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INSTRUCTION FOR USE

FastQ® cff control

Test kit for the detection of a fetal marker as a control for cell-free fetal DNA in maternal blood plasma

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

CE IVD

REF 728214 FastQ® cff control

Version: 1 / 2022 / Issue: 2022-05

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1. INTENDED USE

The intended purpose of the FastQ® cff control kit is to detect cell-free fetal DNA (cffDNA) in maternal plasma using a fetal marker that is present in approx. 50% of the fetuses. This kit should be used in addition to the FastQ® RHD fetal kit which is intended for the analysis of the fetal rhesus D status.

2. PRODUCT DESCRIPTION

The FastQ® cff control enables the non-invasive detection of cffDNA. The detection of a fetal marker, which is only present in about 50% of the samples, included in the kit, can reduce the number of tests to be repeated by about 50%, as it acts as a specific control of the fetal DNA. Thus, in about 50% of the cases that obtain a false-negative or a questionable result in the detection of the RHD exons, but a correct-positive result in the IAC (HGH gene), an insufficient amount of cffDNA can be excluded. Therefore, in addition to the detection for RHD exons 5, 7 and 10 and the internal control for the detection of the maternal and fetal fraction (via the HGH gene) with the FastQ® RHD fetal kit, it is recommended to add the detection of the fetal marker with the FastQ® cff control kit as a fetal control.

The kit can be used from the 11th week of pregnancy (WP) in singleton pregnancies. In case of negative results from samples with WP ≤ 20, the result is to be considered preliminary and the test must be repeated from the 20th WP. The FastQ® cff control 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 were specifically designed for selective amplification of the 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 reaction.

4. MATERIAL

4.1 Content of the kits

- 1x 475 µl Q Mastermix fetal, ready to use, containing dNTPs, Taq Polymerase, reaction buffer. Is available in vials in liquid form.
- 1x 200 µl Q Primermix cff control for the molecular genetic analysis of the fetal marker. The reaction mix contains specific primers and probes 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

Cyclers	RT-PCR reaction tubes	RT-PCR closing system
CFX96 Touch™ & CFX Opus 96 Real-Time PCR Detection System Comp. Bio-Rad	FrameStar® Breakable Vertically PCR Plate, Low Profile Product No. 4ti-1201 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
	Removable 8 Well PCR Tube Strip, Product No. 4ti-0753 Comp. Azenta Life Sciences	

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

4.4 Recommendations for not validated cyclers and reactions tubes

Cyclers	RT-PCR reaction tubes	RT-PCR closing system
LightCycler® 480 II Real-Time PCR Detection System, Comp. Roche Molecular Systems Inc.	LightCycler® 480 Multiwell Plate 96, white, No. 04729692001 Comp. Roche Molecular Systems Inc.	Opitical foil, included in the LightCycler® 480 Multiwell Plate 96
	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 Comp. Azenta Life Sciences

Note: For the mentioned cycler initial tests have been performed but no full validation. The table contains the recommended specifications.

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. 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 cff control 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 cff control
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:

- With the CFX96 Touch™ & CFX Opus 96 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 not validated 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	610	Texas Red	1	10	1

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 a fluorescence signal in the Texas Red channel of the internal control.

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

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 fetal marker result is negative if 1 or no signal is detected when testing the triplicates. 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 when testing the triplicates.

The following fluorophores are used in the FastQ® cff control kit:

Specificity	Fluorophores	Cq-Level	Inspect	Wave length in nm*
Internal amplification control (IAC)	Texas Red	≤ 30	≥ 30	Excitation: 590 Emission: 610
Fetal marker	FAM	≤ 30	≥ 30	Excitation: 495 Emission: 520

* Data according to the synthesis lab

The following table shows the interpretation of the amplification results.

Texas Red (IAC)	FAM (Fetal marker)	Interpretation
Positive	At least 2 out of 3 replicates positive	Positive: Detection of the fetal marker.
Positive	1 out of 3 replicates positive	Negative, repetition is recommended.
Positive	Negative	Negative: No fetal marker was detected. The marker is not present or the sample does not contain a sufficient number of copies of cffDNA.
Negative	Negative	Invalid: Invalid result due to real-time PCR inhibition, reagent failure or unsuitable test material.

Notes:

The predefined Cq value of ≤ 30 applies to the use of the CFX96™ & CFX Opus 96 Real-Time PCR Detection System together with the corresponding PCR tubes and closing systems (see 4.3). The value also applies to the non-validated LightCycler® 480 II real-time PCR detection system together with the corresponding PCR tubes and closing systems (see 4.4).

The amplification signal of samples negative for the fetal marker must be outside the defined Cq value for the specific colour channel.

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 analysis to control for human fetal cffDNA 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 reference material 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 the fetal marker.

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

$R^2 = 0.9959$

8.3 Clinical Evaluation

For the FastQ® cff control 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.

		Reference test	
		Positive	Negative
FastQ® cff control	Positive	46	5
	Negative	8	48

Diagnostic specificity: 90.6%
Diagnostic sensitivity: 85.2%

8.4 Interfering substances

The influence of potentially interfering substances was tested with a sample positive for the fetal marker 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. (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.

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 results should be interpreted in context with other laboratory data and clinical parameters.

10. INTERNAL QUALITY CONTROL

Internal quality controls for new lots are carried out with DNA samples that react positively for all test characteristics. We recommend to use the above mentioned refence sample for this purpose as well. An internal amplification control (IAC) to verify successful amplification is included in each reaction test. The inclusion of negative controls to detect possible contamination is mandatory. For this purpose, a test without cffDNA is prepared (NTC), see point 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 the 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 .
	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 Azenta Life Sciences.

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

13. EXPLANATION OF SYMBOLS USED ON THE LABELS

	Sufficient for n tests
	Consult instructions for use
	Storage temperature / Upper limit of temperature
	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 | cff control	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

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