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Instructions for Use

FastQ® RHD fetal

Electronic instructions for use see www.bag-diagnostics.com

Test kit for the analysis of rhesus D characteristics on cell-free fetal DNA from maternal blood plasma

RUO

For research use only - Not for diagnostic purposes

REF 728210 FastQ® RHD fetal

Contents

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1. Application

The FastQ® RHD fetal Kit enables the analysis of rhesus D characteristics on cell-free fetal DNA (cffDNA) in maternal blood plasma. The identification is based on molecular genetic typing using SSP PCR technique and real-time detection (real-time PCR) of amplification. The kit can be used for analysis from 11 weeks of gestation in singleton pregnancies. In case of RHD-negative results, this should be considered as preliminary result and the test must be repeated from the 20th week of gestation .

There is no differentiation between maternal cell-free DNA and fetal cell-free DNA.

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2. Background

Pregnancy-related alloimmunization to the D antigen (D immunization) in D-negative women is a cause of fetal and neonatal hemolytic disease. For a long time, it was common practice for all D-negative pregnant women to receive prenatal prophylaxis because without pretesting of the fetus, the fetal rhesus D characteristics remained unknown until birth and inasive procedures resulted in an increased risk of miscarriage. A noninvasive procedure ensures that prophylaxis is administered only to those D-negative women who carry a D-positive fetus to term (60%). This prevents unnecessary anti-D treatments. Up to 40% [2] of all anti-D injections can be saved in this way.

3. Product description

The FastQ® RHD fetal kit enables the molecular genetic analysis of three different fetal Rhesus D characteristics from maternal plasma. The FastQ® RHD fetal kit contains all components required for the PCR reaction.

4. Test principle

The test is performed using cffDNA. Scientific studies have shown that the blood plasma of pregnant women contains small amounts of cell-free fetal DNA. The amount of cffDNA increases during pregnancy. [3] Thus, cffDNA can be isolated from the blood plasma of RhD-negative pregnant women to identify the fetal RHD gene, if the fetus has it.
The fetal RHD gene is detected by RT-PCR over three different exons of the RHD gene. These are exons 5, 7 and 10. The cffDNA is amplified in a PCR with sequence-specific primers (SSP). The primers were specifically designed for selective amplification of sections of specific Rhesus D characteristics. The amplicons are detected using fluorescent dye-labeled hydrolysis probes (TaqMan® probes), whereby the sensitivity and specificity of the test allows the detection of low copy numbers in the cffDNA compared to classical methods (SSP-PCR).
When amplicons are present, the probes are hydrolyzed by Taq polymerase, resulting in a fluorescent signal that increases in proportion to the amount of PCR product. The fluorescence signals are measured by the optical detection unit of the RT-PCR cycler.
The test is performed in a PCR reaction in which the internal control (human HGH gene) is also detected using various fluorescent dyes.

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4. Material

4.1 Content of the kits

- **2x 500 µl Q Mastermix fetal**, ready to use, containing dNTPs, Taq Polymerase, reaction buffer. Is available in vials in liquid form.
- **380 µl Oligomix FastQ® RHD fetal** for the molecular genetic determination of fetal rhesus D characteristics. The reaction kit contains specific primers and probes for the detection of exons 5, 7 and 10 of the rhesus D gene as well as HGH-specific control primers and probes (oligomix).

4.2 Additionally required reagents and devices

- Reagents for cffDNA isolation
- RT-PCR-Cycler (recommended cyclers see 4.3)
- Aqua dest.
- Variable pipettes (0.5 - 1000 µl) and pipette tips
- Suitable plastic goods
- Plate centrifuge

4.3 Recommended cyclers and reaction tubes

Cyclers	RT-PCR reaction tubes	RT-PCR closing system
CFX96™ Real-Time PCR Detection System	FrameStar® Break-A-Way PCR Plate 96 white wells, black frame/Product No. 4ti-1201 Fa. 4titude / Brooks Life Sciences	Crystal Strips, Product No. 4ti-0755 Optically clear adhesive film, Product No. 4ti-0560 Comp. Fa. 4titude / Brooks Life Sciences
LightCycler® 480 II Real-Time PCR Detection System, Roche Molecular Systems Inc.	LightCycler® 480 Multiwell Plate 96, white, No. 04729692001 Fa. Roche Molecular Systems Inc.	Optical foil, included in the LightCycler® 480 Multiwell Plate 96

Note: If other real-time thermocyclers, reaction vessels and sealing systems are used, they must be tested and approved by the user for compatibility.

When using the LightCycler® 480 II Real-Time PCR Detection System, a colour compensation is recommended (RHD fetal CC LC480, REF 726323).

5. Storage and stability

Upon receipt, all reagents must be stored at ≤ -20°C in temperature-monitored equipment. The expiry date is indicated on the label of the respective reagents. The expiration date indicated on the outer label refers to the kit component with the shortest shelf life. It is recommended to aliquot the reagents if necessary.

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6. Test procedure

6.1 Precautions and special Remarks

Molecular genetic techniques are particularly sensitive methods and should only be performed by qualified personnel with experience in molecular genetic techniques.

Special precautions must be followed to avoid contamination and thus false reactions:

- Principally wear gloves during work (preferably powder-free).
- Use new tips with each pipetting step (with filter insert or integrated stamp).
- Work in two separate areas for pre-amplification (DNA-isolation, preparation of the reactions) and post-amplification (detection); use two separate rooms if possible.
- Work in two separate areas for pre-amplification (DNA-isolation, preparation of the them.

6.2 Amplification

Note:

- The reaction volume for each RT-PCR-preparation is 20 µl (each well).
- For the current test, the cffDNA is used undiluted after isolation.

Pipetting Process:

For each specimen (triplet-testing) a pre-mix is created:

12 µl Q Oligomix RHD fetal
30 µl Q Mastermix fetal
15 µl specimen cffDNA
3 µl Aqua dest.

Pipette 20 µl from this pre-mix into each of the 3 wells.

For single testing, pipette 4 µl Q Oligomix RHD fetal, 10 µl Q Mastermix fetal, 5 µl specimen cffDNA and 1 µl Aqua dest. into the reaction tube.

For a **negative control** (NTC), prepare a test with Aqua dest. instead of the specimen cffDNA.
Close the reaction tubes and briefly centrifuge the liquid down. Ensure that the PCR plate is **completely sealed** by the lids. Make sure that there are **no bubbles** in the reaction tubes. If bubbles appear, gently tap the tubes on the lab bench to remove them. Then perform the PCR reaction using the following program.

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Program Step	Time [s]	Temperature [°C]	Ramp rate [°C/s]	Plate read	Number of Cycles
Initial Activation	600	95	2,5	-	1
Denaturation	10	95	2,5	-	45
Annealing + Extension	60	60	2,2	-	
	15	72	-	yes	

The following real-time devices are used for testing:
Bio-Rad: CFX96™ Real-Time PCR Detection System
Roche: LightCycler® 480 II Real-Time PCR Detection System

Note:

- With the CFX96 Touch™ Real-Time PCR Detection System, a modified heating rate of the device (ramp rate) must be used. These are listed in the PCR program table above ("Ramp rate" column). Before starting the run **"All Channels"** must be selected and the lid temperature must be set to 105°C.
- When using the LightCycler® 480 II system a colour compensation is required (provided by BAG Diagnostics GmbH).

Use the following channel set up.

Excitation Filter	Emission Filter	Name	Melt Factor	Quant Factor	Max Integration Time (Sec)
465	510	FAM	1	10	1
533	580	VIC	1	10	1
533	610	Texas Red	1	10	1
618	660	Cy5	1	10	1

Other cyclers may require a different heating rate setting. Therefore, validation by the user is required.

6.3 Evaluation, assessment, interpretation of results

All tests with human cffDNA must show fluorescence signals in the FAM channel of the internal control.

Specific positive samples show a positive colour signal in the corresponding colour channels.

Positive results have a Cq value of ≤ 40. This value depends on the amount of cffDNA or the time of collection of the sample material (WG).

Cq values of ≥ 40 are to be regarded as inconclusive results and must be checked and repeated if necessary.
The result of the RHD characteristics is negative if no signal is determined for all RHD exons 5, 7 and 10.
The result of the RHD characteristics is defined as positive if all of the three exons have an Cq value ≤ 40. Exception: The missing amplification of exon 5 (Cy5 channel) is used to detect the RHD pseudogen (PSI). If in the tested samples (triplicate) the detections of IAC, exon 7 and exon 10 have a Cq value ≤ 40 and only the signal of exon 5 is not detectable, it may be a PSI-positive sample. In this case, further testing is recommended.

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The following fluorophores are used in the FastQ® RHD fetal kit:

Specificity	Fluorophores	Wave length in nm*
Internal amplification control (IAC)	FAM	Excitation: 494 Emission: 520
Exon 7	VIC	Excitation: 538 Emission: 554
Exon 10	Texas Red	Excitation: 583 Emission: 603
Exon 5	Cy5	Excitation 649 Emission: 670

* Data according to the synthesis lab

Note

The predefined Cq value of ≤ 40 only applies to the use of the CFX96 and the LightCycler® 480 II cycler together with corresponding PCR tubes (see 4.3).
The amplification signals of negative controls (RHD features negative) must be outside the defined Cq values for each of the three colour channels.

A negative control (NTC) with Aqua dest. does not develop any fluorescence signals over the entire RT-PCR run and serves as a contamination control. If the negative control with Aqua dest. shows fluorescence signals within the defined Cq values, this indicates a contamination. Fluorescence signals outside the defined Cq values may occur due to the very sensitive test method in case of inaccurate pipetting. Detailed analysis is recommended - if necessary, the workplace must be decontaminated from cffDNA or DNA and the reagents must be exchanged.

7. Warnings and Precautions

The kits should only be used by specially trained personnel and qualified personnel. All work should be performed using Good Laboratory Practice.

Biological material used for extraction of cffDNA, e.g. blood, should be handled as potentially infectious. When handling biological material appropriate safety precautions are recommended (do not pipet by mouth; wear disposable gloves while handling biological material and performing the test; disinfect hands when finished the test).
Biological material should be inactivated before disposal (e.g. in an autoclave). Disposables should be autoclaved or incinerated after use.

Spillage of potentially infectious materials should be removed immediately with absorbent paper tissue and the contaminated areas swabbed with a suitable standard disinfectant or 70% alcohol.
Material used to clean spills, including gloves, should be inactivated before disposal (e.g. in an autoclave).

Disposal of all samples, unused reagents and waste should be in accordance with country, federal, state and local regulations.

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Microbial contamination of the reagents while taking aliquots should be avoided. It is recommended to use sterile one way pipettes and tips. Reagents that look cloudy or show any signs of microbial contamination must not be used.
A safety data sheet or a declaration on safety data sheets (SDS) can be downloaded from www.bag-diagnostics.com.

8. Performance Characteristics

The combination of primers and probes allows analysis of human fetal Rhesus D characteristics from maternal plasma according to lot-specific specifications. The accuracy and reproducibility of the test kit reactivity will be verified for each lot using control samples.

9. Limitations of the Method

Since the RT-PCR method is very sensitive to cross-contamination of cffDNA or DNA, care should be taken during isolation. Special care should be taken to avoid contamination of the kit reagents and other laboratory materials with amplicons or DNA. The use of a negative control with Aqua dest. is recommended. No fluorescence signal should be detected in the negative control with Aqua dest. (Cq > N.A.). In case of a signal development in the negative control, the PCR lab station must be decontaminated from cffDNA and DNA if necessary and the reagents must be exchanged. All devices (e.g. pipettes, real-time devices) must be calibrated according to the manufacturer's specifications.

10. Internal Quality Control

Internal quality controls for new lots will be performed using a combination of DNA samples with known type. An internal amplification control (IAC) to verify successful amplification and the inclusion of negative controls to detect possible contamination is recommended. For this purpose, a test without cffDNA is set up (NTC), see item Amplification.

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11. Troubleshooting

Symptom	Possible reason	Potential solution
Poor or no signal	Presence of an inhibitor in the PCR-reaction	Use fresh reagents
	Insufficient amount of cffDNA in the reaction	Repeat test with correct amount of DNA Inappropriate time of sampling (WG)
	Insufficient amount of cffDNA in the reaction	Inappropriate isolation method
	Wrong amplification parameters	Check PCR program and ramp rate
	Contaminated or degraded cffDNA	Check concentration and quality of the cffDNA Check cffDNA on a gel Repeat cffDNA isolation
	Degraded fluorescent probes or primers	Use fresh Q Oligomix Avoid exposure to light and frequent thawing and freezing Pay attention to storage conditions
	Bubbles in the PCR reaction, residual liquid at the inner wall of the tube	Careful pipetting Spin down PCR plate
	Incompatible or low quality RT-PCR plastics	Use compatible and high quality plastics
	Evaporation of the reagents due to improper sealing of the PCR tubes	Check for correct sealing. In the case of adhesive foils, the edge area of the PCR plate must be checked for tightness
Signal in the negative control	Contamination with cffDNA or DNA in the negative control	Repeat the test - decontaminate the workplace

12. Trademarks used in this Document/Product

TaqMan® and the LightCycler® are trademarks of Roche Molecular Systems Inc.

FrameStar® is a trademark of 4titude® Ltd.

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13. Expanation of Symbles used on the Labels

	Sufficient for n tests
	Storage temperature / Lower limit of temperature
	Manufacturer
	Use by
RUO	For research use only
IFU	Instruction for use
eIFU	Electronic Instruction for use
REF	Catalogue number
LOT	Batch code
Q Oligomix RHD fetal	Liquid oligomix (specific primers and probes and HGH-specific control primers and probes).
Q Mastermix fetal	Mastermix, contains dNTPs, Taq polymerase, reaction buffer

14. Literature

1. Gemeinsamer Bundesausschuss. *Richtlinien des Gemeinsamen Bundesausschusses über die ärztliche Betreuung während der Schwangerschaft und nach der Entbindung (Mutterschafts-Richtlinie)*, 2020
2. F. B. Clausen, M. B. Damkjær, M. H. Dziegiel et al. *Noninvasive fetal RhD genotyping*, 2014
3. Y. Zhou, Z. Zhu, Y. Gao et al. *Effects of Maternal and Fetal Characteristics on Cell-Free Fetal DNA Fraction in Maternal Plasma*, 2015.

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