 300 µl	1 x 1000 µl	-25 to -15 °C
FetoGnost® Kit Reaction Mix (white cap)	PCR reaction mix for DNA amplification	2 x 750 µl	5 x 1500 µl	**-25 to -15 °C, after first opening 2 to 8 °C**

FetoGnost® Kit Reaction Mix

The included DNA amplification mix is designed for reliable, highly sensitive real-time PCR. The Master Mix contains a highly purified Taq Polymerase for rapid hot-start PCR, dNTPs with dUTP and Uracil-N glycosylase (UNG) to eliminate amplicon carryover, ROX™ dye (passive reference) and buffer components – additives optimized to handle RT-PCR inhibitors.

Delivery and Storage

The kit is delivered with coolpacks. When stored properly, the kit components are stable until the specified expiration date. This also applies after opening. Store kit protected from light.
Components stored at -25 to -15 °C can be frozen and thawed up to 9 times without loss of quality.

Quality Control Release Testing

In accordance with the ISO 13485 certified Quality Management System of ingenetix, each lot is tested against predetermined specifications to ensure consistent product quality.
Quality control is performed with plasmids containing parts of the human *RHD* gene.

6 Additionally required materials and devices

- Reagents and laboratory equipment for DNA extraction suitable for the extraction of cfDNA from plasma (see 9. Preparation of the samples)
- Nuclease-free water
- Powder-free disposable gloves
- Pipettes (adjustable)
- Filter pipette tips
- Real-time PCR instrument detecting and differentiating fluorescence in FAM, VIC, NED, ROX and Cy5 channel (see 2. Product description)
- Appropriate 96 well optical reaction plates or reaction tubes with corresponding (optical) closing material
- Optional: PCR workstation, face mask

7 Precautions and safety information

7.1 General information

- *in vitro* diagnostic medical device: this product may only be used by qualified personnel instructed in real-time PCR and *in vitro* diagnostic procedures.
- Transportation of clinical specimens must comply with local regulations for the transport of biological materials.
- Specimens should be handled as if infectious in accordance with safe laboratory procedures. Wear protective powder-free disposable gloves when handling kit reagents and specimens.
- Discard specimens, reagents, and waste according to your local safety regulations.
- Improper collection, transport or storage of specimens may hinder the ability of the assay to detect the target sequences.
- Quality of DNA has a profound impact on the test performance. Ensure that the used DNA extraction system is compatible with real-time PCR technology.
- The real-time PCR instrument should be calibrated, serviced, and cleaned regularly.
- Protect kit components from light.
- Avoid mixing reagents of different kits and lots and check expiry date of the kit.

7.2 Specific information

A workflow must be followed to avoid false positive results due to detection of contaminating DNA.

Recommended measures to avoid DNA contamination

- Use separated workspaces for specimen preparation, set-up of real-time PCR and amplification. Supplies and equipment must be dedicated to each of these separate workspaces to ensure workflow in the laboratory from pre- to post-PCR.
- Laboratory benches and devices must be cleaned regularly.
- Preparation of real-time PCR should be done in a PCR workstation.
- If possible, leave consumables and pipettes in the PCR workstation.
- The use of sterile aerosol-resistant pipette tips is required.
- Use only DNA-free consumables.
- Wear lab coat.
- Work only with powder-free disposable gloves, do not touch the outer surface of the palm and fingers of the gloves when putting them on. Change gloves frequently. To avoid skin contact, wear gloves over the sleeves of the lab coat. Use disposable sleeve protectors if possible.
- Do not touch the rim or threads of open vials.
- Caution is advised when handling specimens and positive control to avoid cross-contamination.
- Store positive and potentially positive material separately from all other reagents.
- For a valid interpretation of results, a negative control shall be included during DNA-extraction (e.g., extraction of water instead of sample material), to exclude false-positive results due to contamination with human DNA during extraction.

8 Limits of detection method

- Reliable results with this test can only be guaranteed when appropriate methods for blood collection, transport, storage (depending on the collection tubes) and processing of samples as well as an appropriate DNA extraction procedure are used.
- Cell-free fetal DNA is present in very low concentration in maternal plasma; therefore, extraction is a critical step in the analysis. It must be ensured by the user that fetal DNA can be obtained with the selected extraction method.
- The test is not suitable for samples taken before gestational age 11+0.
- **Up to now**, the test has only been validated with human plasma samples collected with EDTA tubes (with or without separating gel).
- The residual risk remains that negative test results do not exclude an *RHD*-positive fetus. Faulty sample collection, technical errors, mix-up of samples or fetal DNA below the limit of detection in maternal plasma can all compromise the test results. PCR inhibitors can also generate an invalid test result.
- In the case that no prophylaxis is given to a RhD-negative mother of a RhD-positive child the RhD-positive foetus in the subsequent pregnancy suffers from various degrees of anaemia which can lead to further pathologies and can be lethal in rare cases.
- As the usage and transport of the product is partially outsourced to downstream parties, the probability of occurrence cannot be completely eliminated. However, the risk of occurrence is reduced by various control measures, both during pregnancy and during testing (e.g. use of internal extraction control) and also directy after birth (determination of the blood group of the newborn to possibly administer RHD prophylaxis to the mother post-partally).
- Rare clinical subtypes or weak D genotype could result in single exons not being detected, but this does not affect the overall analysis based on multiplex detection of three exons.
- Please note that in rare cases an RhD-negative phenotype may be associated with *RHD* gene inactivation due to genetic variations. This can lead to the detection of single exons of the *RHD* gene of a serologically RhD-negative mother or fetus. This also applies to a weak D genotype of the mother or fetus.
- The fetal genotype may also correspond to a silent *RHD* variant.
- Always interpret results in context with other laboratory test results and clinical parameters.

9 Preparation of samples

FetoGnost® Kit RHD has been validated for the detection of cell-free fetal DNA obtained from maternal plasma. The primary clinical specimen is whole blood from which plasma is separated according to standard procedures (see below).

9.1 Sampling and transport

The temperature and duration of sample storage should be documented. The sample must be sent to the testing laboratory in a timely manner. Transport and store samples at approximately 10-25 °C, do not freeze the sample prior to plasma collection to prevent cell lysis.

Blood samples of gestational age ≥ 11+0 can be collected with different collection systems:

- EDTA blood collection tubes (recommended) with or without separating gel.
- Samples in EDTA blood collection tubes with separating gel must not be refrigerated, as this will cause the separating gel to become brittle.
- If special blood collection tubes for stabilization of cell-free DNA are used, this should be validated accordingly.

9.2 Plasma collection

Plasma must be separated within 6 days after sample collection (Clausen et al., 2013; Legler 2020; Müller et al., 2011).

<u>Conventional EDTA blood collection tubes</u>
Centrifuge the samples using the protocol established in your laboratory for plasma collection.

9.3 DNA extraction from plasma

Store plasma samples at approximately 10-25 °C. Plasma samples that are not extracted on the same day should be stored frozen (Londero et al., 2019; clinical trial validation data). After thawing, plasma must be extracted immediately.

DNA extraction can be performed manually or with automated extraction procedures suitable for the isolation of short DNA fragments (Legler et al., 2007, Yang et al., 2019). Please note that an **extraction negative control** (e.g., extraction of *RHD*-negative tested plasma or water instead of sample material) must be included per extraction batch to exclude false-positive results due to contamination with *RHD*-positive DNA during extraction.

Plasma from a haemolyzed blood sample must not be used for the analysis. Before nucleic acid extraction, a visual inspection for haemolysis must take place after centrifugation of the sample. In case of a haemolyzed sample, a new sample must be collected.

It is recommended to perform an evaluation of cffDNA extraction from maternal plasma with FetoGnost® Kit Control (HUFG050) to ensure that cffDNA can be obtained using the chosen extraction method.

9.3.1 Recommended extraction methods

Extract 1-2 ml of plasma per sample according to the manufacturer's instructions and elute DNA in up to 100 µl. For samples collected before the 16th week of pregnancy, extraction of at least 1 ml of plasma is recommended. From ≥ 16 gestational age, a sample volume of 0.5 ml of plasma eluted in up to 100 µl is sufficient.

FetoGnost® Kit IPC Target

- The target is added during extraction (extraction control and control for potential PCR inhibition). The FetoGnost® Kit IPC Target must not be pipetted directly to the sample material but must be added to the lysis buffer or to the sample in lysis buffer.
- Add 1-5 µl FetoGnost® Kit IPC Target (orange cap) to the plasma sample after the lysis buffer has been added. It is also possible to add the IPC Target directly to the lysis buffer. The IPC target remains stable in the lysis buffer.

Note

The Cq values of the IPC depend on the extraction method. It must have been ensured by a previous verification of the extraction system that the IPC reaches a Cq value of approx. 28 - 36. If the IPC has smaller Cq values than 28, the amount of the target must be reduced accordingly.

Elution

The elution volume should be selected so that 10 µl of eluate corresponds to at least 100 µl of plasma.

- Per ml plasma: elution in ≤ 100 µl.

DNA samples can be refrigerated for one day or stored long-term at -15 to -25 °C until analysis.

For manual extraction recommended

- QIAamp® DSP Virus Kit (QIAGEN) for 500 µl plasma. Add 1 µl FetoGnost® Kit IPC Target (orange cap) to the lysis buffer. Elute in 60 µl.
- QIAamp Circulating Nucleic Acid Kit (QIAGEN) for 1-2 ml plasma. Add 1 µl FetoGnost® Kit IPC Target (orange cap) to the lysis buffer. Elute in up to 100 µl.

For automated extraction recommended

- QIAsymphony DSP Circulating DNA Kit (QIAGEN) with the QIAsymphony® SP instrument using a QIAGEN adapted protocol for 1-2 ml plasma and elution in 80-100 µl. Per sample add 1 µl of FetoGnost® Kit IPC Target (orange cap) to the required Proteinase K solution.
- QIAsymphony DSP Virus/Pathogen Midi Kit (QIAGEN) for 1 ml plasma with the QIAsymphony® SP instrument using a QIAGEN adapted protocol for elution in 80-100 µl. Add 1-3 µl of FetoGnost® Kit IPC Target (orange cap) to the Carrier RNA per sample. Use 0.5 µg Carrier RNA per sample.
- chemagic cfDNA 2k Kit H24 (Perkin Elmer®) for 1-2 ml plasma with the Chemagic™ 360 instrument (Perkin Elmer®). To 1- 2 ml plasma add first Proteinase K, then Poly(A) RNA and the Lysis Buffer 1 followed by 1 µl of FetoGnost® Kit IPC Target (orange cap). Elution in 80-100 µl.
- Mag-Bind® cfDNA Kit (Omega) for 1 ml plasma with the KingFisher instrument (Thermo Fisher Scientific) with 24-pin magnetic head using the protocol for cfDNA. Addition of 67 µl DSP, and 15 µl Proteinase K. After Proteinase K digestion, add 1 µl of FetoGnost® Kit IPC Target (orange cap) to each sample. Elution in 80-100 µl.
- Maxwell® RSC ccfDNA Plasma Kit (Promega) for 1 ml plasma with the Maxwell® CSC instrument (Promega) using the appropriate protocol. Add 1 µl FetoGnost® Kit IPC Target (orange cap) to the lysis buffer and then pipette plasma. Elution in 80-100 µl elution buffer.

The detection of fetal DNA can be evaluated with the FetoGnost® Kit Control (order no. HUFG050, 50 reactions for up to 30 plasma samples, RUO). Analysis of a sample with both kits can be performed in one PCR run.

10 Preparation of real-time PCR

10.1 Pipetting scheme

		Per sample
Preparation of Master Mix (mix well)	FetoGnost® Kit Reaction Mix	15.0 µl
	FetoGnost® Kit RHD Assay Mix	5.0 µl
	Total volume Master Mix	20.0 µl
Preparation of PCR reaction	Master Mix	20.0 µl
	Sample	10.0 µl
	Total volume	30.0 µl

- Make sure that one extraction negative control, one positive control (FetoGnost® Kit RHD Positive Control, red cap), and optionally one extraction positive control (e.g., *RHD* positive plasma pool) are included per PCR run.
- **Each sample and all controls shall be analysed <u>in triplicates</u> with 10 µl sample/well.**
- Thaw kit components completely at room temperature. When thawed, mix components carefully, centrifuge briefly with low speed.
- Mix the DNA Reaction Mix gently to ensure homogeneity of solution.
- Prepare the Master Mix according to the number of samples, calculating an additional volume of approx. 10% for pipetting loss.
- Pipette 20 µl of the prepared Master Mix per sample into the well of the optical reaction plate.
- Then add 10 µl of the extracted sample or controls. Pipette the positive control at last.
- Seal the plate properly with a suitable optical sealing material.
- Vortex the sealed plate for 1-2 seconds and briefly centrifuge the plate.

10.2 Programming of the temperature profile

Further information on programming the real-time PCR instrument can be found in the respective operator's manual. Please, keep in mind that some PCR-platforms first must be calibrated with the corresponding dye before performing multiplex-PCR.

Select detection channels:

RHD exon 5	VIC-NONE
RHD exon 7	FAM-NONE
RHD exon 10	NED-NONE
IPC	Cy5-NONE

Passive reference dye: ROX (e.g., for ABI® 7500, QuantStudio™ 5/6/7)

Reaction volume: 30 µl

Ramp speed: without "fast cycling" parameter for ABI® 7500 Instrument or QuantStudio™ 5/6/7 (Thermo Fisher Scientific)

Program 1 Cycles: 1 Analysis: None	**Program 2** Cycles: 1 Analysis: None	**Program 3** Cycles: 45 Analysis: Quantification Acquisition at 60°
50 °C 2 min	95 °C 5 min	95 °C 5 sec 60 °C 1 min

11 Interpretation of data

Reactions with positive Cq values are considered positive, those with negative or Cq ≥ 45 (cut-off) as negative.

Important: In addition to the Cq values, also check the amplification curves and adjust the threshold if necessary. Samples should be checked in both logarithmic and linear views and compared to the negative control.

Table 2 shows the criteria for valid positive and negative controls. Table 3 shows interpretation of data with clinical samples.

11.1 Controls

Table 2 Criteria for valid controls

Controls (in triplicates)	VIC channel Exon 5	FAM channel Exon 7	NED channel Exon 10	Cy5 channel IPC	Interpretation	Action
Positive Control	At least 6 out of 9 replicates positive (Cq < 31)			Negative*	Valid	-
Positive Control	Less than 6 out of 9 replicates positive			Negative	Invalid	See 12.1
NTC**	A maximum of 2 out of 9 replicates positive			28-36	Valid	-
NTC**	Negative	Negative	Negative	Negative	Invalid	See 12.1

*A background signal of Cy5 >Cq 38 is still valid

**NTC = negative control of the extraction

Assessment of clinical specimen test results should be performed after the positive and negative controls have been examined and determined to be valid. If results of the controls are not valid, the results of clinical samples cannot be interpreted.

11.2 Clinical samples

In the case of an *RHD* positive fetus, Cq values between 32-40 can be expected depending on the progress of the pregnancy.

Table 3 Interpretation of samples

Sample (triplicates)	VIC channel Exon 5	FAM channel Exon 7	NED channel Exon 10	Cy5 channel* IPC	Interpretation of fetal *RHD* genotype	
Sample	At least 4 out of 9 replicates positive (Cq > 31)**			Positive	Positive	I
Sample	3 out of 9 replicates positive			Positive	No interpretation possible, repeat analysis	II
Sample	A maximum of 2 out of 9 replicates positive			Positive	Negative	III
Sample	Negative	Negative	Negative	Negative	Invalid, see 12.4	V

*The Cq values of the IPC in the PCR run should show comparable results. IPC Cq values depend on the extraction method and should show Cq values between 28-36. A shift in Cq values towards later (higher) Cq values may indicate partial inhibition of the PCR reaction. If the IPC has Cq values < 28, the amount of the target must be reduced accordingly.

** Very low Cq values compared to other results (Cq < 30) maybe maternal *RHD* variant. (see Interpretation Details IV.)

Interpretation Details

- **I. *RHD*-positive fetus:** At least 4 out of the 9 replicates are positive. Cq values between 32-40 can be expected, depending on the gestational age. If only one or two exons are amplified, a fetal partial D *RHD* genotype could be suspected. Targeted anti-D prophylaxis must be given.
- **II. Interpretation of the fetal *RHD* status is not possible:** If 3 out of the 9 replicates are positive, the fetal *RHD* genotype cannot be determined, and the analysis should be repeated. Targeted anti-D prophylaxis must be given.
- **III. *RHD*-negative fetus:** exons 5, 7 and 10 are undetectable (at least 7 of the 9 replicates are negative).
- **IV. Interpretation of the fetal *RHD* status is not possible:** If Cq values are in the range of the positive controls (Cq < 30), maternal *RHD* DNA was most likely detected. In this case, the fetal *RHD* genotype cannot be determined. Targeted anti-D prophylaxis must be given.

  → In a RhD-negative status, there is usually a complete deletion of the *RHD* gene. Less frequently, minor genetic alterations (e.g., point mutations, insertions, deletions, and gene rearrangements) result in a non-functional or weak RhD antigen.

  This can lead to the PCR-detection of single exons of the *RHD* gene from serologically RhD-negative or serologically RhD-weak positive mothers or fetuses. Concerning the mother in this case, the test detects maternal DNA for individual exons, which show Cq values < 30.

  Example: Partial D *RHD* genotype.

  In the case of *RHD-CE-D* hybrid genes of the mother or child, some sections of *RHD* are replaced by *RHCE*.

  Example: *RHD* pseudogene (*RHDΨ*). *RHDΨ* carriers are serologically RhD-negative.

  - In the case of a *RHDΨ* mother, exons 7 and 10 are positive with the test (Cq < 30). Exon 5 shows a shift of about 3 Cq values towards higher Cq values compared to exons 7 and 10.

  - In the case of an *RHDΨ* fetus, exons 7 and 10 are positive with the test (Cq > 31). Exon 5 is either negative or shows a shift of approximately 3 Cq values towards higher Cq values compared to exons 7 and 10.
- **V. In case of invalid data:** See 12. Troubleshooting.

12 Troubleshooting

12.1 No signal in FAM, VIC, NED and Cy5 channels with controls and sample

- Incorrect programming of the temperature profile or incorrect assignment of detection channels on the real-time PCR instrument.
  → Compare the temperature profile and assignment of detection channels with the protocol.
- Incorrect configuration of the PCR reaction.
  → Check your pipetting steps with the scheme and repeat the PCR, if necessary.
- No positive control was added.
  → Check your pipetting steps and repeat the PCR.
- To control the DNA extraction and real-time PCR, the IPC Target must be added to the lysis buffer (not directly to the sample) during the extraction. If the IPC is negative:
  → Repeat the extraction.

12.2 *RHD*-specific signal with negative control of extraction

- A contamination occurred during extraction.
  → Repeat the extraction and PCR with new reagents.
  → Strictly pipette the positive controls at last.
  → Make sure that workspace and instruments are decontaminated at regular intervals.

12.3 Valid results with controls, no IPC specific signals with sample

- Incorrect assignment of detection channels in sample.
  → Please verify the correct assignment of detection channels.
- If FetoGnost® Kit IPC Target was added during extraction:
  - Inhibition of PCR may have occurred.
  - DNA extraction was unsuccessful.
  - The IPC target was not added to the lysis buffer of the sample.
  - The extracted DNA was not added to the PCR-reaction.

  → No statement is possible. Verify that an appropriate DNA extraction method was used and review the DNA extraction steps.

13 Specifications and performance evaluation

13.1 Testperformance on different real-time PCR instruments

FetoGnost® Kit RHD has been validated with an ABI® 7500 instrument (Thermo Fisher Scientific) and QuantStudio™ 7 Pro (Thermo Fisher Scientific).

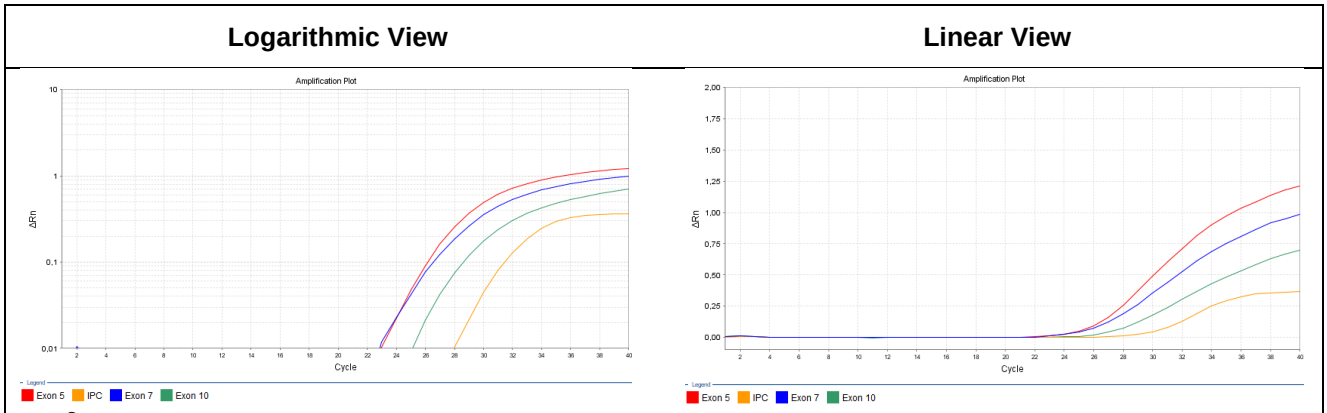

Figure 1 Performance of FetoGnost® Kit RHD

13.2 Analytical sensitivity – limit of detection

The limit of detection (LoD95% = smallest number of copies of target DNA which can be detected in 95% of cases) was determined by testing AmpFl STR® Control DNA 007 (stock concentration 100 pg/µl, Thermo Fisher Scientific). Twelve replicates at eight different concentrations around the detection limit were tested (50, 34, 28, 20, 14, 4, 2.8, 1.4 copies). Calculation was performed with a non-linear (logistic) curve fit using GraphPad Prism Software. The LoD95% for exon 5, 7, and 10 detection is 13, 9, and 7 target copies/reaction, respectively (Table 4).

Table 4

	Exon 5	Exon 7	Exon 10
50 *RHD* copies	12/12 positive	12/12 positive	12/12 positive
34 *RHD* copies	12/12 positive	12/12 positive	12/12 positive
28 *RHD* copies	12/12 positive	12/12 positive	12/12 positive
20 *RHD* copies	12/12 positive	12/12 positive	12/12 positive
14 *RHD* copies	11/12 positive	12/12 positive	11/12 positive
4 *RHD* copies	11/12 positive	8/12 positive	10/12 positive
2.8 *RHD* copies	10/12 positive	7/12 positive	7/12 positive
1.4 *RHD* copies	10/12 positive	5/12 positive	6/12 positive

13.3 Linearity and dynamic measuring range

Linearity was determined testing a 10-fold dilution series of plasmid DNA.
Exon 5: The test shows linearity between 100 - 1,000,000 target copies/reaction, with a slope of -3.786 ± 0.06449 and a coefficient of determination $R^2 > 0.98$ (Figure 2).
Exon 7: The test shows linearity between 100 - 1,000,000 target copies/reaction, with a slope of -3.827 ± 0.07239 and a coefficient of determination $R^2 > 0.98$ (Figure 2).
Exon 10: The test shows linearity between 100 - 1,000,000 target copies/reaction, with a slope of -3.820 ± 0.05859 and a coefficient of determination $R^2 > 0.98$ (Figure 2).

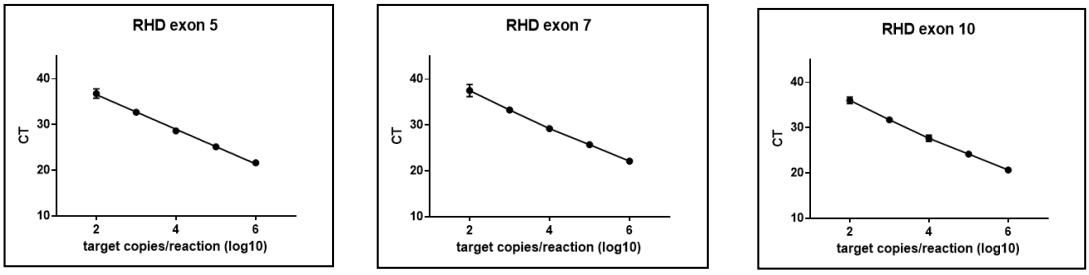

Figure 2

13.4 Analytical specificity and cross-reactivity

Analytical specificity is ensured by careful selection of primers and probes. *In silico* analyses in the NCBI database and on http://rhesusbase.info/I_RHD.htm validated the presence of allelic variants and the presence of single nucleotide polymorphisms (SNP) in the amplification targets, respectively. Few rare clinical subtypes may lead to non-detection of the corresponding exon, but this does not affect the overall analysis based on multiplex detection of three exons.

To investigate potential cross-reactivity, primers and probes were screened for potential homologies to currently published sequences. This database analysis (BLAST analysis) validated the specific detection of *RHD* exons 5, 7, and 10. There is no cross-reactivity to the closely related *RHCE* gene.

13.5 Inter-assay precision

Inter-assay precision is defined as the reproducibility of a sample's results between PCR runs on different days.
The inter-assay precision of FetoGnost® Kit RHD (exon 5, 7, 10) was determined from 10-fold plasmid DNA dilutions (E+6 to E+1 target copies/reaction) in three independent experiments performed on different days in triplicates (two) and quadruplicates (one). Arithmetic mean ($\bar{x}$), standard deviation (SD) and coefficient of variation (CV %) between the replicate runs were calculated.

For **exon 5**, inter-assay coefficients of variation ranged from 0.43% to 3.03%, with a mean overall inter-assay precision of 1.56%.
For **exon 7**, the inter-assay coefficients of variation were in the range of 0.73% to 2.88%, with a mean overall inter-assay precision of 1.58%.
For **exon 10**, the inter-assay coefficients of variation ranged from 0.61% to 3.72%, with a mean overall inter-assay precision of 1.89%.

Inter-assay precision was further analysed using 9 replicates from a plasma pool of pregnant women on 6 different days. Testing of the 9 replicates on 6 different days showed 100% positive Cq values for exon 5, 7, and 10.
Based on the common mean value 36.14 and a standard deviation of 1.16, a CV of 3.2% was obtained.

13.6 Intra-assay precision

Intra-assay precision is defined as the reproducibility of the results of a sample within a PCR run.
Intra-assay precision of FetoGnost® Kit RHD was determined from one of the replicate runs described in 13.3. Arithmetic mean ($\bar{x}$), standard deviation (SD) and coefficient of variation (CV %) of the replicates were calculated.

For **exon 5**, intra-assay coefficients of variation were in the range of 0.74% to 1.98%, with a mean overall intra-assay precision of 1.01%.
For **exon 7**, inter-assay coefficients of variation ranged from 0.33% to 5.03%, with a mean overall inter-assay precision of 1.89%.
For **exon 10**, intra-assay coefficients of variation ranged from 0.05% to 2.72%, with a mean overall inter-assay precision of 0.81%.

Intra-assay precision was further determined with a test run in 8 replicates with a plasma pool of pregnant women. For exon 5, 7 and 10 considered together, results gave a Cq mean of 36.05 and a standard deviation of 1.48, from which a coefficient of variation (CV) of 4.1% was determined.

13.7 Inter-lot precision

The inter-lot precision describes the conformity of performance between different manufactured kit lots. The inter-lot precision is presented as a percentage conformity between results.
Inter-Lot variability was determined from two different kit lots with 10-fold plasmid DNA dilutions (E+05 bis E+03 target copies/reaction). Arithmetic mean ($\bar{x}$), standard deviation (SD) and coefficient of variation (CV %) of the replicates were calculated.

For **exon 5**, inter-lot coefficients of variation were in the range of 2.01% to 0.15%, with a mean overall intra-assay precision of 0.80%.
For **exon 7**, inter-assay coefficients of variation ranged from 1.48% to 0.41%, with a mean overall inter-assay precision of 0.89%.
For **exon 10**, intra-assay coefficients of variation ranged from 0.88% to 0.55%, with a mean overall inter-assay precision of 0.73%.

13.8 Validation with plasma samples

Different volumes of plasma samples (400 µl, 267 µl, 200 µl) of two women in the 12th week of gestation were extracted and eluted in 100 µl elution buffer (60 µl eluate). Extraction was performed with the automated nucleic acid purification instrument EZ1 (Qiagen) with the EZ1 DSP Virus Kit. Different volumes of DNA extract (10 µl, 6.7 µl and 5 µl) were analysed in triplicates with QuantStudio™ 6 (Thermo Fisher Scientific). The sample was interpreted *RHD* positive in the presence of at least 4 of 9 positive replicates. The fetal *RHD* status of all samples except sample 5 were determined correctly (Table 5).

Table 5

Sample (number of positive replicates)		**Cq $\bar{x}$ (number of positive replicates)**			
		Exon 5	**Exon 7**	**Exon 10**	**IPC**
No. 1 (patient 1) (7/9)	400 µl plasma extracted 10 µl DNA extract in real-time PCR	35.78 (3/3)	36.89 (2/3)	36.60 (2/3)	28.56 (3/3)
No. 4 (patient 1) (7/9)	400 µl plasma extracted 6.7 µl DNA extract in real-time PCR	35.62 (3/3)	37.48 (3/3)	36.18 (1/3)	28.99 (3/3)
No. 5 (patient 1) **(3/9)**	400 µl plasma extracted 5 µl DNA extract in real-time PCR	36.49 (2/3)	38.01 (1/3)	Neg (3/3)	29.61 (3/3)
No. 2 (patient 1) (5/9)	267 µl plasma extracted 10 µl DNA extract in real-time PCR	36.28 (2/3)	37.59 (1/3)	35.88 (2/3)	28.49 (3/3)
No. 3 (patient 1) (5/9)	200 µl plasma extracted 10 µl DNA extract in real-time PCR	36.26 (1/3)	37.34 (2/3)	36.22 (2/3)	28.27 (3/3)
No. 6 (patient 2) (9/9)	400 µl plasma extracted 10 µl DNA extract in real-time PCR	35.72 (3/3)	37.05 (3/3)	37.34 (3/3)	28.61 (3/3)
No. 9 (patient 2) (5/9)	400 µl plasma extracted. 6.7 µl DNA extract in real-time PCR	35.14 (1/3)	38.57 (2/3)	35.81 (2/3)	29.06 (3/3)
No. 10 (patient 2) (5/9)	400 µl plasma extracted 5 µl DNA extract in real-time PCR	35.94 (2/3)	36.73 (1/3)	36.82 (2/3)	29.53 (3/3)
No. 7 (patient 2) (7/9)	267 µl plasma extracted 10 µl DNA extract in real-time PCR	36.79 (2/3)	38.16 (3/3)	36.94 (2/3)	28.15 (3/3)
No. 8 (patient 2) (5/9)	200 µl plasma extracted 10 µl DNA extract in real-time PCR	36.80 (2/3)	38.00 (2/3)	38.28 (1/3)	28.60 (3/3)

13.9 Testing of the WHO reference material

The WHO standard RhD/SRY Plasma DNA (code 07/222, National Institute for Biological Standards and Control (NIBSC), Potters Bar, Hertfordshire, UK) is used as an international reference for the detection of *RHD* DNA in plasma. According to the manufacturer, in a WHO study dilution series of undiluted, 1:2, 1:4, 1:8, and 1:16 were analyzed, to determine the highest possible dilution at which *RHD* is still detectable. Most laboratories participating in the study were able to detect *RHD* up to a 1:2 dilution in real-time PCR.
With FetoGnost® Kit RHD, the dilution level 1:2 was positive for *RHD* in all exons.

13.10 Clinical sensitivity

A previous retrospective study was performed with the precursor test FetoGnost RhD & Control assay (order no. FRT5RC, ingenetix, test did not include an Internal Positive Control and DNA reaction mix) at the University Hospital Göttingen, Department of Transfusion Medicine, Germany and the Department of Blood Group Serology and Transfusion Medicine, Medical University of Vienna, Austria during the years 2009 – 2020. Diagnostic accuracy was calculated from 2244 cases with the newborn's serological RhD status reported. A sensitivity of 99.93% (95% CI 99.61% - 99.99%) and a specificity of 99.61% (95% CI 98.86% - 99.87%) were achieved (Legler et al., 2021).

A prospective multicentre clinical trial for the FetoGnost® Kit RHD has been performed at the University Medical Center Göttingen, Department of Transfusion Medicine, Germany, during 26 April 2022 – 31 May 2023. The established gold standard was the serological status of the new-born. The purpose of the study was to evaluate the diagnostic accuracy for non-invasive prenatal determination of the fetal *RHD* status (NIPT-RHD).

During 04/28/2021- 05/31/2023, a total of 1521 noninvasive prenatal tests were performed to determine fetal RhD status. NIPT-RhD testing revealed that 61.1% of fetuses were RhD-positive and 36.9% were RhD-negative. In 0.5% of cases, the indication for NIPT-RhD was questionable because the mother tested serologically RhD-positive. In addition, 1.0% mothers and 0.4% fetuses had a variant RhD, so that a definite statement on the fetal RhD status was not possible. By May 31, 2023, 38.9% (n=462) of women contacted provided feedback on the RhD status of their newborn. 59.5% of the newborns had tested RhD-positive consistent with our analyzed RhD result, and 39.8% had tested RhD-negative consistent with our result. In 2 cases, the NIPT-RhD was false positive, and in one case, it was false negative. The cause of the false-negative result could be both a very low DNA concentration in the NIPT-RhD sample and sample mix-up during blood collection in pregnancy. Both cross-contamination during nucleic acid extraction and sample mix-up during blood collection during pregnancy are possible causes of the false-positive results. Accordingly, the sensitivity of NIPT-RhD was 99.64% and the specificity was 98.92% (Table 6).
Since recruitment in this study was not as extensive as planned (1200 study participants), data will be continually collected to determine the sensitivity and specificity of the NIPT-RhD based on serological blood grouping in new-borns previously tested at the University Medical Center Göttingen.

Table 6 Summary of clinical study, reporting period 26 April 2022 – 31 May 2023 of clinical study, including 462 samples with known fetal RhD status

	Value	95% confidence interval (CI)
Sensitivity	99.64%	98.00% to 99.99%
Specificity	98.92%	96.17% to 99.87%
NPV	99.46%	97.03% to 99.99%
PPV	99.28%	97.42% to 99.91%
Prevalence	59.74%	55.11% to 64.25%
Accuracy	99.35%	98.11% to 99.87%

13.11 Summary of Performance Data

A summary of the performance data can be found in Table 7.

Table 7 Performance data

Parameter	Performance data
LoD95%	**Results obtained with genomic DNA dilutions** *RHD* exon 5: 13 copies/reaction *RHD* exon 7: 9 copies/reaction *RHD* exon 10: 7 copies/reaction
Linearity and dynamic measuring range	R^2: 0.98. Slope: -3.8 The test shows linearity over the range of 100 to 1,000,000 target copies/reaction.
Cut-off Cq value	Cq ≥45
Inter-Lot-Precision	**Results obtained with dilutions of target plasmid DNA** exon 5: mean 0.80% exon 7: mean 0.89% exon 10: mean 0.73%
Intra-Assay-Precision	**Results obtained with dilutions of target plasmid DNA** exon 5: mean 1.01% exon 7: mean 1.89% exon 10: mean 0.81% **Results obtained with pooled plasma samples** Cq mean: 36.05 Standard deviation: 1.48 Coefficient of variation (CV): 4.1%
Inter-Assay-Precision	**Results obtained with dilutions of target plasmid DNA** exon 5: mean 1.56% exon 7: mean 1.58% exon 10: mean 1.89% **Results obtained with pooled plasma samples** Cq mean: 36.14 Standard deviation: 1.16 Coefficient of variation (CV): 3.2%
Cross-reactivity	No cross-reactivity with *RHCE* gene
Analytical specificity	100% specific A few clinical *RHD* subtypes are not detected due to SNPs in single exons. However, this does not affect the overall analysis as a total of three exons are detected.
Real-time PCR system comparison with • **ABI® 7500 instrument** • **QuantStudio™ 7 Pro**	100% comparable
Stability	FetoGnost® Kit RHD is stable for up to 19 months
Robustness	The test is robust to fluctuations in annealing temperatures and reagent concentrations.
Testing of WHO reference material (07/222)	100% positive for the dilution 1:2
Diagnostic sensitivity*	99.64% (95% CI 98.00% to 99.99%)
Diagnostic specificity*	98.92% (95% CI 96.17% to 99.87%)

*Reporting period 26 April 2022 – 31 May 2023 of clinical study, including 462 samples with known fetal RhD status

14 Literature

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15 Revision history

Revision	Date	Description
V2.0en	30 August 2023	**Change of the company address** The note to the buyer: Limited license has been moved to the end of the document. **1 Intended purpose** The term "intended use" has been replaced by the term "intended purpose". Inclusion of exons in the Intended purpose statement: “The test detects exons 5, 7 and 10 of the *RHD* gene.” Furthermore, the intended user was included: “The test is intended for professional use and is limited to qualified personnel instructed in the procedures of real-time PCR and *in vitro* diagnostic procedures.” **2 Product description** Inclusion of kit contents: The kit content of 100 reactions allows the analysis of 30 plasma samples including controls in triplicates on a fully loaded 96-well reaction plate. Inclusion of the Passive Reference Dye ROX among "compatible real-time PCR instruments”. **3 Background** Revised- ethnic groups with percentages removed. **5 Contents of the kit, stability, and storage** Preceding version “Contents of the FetoGnost® Kit RHD”.

		Changed filling volume of FetoGnost® Kit IPC Target and positive control. FetoGnost® Kit IPC Target is no longer diluted in DNA/RNA stabilizer. Therefore, there is no longer a hazard label. Furthermore, under this item, inclusion of the description for "Delivery and storage" and "Quality control release testing". **6 Additionally required materials and devices** Revised or updated. Inclusion of nuclease-free water and the passive reference dye ROX for real-time PCR instruments. The term cffDNA has been replaced with cfDNA, and the reference to extraction kits has been omitted. **7 Precautions and safety information** Header in previous version “General Precautions” Section updated, subdividing it into 7.1 General information and 7.2 Specific information. Inclusion of additional information on sample collection and storage, processing of samples after collection, storage conditions of samples, transport and storage conditions of purified DNA. Recommended measures to avoid DNA contamination inserted. **9 Preparation of samples** ISO 20186-3:2019 deleted. Item 9.3 DNA extraction from plasma revised. New item 9.3.1 Recommended extraction methods - Revision of extraction methods for manual extraction and automated extraction procedures. **10 Preparation of real-time PCR** Update under 10.1 Pipetting scheme. Replaced sample volume by reaction volume under item 10.2 Programming of the temperature profile. **11 Interpretation of data** In the heading "real-time PCR" removed. Split into sub-items 11.1 Controls - with new definition of positive control and removal of indication of Cq values and sub-item 11.2 Clinical specimens - removal of Interpretation IV. Partial *RHD D*. **13 Specifications and performance evaluation** New heading under 13.1 Test performance on various real-time PCR - previously just 13.1 Test performance. **13.8 Validation with plasma samples** Previous heading “Validation with different volumes of plasma samples and DNA extracts”. **13.10 Clinical sensitivity** New data set **13.11 Summary Performance Data** Introduction of a new subitem 13.11. Update in Table 7 of "Diagnostic Sensitivity" and "Diagnostic Specificity" due to new clinical data.

Note

Any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.

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