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INSTRUCTION FOR USE

FastQ[®] RHD fetal plus

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

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



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FastQ[®] RHD fetal plus

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1. INTENDED USE

The intended purpose of the FastQ® RHD fetal plus kit is the molecular genetic analysis of the fetal rhesus D status using cff DNA isolated from maternal plasma. For the analysis exon 5, exon 7 and exon 10 are used.

2. PRODUCT DESCRIPTION

Pregnancy-related alloimmunization to the rhesus D antigen (D immunization) in D-negative women carrying a D-positive fetus 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 status remained unknown until birth. A noninvasive procedure ensures that prophylaxis is administered only to those rhesus D-negative women who carry a D-positive fetus to term (60%). This prevents unnecessary anti-D treatments. Up to 40% (Clausen et al., 2014) of all anti-D injections can be saved in this way.

The FastQ® RHD fetal plus kit allows the analysis of rhesus D status from cell-free fetal DNA (cffDNA) in maternal blood plasma, with additional detection of a fetal marker as a control for cffDNA, which reacts positively in about 50% of cases. The identification is based on molecular genetics using the SSP PCR technique and real-time detection (real-time PCR) of amplification. The kit can be used for analysis from the 11th week of pregnancy (WP) in singleton pregnancies. In the case of negative results for the fetal marker from samples with WP ≤ 20, the result is to be regarded as preliminary and the test must be repeated from the 20th WP. The FastQ® RHD fetal plus kit contains all components required for the PCR reaction.

3. TEST PRINCIPLE

The test is performed with human cffDNA isolated from maternal plasma. The DNA is amplified in a real-time PCR with sequence-specific primers (SSP). The primers are designed for the selective amplification of specific sequences of the RHD alleles and a fetal marker. The amplicons are detected with fluorescent dye-labelled hydrolysis probes (TaqMan® probes). If amplicons are present, the probes bind to the amplicons and are hydrolyzed by the 5' to 3' exonuclease activity of the Taq polymerase. A fluorescence signal is thus generated that increases proportionally to the amount of the amplicons. The fluorescence signals are measured by the optical detection unit of the real-time PCR-Cycler.

An internal amplification control (human HGH gene) is included in the multiplex PCR reaction which is detected in a different colour channel than the specific reactions for RHD and fetal marker.

4. MATERIAL

4.1 Content of the kits

- 2x 475 µl Q Mastermix fetal, ready to use, containing dNTPs, Taq Polymerase, reaction buffer. Is available in vials in liquid form.

- 380 µl Q Primermix RHD fetal plus for the molecular genetic analysis of the fetal rhesus D status. The reaction mix contains specific primers and probes for the detection of exons 5, 7 and 10 of the rhesus D gene, for the fetal control marker as well as HGH-specific control primers and probes.
- **Electronic instructions for use**, available from the download server www.service.bag-diagnostics.com, for further information see accompanying information sheet in the kit.

4.2 Additionally required reagents and devices

- Reagents for cffDNA isolation (validated extraction kits see 6.2)
- RT-PCR-Cycler (validated cyclers see 4.3)
- Aqua dest.
- Variable pipettes (0.5 - 1000 µl) and pipette tips
- RT-PCR reaction tubes and closing system (validated products see 4.3)
- Plate centrifuge

4.3 Validated RT-PCR cyclers and reaction tubes

Cycler	RT-PCR reaction tubes	RT-PCR closing system
QuantStudio™ 6 Flex Real-Time PCR-System 96-Well Fast, laptop Comp. Applied Biosystems	Removable 8 Well PCR Tube Strip, Product No. 4ti-0753 Comp. Azenta Life Sciences	Strip of 8 Flat Optical Caps Crystal Clear, Product No. 4ti-0755 qPCR Adhesive Seal, Product No. 4ti-0560 Comp. Azenta Life Sciences
	FrameStar® 96 Well Semi-Skirted, PCR Plate, ABI® FastPlate Style, white wells, clear frame Product No. 4ti-0911 Comp. Azenta Life Sciences	
QuantStudio™ 5 Dx Real-Time PCR-System, 96-Well, 0.2 ml Comp. Applied Biosystems	Saphire PCR Microplates, 96 Well, semi skirted, ABI design REF 652260 Comp. Greiner BIO-ONE®	qPCR Adhesive Seal, Product No. 4ti-0560 Comp. Azenta Life Sciences

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

5. STORAGE AND STABILITY

Upon receipt, all reagents must be stored at $\leq -20^{\circ}\text{C}$ 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. The freeze-thaw cycle testing has shown that up to 12 cycles for the Q Mastermix fetal and up to 15 cycles for the Q Primermix RHD fetal plus have no detrimental effects on the quality of the kit. It is recommended to aliquot the reagents if necessary.

The pipetted reaction mixture before or after addition of the DNA sample can be stored protected from light at 2...8°C for up to 16 hours before starting the PCR run. If the protective packaging is damaged, please contact the customer service.

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:

- Wear gloves during work (preferably powder-free).
- Use new tips with each pipetting step (with filter insert or integrated stamp).
- If possible, use two separate areas for pre-amplification (cffDNA-isolation and PCR set up) and post-amplification (detection).
- Use devices and other materials only at the respective places and do not exchange them.

6.2 cffDNA Isolation

The test is performed using cell-free fetal DNA (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 (Zhou et al., 2015). Thus, cffDNA can be isolated from the blood plasma of rhesus D-negative pregnant women to analyse the fetal rhesus D status.

The sample material for the isolation of cffDNA must be sent in appropriate blood collection systems. For the test EDTA blood is required. The presence of heparin potentially inhibits PCR; therefore blood collection systems with heparin are not suitable (Beutler et al., 1990) and must not be used. Transport of the whole blood sample is possible under clearly defined conditions, e.g. up to 6 days at room temperature. The plasma must be separated within these 6 days after sample collection (Clausen et al., 2013; Müller et al., 2011). The plasma must then be used directly for the isolation of cffDNA or the samples can be stored at approx. -20°C until DNA extraction (Londero et al., 2019).

It is recommended to use **CE** IvD certified kits for the DNA isolation.

Validated DNA isolation kits:

- QIASymphonie®; DSP Circulation DNA Kit
- Qiagen QIAMP®; MinElute® ccfDNA Mini Kit

If the established standard method of the lab is used for cffDNA isolation and this is not one of the validated kits above, it must be validated by the user.

6.3 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:

Each sample has to be performed as triplicate.

For single testing, pipette the following volumes into the reaction tube:

4 µl Q Primermix RHD fetal plus
 10 µl Q Mastermix fetal
 1 µl Aqua dest.

Prepare the pre-mix according to the number of samples and calculate an extra volume of 10% to account for pipetting loss. Pipette 15 µl from the pre-mix into each well and add 5 µl specimen cffDNA.

A negative control (NTC) is mandatory, for this a test must be prepared with Aqua dest. instead of the sample 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.

Program Step	Time [s]	Temperatur [°C]	Ramp rate [°C/s]	Number of Cycles
Initial Activation	10 min	95	2,5	1
Denaturation	10 sec	95	2,5	10
Annealing + Extension	1 min	60	2,2	
	15 sec	72	-	
Denaturation	10 sec	95	2,5	35
Annealing + Extension	1 min	60	2,2	
	15 sec + plate read	72	-	

Note:

When using the QuantStudio™ 6 Flex & 5 Dx Real-Time PCR-System, the following steps should be followed.

Step 1: "Experiment properties"

When working with Applied Biosystems QuantStudio™ 6 Flex Real-Time PCR System 96-Well Fast or a QuantStudio™ 5 Dx System, make the following basic settings:

Experiment properties*	QuantStudio™ 6 Flex Real-Time PCR-System 96-Well Fast	QuantStudio™ 5 Dx Real-Time PCR-System, 96-Well, 0.2 ml
Instrument	Quantstudio™ 6 Flex System	Quantstudio™ 5 Dx
Block	Fast 96-Well (0.1mL)	96-Well 0.2-mL
Experiment Type	Comparative Ct	Comparative Ct
Chemistry	TaqMan® Reagents	TaqMan® Reagents
Run Mode	Standard	Standard
Reaction Volume	20 µL	20 µL
Heated Cover Temperature	105.0°C	105.0°C
Passive Reference	None	None

*names can be different depending on Software

Step 2: "Define"/ "Plate" Selection of reporters and quenchers

On this RT-cyclers, the reporters and quenchers can be selected. A reporter can only be selected if the dye has been calibrated beforehand. The reporters must be selected on a kit-specific basis and depend on the probes to be detected in the respective kit. The reporter name and colour is selected by each laboratory during calibration. For the quencher, NFQ-MGB should be selected for all kits.

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

6.4 Evaluation, assessment, interpretation of results

All tests with human cffDNA must show fluorescence signals in the Cy5 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 ≤ 30 . This value depends on the amount of cffDNA or the time of collection of the sample material (WP).

Cq values of ≥ 30 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 traits is defined as positive if at least 4 of 9 possible specific detections (triplicates x 3 exons, see table below) have a Cq value of ≤ 30 . Exception: The missing amplification of exon 5 (FAM channel) is used for the detection of the RHD pseudogene (PSI). If in the tested samples (triplicate), the detections of IAC, exon 7 and exon 10 show a Cq value of ≤ 30 and only the signal of exon 5 is not detectable, it may be a PSI-positive sample. In this case, further testing is recommended.

The fetal marker result is negative if 1 or no signal is detected when testing the replicates. This is an indication that either the fetus does not carry the marker or that there is not sufficient cffDNA present in the sample to detect the marker.

The fetal marker result is positive if 2 or 3 signals are detected during replicate testing.

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

Specificity	Fluorophores	Cq-Level	Inspect	Wave length in nm*
Internal amplification control (IAC)	Cy5	≤ 30	≥ 30	Excitation: 494 Emission: 520
Exon 7	VIC	≤ 30	≥ 30	Excitation: 538 Emission: 554
Exon 10	Texas Red	≤ 30	≥ 30	Excitation: 583 Emission: 603
Exon 5	FAM	≤ 30	≥ 30	Excitation 649 Emission: 670
Fetal marker	TAMRA	≤ 30	≥ 30	Excitation: 557 Emission: 583

* Data according to the synthesis lab

The following table shows the interpretation of the amplification results:

Cy5 (IAC)	TAMRA (Fetal marker)	VIC (Ex 7)	Texas Red (Ex 10)	FAM (Ex 5)	Interpretation
Positive	At least 2 out of 3 replicates positive	At least 4 out of 9 replicates positive			Positive.
Positive	1 out of 3 replicates positive	1-3 out of 9 replicates positive			Negative, repetition is recommended.
Positive	2-3 replicates positive	Very early Cq-values compared to other results			Not assessable, maternal RHD variant possible - normal or weakly expressed D antigen or silent variant of the RHD gene*. The fetal RHD genotype cannot be determined.
Positive	2-3 replicates positive	Negative	2-3 replicates positive	Negative	Partial rhesus D genotype. Mother* or child carry 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. 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.
Positive	2-3 replicates positive	2-3 replicates positive	2-3 replicates positive	Negative	It may be a PSI-positive sample. In this case, further testing is recommended*.
Positive	Negative	Negative	Negative	Negative	Negative: No specific exon was detected. Fetus RHD negative and the fetal marker not present or the sample does not contain detectable or sufficient copy numbers (LOD) of the cffDNA.
Positive	2-3 replicates positive	Negative	Negative	Negative	Negative: Sufficient cffDNA was entered in the test
Negative	Negative	Negative	Negative	Negative	Invalid: Invalid result due to real-time PCR inhibition, reagent failure or unsuitable test material.

* It is recommended to confirm the rhesus D genotype of the mother (BAG Diagnostics ERY Q® RH, REF 728405; ERY Q® Weak D, REF 728401; ERY Q® Partial D, REF 728403).

Notes:

The predefined Cq value of ≤ 30 only applies to the use of the QuantStudio™6 Flex & 5 Dx cycler together with corresponding PCR tubes and closing systems (see 4.3).

The amplification signals of samples negative for RHD and the fetal marker must be outside the defined Cq values for each of the four colour channels.

A negative control (NTC) with Aqua dest. does not develop any fluorescence signal 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 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.

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 the analysis of human fetal rhesus D status from maternal plasma according to lot-specific specifications. The accuracy and reproducibility of the specificity of the test kit is verified for each lot with pre-typed reference samples.

8.1 Reference material

As referencematerial was used the WHO Reference Reagent RHD/SRY DNA sensitivity standard NIBSC code 07/222.

8.2 Analytical Evaluation

Limit of detection:

The analytical limit of detection was determined with the WHO reference sample (NIBSC code 07/222). The sample was tested undiluted and in a dilution 1:2. Both were tested in 4 replicates and determined positive.

Analytical sensitivity / specificity:

Sensitivity testing has shown that the kit detects a genomic DNA concentration of 0.1 ng/ml in plasma, with 3 out of 3 triplicates being positive within the specified limits (positive results have a Cq value of ≤ 30).

Specificity was demonstrated by testing negative samples. No background signal was observed, suggesting that the primers and probes were selected to be specific for RHD exon 5, 7, 10 and the fetal marker.

Linearity:

The Kit shows linear reactions from a DNA concentration of 100 ng/ml plasma to 0.1 ng/ml plasma.

Exon 5: $R^2 = 0.9977$

Exon 7: $R^2 = 0.9917$

Exon 10: $R^2 = 0.9949$

Fetal marker: $R^2 = 0,9944$

8.3 Clinical Evaluation

For the FastQ® RHD fetal plus kit a performance evaluation study was performed with pre-typed samples. The results from the study were compared to the results that were obtained by a CE IVD certified reference test. The evaluation of the results for all samples was used for the calculation of the diagnostic specificity and sensitivity of the test.

Diagnostic specificity and sensitivity only for the fetal marker. Due to lack of sample material, retesting was not possible:

		Reference test	
		Positive	Negative
FastQ® RHD fetal plus	Positive	52	5
	Negative	8	49

Diagnostic specificity: 90.7%

Diagnostic sensitivity: 86.7%

Diagnostic specificity and sensitivity only for the rhesus D analysis:

		Referenztest	
		Positiv	Negativ
FastQ® RHD fetal plus	Positive	64	0
	Negativ	0	55

Diagnostic specificity: 100%

Diagnostic sensitivity: 100%

8.4 Interfering substances

The influence of potentially interfering substances was tested with a rhesus D positive sample without interfering substances and spiked with the interfering substances at the concentrations (conc.) listed in the table below. Tests with interfering substances were carried out in triplicates. No inhibiting effect was observed. Concentration 3 was determined as the maximum concentration and should not be exceeded.

Substances	Conc. 1	Conc. 2	Conc. 3
Bovine Serum Albumin (BSA)	0.1 mg/ml	0.2 mg/ml	0.4 mg/ml
EDTA	0.2 mM	0.6 mM	1.0 mM
Tris buffer	2 mM	6 mM	10 mM
Sodium chlorite (NaCl)	5 mM	15 mM	25 mM
Isopropanol	0.3% v/v	0.9% v/v	1.5% v/v
Ethanol	0.3% v/v	0.9% v/v	1.5% v/v

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 mandatory. No fluorescence signal should be detected in the negative control with Aqua dest. ($C_q > N.A.$). 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.

Since cell-free fetal DNA is only present in very low concentrations in maternal plasma, extraction is a critical step in the analysis. It should be ensured that sufficient fetal DNA can be obtained with the extraction method.

The test is not suitable for samples collected before 11 weeks of gestation.

Improper use or PCR inhibitors may lead to false results. The residual risk of a negative test result in an RDH-positive fetus cannot then be excluded.

Rare clinical subtypes may lead to non-detection of the corresponding exon for individual exons. However, this has no influence on the overall analysis of the multiplex mix of the three exons.

Trisomy 21 or trisomy 18 of the fetus may influence the concentration of cffDNA. A twin pregnancy increases the cffDNA concentration (Zhou et al., 2015).

In rare cases, an RHD-negative phenotype may be associated with RHD inactivation due to genetic variations (e.g. point mutations). Thus, a serological RHD-negative mother/fetus may test positive for single exons of the RHD gene. The results should be interpreted in context with other laboratory data and clinical parameters.

10. INTERNAL QUALITY CONTROL

Internal quality controls for new lots are performed using a combination of DNA samples with known type. An internal amplification control (IAC) to verify successful amplification is included in each test. The inclusion of negative controls to detect possible contamination is mandatory. For this purpose, a test without cffDNA is set up (NTC), see item Amplification (6.3).

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. Pay attention to correct pipetting. Inappropriate time of sampling (WP).
	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. Repeat cffDNA isolation.
	Degraded fluorescent probes or primers.	Use fresh Primermix. 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.
Signal in the negative control	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.
	Contamination with cffDNA or DNA in the negative control.	Repeat the test. Decontaminate the workplace.






12. TRADEMARKS USED IN THIS DOCUMENT/PRODUCT

TaqMan® is a trademark of Roche Molecular Systems Inc.

FrameStar® is a trademark of Azenta Life Sciences.

QIAamp®, MinElute® and QIASymphony® are trademarks of Qiagen.

13. EXPLANATION OF SYMBOLS USED ON THE LABELS

	Sufficient for n tests
	Storage temperature / Upper limit of temperature
	Consult instructions for use
	Manufacturer
	Use by
IVD	For in vitro diagnostic use
eIFU	Electronic Instruction for use
FETAL TYPING	Intended use: Test kit for the analysis of cell-free fetal DNA from maternal blood plasma.
REF	Catalogue number
CONT	Contens, contains
LOT	Batch code
Q PRIMERMIX RHD fetal plus	Liquid Primermix (specific primers and probes and HGH-specific control primers and probes).
Q MASTERMIX fetal	Mastermix, contains dNTPs, Taq polymerase, reaction buffer

14. LITERATURE

Gemeinsamer Bundesausschuss. Richtlinien des Gemeinsamen Bundesausschusses über die ärztliche Betreuung während der Schwangerschaft und nach der Entbindung (Mutterschafts-Richtlinie), 2020

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