FetoGnost® Kit RHD

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
REF	Catalogue number		Manufacturer
Σ	Contains sufficient for <n> tests		Store at
CE	This product fulfills the requirements of the European Directive 98/79 EC for *in vitro* diagnostic medical devices	IVD	For *in vitro* diagnostic use
	Corrosion, GHS05		Exclamation mark, GHS07

1. Intended Use

FetoGnost® Kit RHD is a non-automatic *in vitro* diagnostic real-time PCR test for the qualitative detection of fetal RHD DNA from maternal plasma of non-immunized RhD negative women (non-invasive prenatal determination of fetal RHD status, NIPT-RHD).

This test is suitable for women of all ages with gestation 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. The immunization status of the pregnant woman should be known before starting the test.
- The test is not suitable for samples taken before gestation age 11+0. The limited performance of the test prior to gestation age ≥11+0 must be indicated on the report.
- The test is not intended for Rhesus D determination of transfusion recipients and blood donors.

2. Product description

FetoGnost® Kit RHD allows a rapid, sensitive and non-invasive detection of the fetal rhesusfactor D (RHD) gene in samples purified from maternal plasma of RhD-negative pregnant women. The test is based on real-time PCR technology. 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. In addition, an internal positive control (IPC) with detection in Cy5 channel monitors the integrity of kit reagents, serves as a control for DNA extraction and excludes false-negative interpretation of results due to inhibition of real-time PCR. The target for the IPC is added during DNA extraction of maternal plasma samples.
Using a 96-well reaction plate, the DNA of 30 plasma samples including controls can be analysed in triplicates (order no. HUFG100(.

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 Applied Biosystems® 7500 instrument and QuantStudio™ 7 Pro (Thermo Fisher Scientific) (fast cycle parameters are not supported), but is also compatible with other real-time PCR instruments which detect and differentiate fluorescence in FAM, VIC, NED and Cy5 channels. When using PCR-platforms not tested by ingenetix, an evaluation of the multiplex-PCR is recommended.

Before using FetoGnost® Kit RHD we highly recommend an evaluation of the extraction of cell-free fetal (cff) DNA from maternal plasma to ensure that the extraction method yields enough fetal DNA. The detection of fetal DNA can be evaluated with FetoGnost® Kit Control (order no. HUFG050, 50 rxns for up to 30 plasma samples).
Fragmented DNA (<300 bp) of fetal origin represents only a small fraction of the circulating cell-free DNA (ccfDNA) present in maternal plasma and increases during pregnancy. In maternal plasma samples from the second trimester of pregnant women, the concentration of cffDNA can reach 50-200 genome equivalents of cffDNA/ml of blood (Birch et al., 2005).

Please follow the official and country-specific recommendations or guidelines (e.g. recommendation by DGTI, Deutsche Gesellschaft für Transfusionsmedizin und Immunhämatologie) during evaluation of FetoGnost® Kit RHD.
The sampling for the determination of the fetal RHD status shall be after gestation age ≥11+0.

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 54 antigens on the surface of red blood cells. The dominant RHD gene determines whether a person is RhD-positive or -negative. Approximately 85 % of the European population is RhD-positive, around 95 % in sub-Saharan Africa and greater than 99.5 % in eastern Asia. The majority of RhD-negative Caucasians have a complete deletion of the RHD gene. In other populations such as Asians, Japanese and black Africans, the negative phenotype can also be associated with smaller genetic variations (e.g. point mutations, insertions, gene-rearrangements) resulting in non-functional RHD genes.
The prediction of the fetal RHD status is significant for the prevention of fetal hemolytic disease, where a RhD-negative mother becomes sensitized to an RHD-positive fetus causing a maternal immune response to produce IgG anti-D antibodies.

4. Principle of real-time PCR

FetoGnost® Kit RHD is based on multiplex real-time PCR technology by 5´-nuclease-assay technology. Specific DNA sequences in the RHD gene are amplified and the generated PCR-products are detected by oligonucleotide-probes labelled with fluorescent dyes (VIC, FAM und NED). 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 dyes is recorded cycle by cycle in the real-time PCR instrument in the closed reaction tube at different fluorescence wavelengths.
The Ct value (Cycle threshold, Ct = Quantification cycle, Cq = Crossing point, Cp) describes the cycle at which the fluorescence first rises significantly above the background fluorescence.

5. Contents of the FetoGnost® Kit RHD

Labelling	Content	HUFG100	HUFG500	Storage
FetoGnost® Kit RHD Assay Mix (green cap)	Primer and probes (labelled FAM, VIC, NED, Cy5) for detection of RHD exon 5, 7, 10 and IPC	1 x 500 µl	5 x 500 µl	-15°C to -25°C
FetoGnost® Kit IPC Target (orange cap)	Target for IPC (internal DNA Positive Control, 60,000 copies/µl)	1 x 200 µl	5 x 200 µl	-15°C to -25°C
FetoGnost® Kit Reaction Mix (white cap)	DNA Reaction Mix	2 x 750 µl	5 x 1500 µl	**-15°C to -25°C until first use, then at +4°C**
FetoGnost® Kit RHD Positive Control (red cap)	DNA Positive Control (mixture of four DNA target sequences for exon 5, 7, and 10, each approx. 1,000 target copies/µl)	1 x 100 µl	1 x 500 µl	-15°C to -25°C

The components of FetoGnost® Kit RHD are stable until the expiry date stated on the label after first use. Protect kit components from light.

FetoGnost Kit Reaction Mix: The DNA Reaction Mix provided with the kit has been designed for reliable, high-sensitivity 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.

6. Additionally required materials and devices

- Suitable reagents and laboratory equipment for extraction of cffDNA (e.g. for manual extraction recommended: QIAamp® DSP Virus Kit or QIAamp Circulating Nucleic Acid Kit, (QIAGEN) or an automated extraction procedure.
- Disposable powder-free gloves
- Pipettes (adjustable)
- Pipette tips with filters
- Appropriate 96 well reaction plates or reaction tubes with corresponding (optical) closing material
- Real-time PCR instrument which is able to detect and differentiate fluorescence in FAM, VIC, NED and Cy5 channel

7. General Precautions

- For *in vitro* diagnostic use. The use of this kit is limited to qualified personnel instructed in the procedures of real-time PCR and *in vitro* diagnostics.
- This product should only be used by professionals trained in real-time PCR methods.
- Benches and equipment should be decontaminated periodically.
- Always use DNase/RNase-free disposable pipette tips with aerosol barriers and disposable powder-free gloves.
- Samples should always be considered as potential infectious material and be handled appropriately. Wear disposable powder-free gloves when handling clinical samples and kit reagents.
- Spatially separated workspaces for DNA extraction, preparation of real-time PCR and amplification are required. Supplies and equipment have to be dedicated to the separate working areas. The workflow in the laboratory should always proceed from pre- to post-PCR.
- Be careful when handling samples and the positive control in order to prevent cross-contamination. Gloves should be changed after handling of samples and the positive control.
- Store and extract positive material (specimens, controls and amplicons) separated from all other reagents and add it to the reaction mix in a spatially separated workspace.
- The quality of the DNA has a big impact on test performance. It has to be ensured that the DNA extraction method does not cause contaminations and is suitable for the isolation of short DNA-fragments.
- Contamination of equipment and supplies with DNA/RNA, nucleases or amplicons should be avoided using good laboratory practices.
- Protect components of the kit from light and avoid repeated freeze-thaw cycles.
- Thaw all components thoroughly at room temperature before pipetting. When thawed, mix the components and centrifuge briefly.
- For valid interpretation of results always include positive and negative controls to exclude false-positive or false-negative results.
- Sample material, reagents and waste should be discarded according to local safety regulations.
- Do not mix reagents of different kits and lots and check expiry date of the kits.
- **Attention:** The DNA IPC target is stored in stabilizer containing DTT/guanidine thiocyanate/triton X-100 (for MSDS, see www.ingenetix.com).

8. Limits of detection method

- Reliable results can only be guaranteed when appropriate methods for blood collection, transport, storage (depending on the collection tubes) and processing of samples are used.
- Cell-free fetal DNA is found in very low concentration in maternal plasma, therefore extraction is a critical step in the analysis. It must be ensured by the user that sufficient fetal DNA can be obtained with the selected extraction method.
- The test is not suitable for samples taken before gestation 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).
- A 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.
- Rare clinical subtypes or a weak D genotype may lead to non-detection of the corresponding exon, 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 minor genetic variations (e.g. point mutations, insertions, gene rearrangements) and therefore such a serologically RhD-negative mother or fetus or a weak D genotype of the mother or fetus will test positive for the respective exons of the RHD gene.
- The fetal haplotype 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 the plasma is separated according to standard procedures. Please also consider the specifications of the ISO 20186-3: 2019 “Molecular in vitro diagnostic examinations – Specifications for pre-examination processes for venous whole blood – Part 3 Isolated circulating cell free DNA from plasma”.

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.
- Blood samples of gestation age ≥11+0 can be collected with different collection systems:
  - EDTA blood collection tubes with or without separating gel (recommended).
    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; Müller et al., 2011).

9.3 DNA extraction from plasma

- Plasma samples can be stored at approximately -20°C until DNA extraction (Londero et al., 2019).
- Using a 96-well reaction plate the DNA of 30 plasma samples including controls can be analyzed in triplicates. Please note that one **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.
- Important: Plasma from a hemolyzed blood sample must not be used for the analysis. Visual inspection for hemolysis after centrifugation of the sample must be done prior to nucleic acid extraction. In case of a hemolyzed sample, a new sample must be collected.
- DNA extraction can be performed manually (e.g. using the QIAamp® DSP Virus Kit or QIAamp Circulating Nucleic Acid Kit, QIAGEN) or using automated extraction procedures suitable for the isolation of short DNA fragments (Legler et al., 2007, Yang et al., 2019).
- 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 gestation age 16, extraction of at least 2 ml of blood is recommended. Of samples ≥16 gestation age, a sample volume of 0.5 ml of blood eluted in up to 100 µl is sufficient.

  → **The FetoGnost® Kit IPC** Target is added during extraction (extraction control and control for potential PCR inhibition):
  Add 1 µl FetoGnost® Kit IPC Target to the plasma sample after the lysis buffer has been added. When using an automated extraction procedure, add 1 µl FetoGnost® Kit IPC Target per sample to the appropriate amount of lysis buffer. The IPC Target in the lysis buffer remains stable.
  Based on prior evaluation of the extraction method, it has to be guaranteed that the IPC has a Cq-value of approx. 28 -33. If the IPC yields lower Cq-values, the amount of the target has to be reduced accordingly

Caution: 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.

→ **Elution**: The elution volume should be selected so that 10 µl of eluate corresponds to at least 100 µl of plasma.
- Extraction of 1-2 ml plasma: elute in ≤100 µl.
- Of samples ≥16 gestation age, a sample volume of 0.5 ml of blood eluted in up to 100 µl is sufficient.

- DNA samples can be stored at approx. -20°C or for a few hours at approx. +4°C until analysis.

10. Preparation of real-time PCR

Make sure that one extraction negative control, one positive control (FetoGnost® Kit RHD Positive Control, red cap), or one extraction positive control (optional) are included per PCR run.

Each sample and all controls have to be analysed in triplicates with 10 µl sample volume per well.

10.1. Pipetting scheme

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

- Prepare the Master Mix according to the number of samples, taking into account an additional volume of approx. 10% to ensure a sufficient amount of Master Mix.
- 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 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. Keep in mind that some PCR-platforms first have to be calibrated with the corresponding dye before performing multiplex-PCR.

Select dyes:

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

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

Reaction volume: 30 µl

Temperature Profile: Ramp speed: without "fast cycling" parameter for ABI® 7500 Instrument or QuantStudio™ 5/7

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

*) UNG (Uracil-N-glycosylase) is a component of the FetoGnost® Kit RHD Reaction Mix as are dNTPs with dUTP to eliminate future amplicon carryover.

11. Interpretation of PCR-data

Reactions with positive Cq values are considered positive, those with negative or Cq ≥ 45 (cut-off) as negative (quantification cycle (Cq) = cycle threshold (Ct) = crossing point (Cp)).
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 1 shows the criteria for valid controls. Table 2 shows interpretation of data with samples.

Table 1 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	Cq<31	Cq<31	Cq<31	Negative	Valid	-
Positive Control	Negative	Negative	Negative	Negative	Invalid	See 12.1.
NTC	Negative	Negative	Negative	28-33	Valid	-
NTC	Negative	Negative	Negative	Negative	Invalid	See 12.1.

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 patient results cannot be interpreted.

Table 2 Interpretation of the test samples

Sample (in 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			Positive	I. Positive
Sample	3 out of 9 replicates positive			Positive	II. No interpretation possible, repeat analysis
Sample	Very low Cq values compared to other results			Positive	III. No interpretation possible, maybe maternal RHD variant
Sample	Negative	Negative	Positive	Positive	IV. Partial RHD D
Sample	A maximum of 2 out of 9 replicates positive			Positive	V. Negative
Sample	Negative	Negative	Negative	Negative	VI. Invalid, see 12.4.

*) The IPC Cq values in a PCR-run should show comparable results, a shift of Cq values can indicate a partial inhibition of the PCR reaction.

- I. RHD-positive fetus: At least 4 of the 9 replicates are positive. Cq values between 28-40 can be expected, depending on the gestation age. The fetal haplotype may also correspond to a silent RHD variant. If only one or two exons are amplified, a fetal partial D RHD genotype could be suspected, or an insufficient amount of cff DNA is present.
- II. Interpretation of the fetal RHD status is not possible: If only a maximum of 3 of the 9 replicates were positive, the fetal RHD genotype cannot be determined and the analysis should be repeated.
- III. Interpretation of the fetal RHD status is not possible: If Cq values are comparable to the positive controls, either fetal RHD DNA or maternal RHD DNA (mother with normal or weakly expressed D antigen or silent variant of RHD gene) was detected. In this case, the fetal RHD genotype cannot be determined.
- IV. Partial D RHD genotype. In the case of maternal or fetal RHD-CE-D hybrid genes such as RHD-CE (2-9)-D, RHD-CE (3-9)-D, RHD-CE (3-7)-D, RHD-CE (4-7)-D, a section of RHD is replaced by the corresponding section of RHCE. In the case of RHD-CE-D hybrid genes, only exon 10 is positive.
- V. RHD-negative fetus: exons 5, 7 and 10 are undetectable (at least 7 of the 9 replicates are negative).
- VI. In case of invalid data: See 12. Troubleshooting.

12. Troubleshooting

12.1. No specific signal in FAM, VIC, NED and Cy5 channels with positive control 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 (see 10. Preparation of real-time PCR).
- Incorrect configuration of the PCR reaction.
  → Check your work steps (see 10. Preparation of real-time PCR) and repeat the PCR, if necessary.
- No positive control was added.
  → If all clinical samples are also negative, 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 target has been forgotten:
  → 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 in replicates.
→ Strictly pipette the positive controls at last.
→ Make sure that workspace and instruments are decontaminated at regular intervals.

12.3. IPC specific signal with the negative control and the positive control.

- IPC target was added during extraction, but there is IPC specific signal with negative control and positive control: contamination with IPC target.

12.4. 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. Make sure that you use a recommended method for DNA isolation and stick closely to the manufacturer's instructions.

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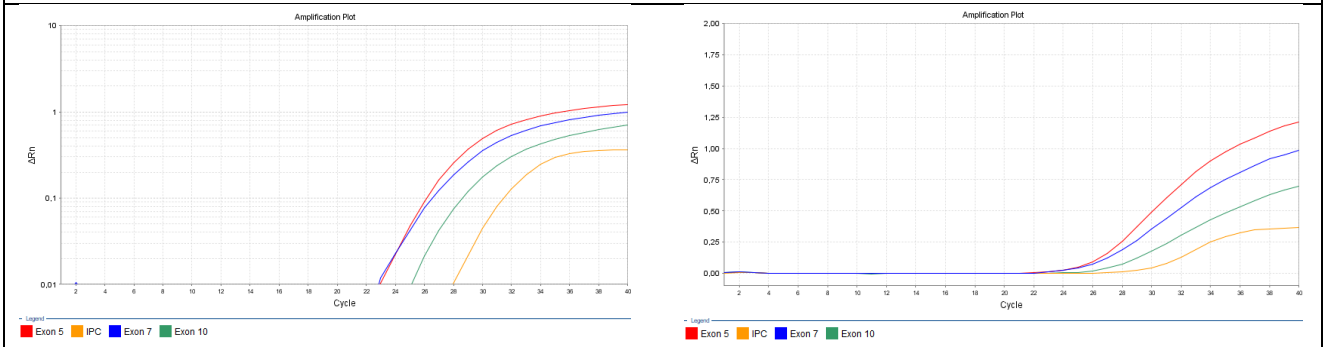
13. Specifications and performance evaluation

13.1. Testperformance

FetoGnost® Kit RHD has been evaluated with an Applied Biosystems® 7500 instrument (Thermo Fisher Scientific) and QuantStudio™ 7 Pro (Thermo Fisher Scientific). For further validation data contact ingenetix GmbH.



ABI® 7500: FAM, VIC, NED and Cy5 channel
FetoGnost® Kit RHD positive control und internal DNA positive control

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 of 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 (Table 3). The 95% LOD for exon 5, 7, and 10 detection is 13, 8, and 7 target copies/response, respectively.

Table 3

	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 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 correlation coefficient $R^2 > 0.98$ (Figure 3).
Exon 7: The test shows linearity between 100 - 1,000,000 target copies/reaction, with a slope of -3.827 ± 0.07239 and a correlation coefficient $R^2 > 0.98$ (Figure 3).
Exon 10: The test shows linearity between 100 - 1,000,000 target copies/reaction, with a slope of -3.820 ± 0.05859 and a correlation coefficient $R^2 > 0.98$ (Figure 3).

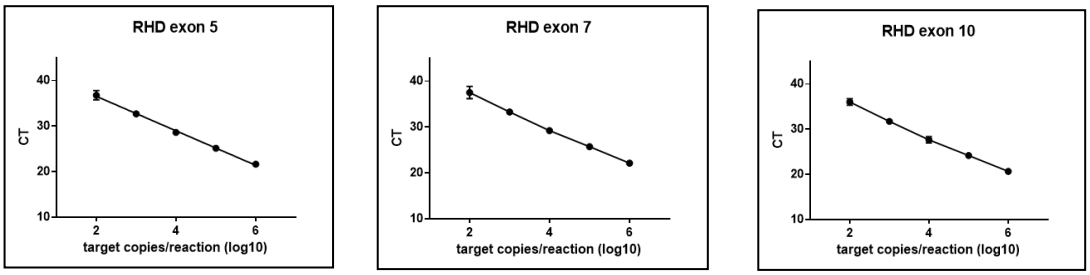


Figure 3

13.4. Analytical specificity

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. Precision inter-assay

Inter-assay precision shows the reproducibility between assays done 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) in three independent experiments performed on different days in triplicates (two times) and quadruplicates (one time). Arithmetic mean ($\bar{x}$), standard deviation (σ) 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% in the range of E+6 to E+1 copies/reaction.
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% in the range of E+6 to E+1 copies/reaction.
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% in the range of E+6 to E+1 copies/reaction.

Inter-assay precision was further analysed using 9 replicates from a 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. Precision intra-assay

Intra-assay precision of FetoGnost® Kit RHD (exon 5, 7, 10) was determined from the replicate runs of point 11.3. Arithmetic mean ($\bar{x}$), standard deviation (σ) 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% in the range of E+6 to E+1 copies/reaction.
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% in the range of E+6 to E+1 copies/reaction.
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% in the range of E+6 to E+1 copies/reaction.

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 Ct 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. Precision inter-Lot

The inter-lot precision describes the conformity of performance between different manufactured kit lots. The lot-to-lot equivalence 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+5 to E+3). Arithmetic mean ($\bar{x}$), standard deviation (σ) 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% in the range of E+5 to E+3 copies/reaction.
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% in the range of E+5 to E+3 copies/reaction.
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% in the range of E+5 to E+3 copies/reaction.

13.8. Validation with different volumes of plasma samples and DNA extracts

Different volumes of plasma samples of two women in the twelfth 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 4).

Table 4

Sample (number of positive replicates)		**Ct $\bar{x}$ (number of positive replicates)**			
		Exon 5	**Exon 7**	**Exon 10**	**IPC**
1 (7/9)	Patient 1: 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)
4 (7/9)	Patient 1: 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)
5 **(3/9)**	Patient 1: 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)
2 (5/9)	Patient 1: 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)

Sample (number of positive replicates)		Ct x̄ (number of positive replicates)			
3 (5/9)	Patient 1: 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)
6 (9/9)	Patient 2: 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)
9 (5/9)	Patient 2: 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)
10 (5/9)	Patient 2: 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)
7 (7/9)	Patient 2: 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)
8 (5/9)	Patient 2: 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, in order to determine the highest possible dilution at which RHD is still detectable. Most laboratories participating in the study were able to detect RHD only just in the dilution 1:2 in real-time PCR.
With FetoGnost® Kit RHD, the dilution level 1:2 was positive for RHD in 4 out of 4 replicates in all exons.

13.10. Clinical sensitivity

A retrospective study on the reliability of prenatal determination of fetal Rh factor from fetal DNA in maternal plasma was performed at the University Medical Center Göttingen, Department of Transfusion Medicine, Germany and Department of Blood Group Serology and Transfusion Medicine, Medical University of Vienna, Austria (**Legler et al., 2021**).

FetoGnost® Kit RHD is the successor product of FetoGnost RhD assay (ingenetix), which did not include the Internal Positive Control and the DNA reaction mix. Due to the same components for the detection of RHD exon 5, 10 and 10 (Assay Mix), the present results are also applicable for the FetoGnost® Kit RHD.

The purpose of the study was to evaluate the diagnostic accuracy of FetoGnost RhD assay for noninvasive prenatal determination of the fetal RhD status (NIPT-RhD) with a focus on early gestation and multiple pregnancies.
The FetoGnost RhD assay (ingenetix) was routinely applied for clinical decision making either in woman with anti-D alloimmunization or in order to target the application of routine antenatal anti-D prophylaxis (RAADP) to women with a RhD positive fetus. Based on existing data in the laboratory information system the newborn's serological RhD status was compared with NIPT RhD results.
Since 2009 NIPT RhD was performed in 2,968 pregnant women between week 5+6 and 40+0 of gestation (median 12+6) and conclusive results were obtained in 2,888 (97.30%) cases. Diagnostic accuracy was calculated from those 2244 (77.70%) cases with the newborn's serological RhD status reported. **The sensitivity of the FetoGnost RhD assay was 99.93% (95% CI 99.61% - 99.99%) and the specificity was 99.61% (95% CI 98.86% - 99.87%).** No false positive or false negative NIPT RhD result was observed in 203 multiple pregnancies.
As conclusion, NIPT RhD results are reliable when obtained with FetoGnost RhD assay. Targeted routine anti-D-prophylaxis can start as early as 11+0 weeks of gestation in singleton and multiple pregnancies

Table 5 Summary of performance data

Parameter	Performance data
LoD95	**Results obtained with genomic DNA dilutions:** RHD exon 5: 13 copies/reaction RHD exon 7: 8 copies/reaction RHD exon 10: 7 copies/reaction
LoD50	RHD exon 5: 2 copies/reaction RHD exon 7: 4 copies/reaction RHD exon 10: 3 copies/reaction
Linearity and 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 target plasmid DNA dilutions:** For exon 5: mean overall intra-assay precision of 0.80% For exon 7: mean overall inter-assay precision of 0.89% For exon 10: mean overall inter-assay precision of 0.73%
Intra-Assay-Precision	**Results obtained with target plasmid DNA dilutions:** For exon 5: mean overall intra-assay precision of 1.01% For exon 7: mean overall inter-assay precision of 1.89% For exon 10: mean overall inter-assay precision of 0.81% **Results obtained with pooled plasma samples:** Ct mean: 36.05 Standard deviation: 1.48 Coefficient of variation (CV): 4.1%
Inter-Assay-Precision	**Results obtained with target plasmid DNA dilutions:** For exon 5: mean overall inter-assay precision of 1.56% For exon 7: mean overall inter-assay precision of 1.58% For exon 10: mean overall inter-assay precision of 1.89% **Results obtained with pooled plasma samples:** Ct mean: 36.14 Standard deviation: 1.16 Coefficient of variation (CV): 3.2%
Cross-reactivity	No cross-reactivity with RHCE
Analytical specificity	100% specific, a few clinical subtypes (SNPs) of exons are not detected, which has no influence on overall analysis based on analysis of three exons
Real-time PCR system equivalence with • **ABI® 7500 instrument** • **QuantStudio™ 7 Pro**	100%
Robustness	The test is robust to fluctuations of annealing temperatures +/1 1°C and of reagent concentrations
Stability	FetoGnost® Kit RHD is stable up to 37 months
Testing of WHO reference material (07/222)	100% positive for the dilution level 1:2 (4 of 4 replicates positive in all exons)
Diagnostic sensitivity	99.93% (95% CI 99.61% - 99.99%)
Diagnostic specificity	99.61% (95% CI 98.86% - 99.87%)

14. Literature

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15. Revision history

Revision	Date	Description

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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