NIMoTest®Fetal RHD qPCR kit 96

For the determination of fetal RHD genotype from maternal plasma

Instructions For Use

For *in vitro* use

Réf: NIMRHDQ96

Store ≤ -20°C

Updates:

Version 1:	March 2021. Establishment
Version 2:	April 2021. Completion of logos, of limitations of use and performances
	Modification of the volume of extraction control (EC)
	Addition of qPCR software versions
Version 3:	July 2021. Removal of analysis software related items
Version 4:	October 2021. Claim for the M2000rt qPCR device (Abbott)
	Rewording the test performance
	Performance validation details
	Addition of storage temperature
Version 5:	December 2021. Replaced the preservation statement "at -20°C" with "≤ -20°C"
	Update of the "limitations of use" paragraph
Version 6:	December 2021. Rewording the results in patient with *RHD* gene
Version 7:	April 2022. Update of suppliers
Version 8:	September 2023. Replacement of the illustration on page 1
	Addition of fidelity results
	Update of general information
	Addition of the statement "The test is not automated."
	Addition of the EC diluent
	Consistency between §6 and 10.d
	Editing the examples in the annex

Content

1. Key to symbols 5
2. Kit content 5
3. Shipping and storage 6
4. Warnings and precautions 6
5. Intended use 6
6. Limitations of use 7
7. Principle 7
8. Equipment 8
a. Equipment required but not supplied 8
b. Validated devices 9
9. Sample collection and preparation 9
a. Plasma samples 9
b. cffDNA extraction 9
10. Protocol 10
a. Sample setup sheet 10
b. PCR preparation 10
c. Thermal cycler configuration and data analysis 11
d. Results interpretation 12
11. Test performances 14
a. Limit of detection (LOD) 14
b. Fidelity 14
c. Diagnostic sensibility and specificity 15
12. References 16
13. Troubleshooting guide 16
14. General information 19
15. Appendices 20
a. Overview of an experiment 20
b. Interpretation examples 21

Warning

Despite our efforts to validate the information in this notice, if you find information in this manual that is incorrect, misleading, or incomplete; we would appreciate your comments and suggestions. Please send them to support@bedia.fr.

Any serious incident related to this device will be notified to the competent authority (ANSM).

1. Key to symbols

ELEMENTS	SYMBOL
Legal Manufacturer	
Catalogue number	REF
Batch code/Lot number	LOT
Use-by date	
Contains reagents for n tests	Σ
Consult instructions for use	www.bediagenomics.com
In vitro diagnostic device	IVD
CE marked	CE
Warning	
Light sensitive	
Storage temperature	-20°C

2. Kit content

The NIMoTest®Fetal RHD qPCR kit 96 is a sealed kit containing all the reagents for the determination of fetal *RHD* genotype from maternal plasma. Twenty-one plasmas can be analyzed in one or two experiments. All reagents are ready to use, they can undergo two freeze/thaw cycles. The test is not automated.

Included reagents

	Size	Feature
Extraction Control	145 µl	1 vial - red cap
Reaction Mix Extraction Control	450 µl	1 vial - blue cap
Reaction Mix *RHD*	2 x 675 µl	2 vials - green cap
Positive Control	45 µl	1 vial - purple cap
Negative Control	45 µl	1 vial - black cap
Water	100 µl	1 vial - colourless cap

3. Shipping and storage

- The NIMoTest®Fetal RHD qPCR kit is shipped on dry ice and should be stored ≤ -20°C upon arrival. Contact us if the cold chain has been compromised.
- The kit is stable until the kit expiration date (kit label) when stored ≤ -20°C.
- Products should be kept in the dark as much as possible when in use.

4. Warnings and precautions

Positive and negative controls are human DNAs. Samples were tested negative for HBV (Hepatitis B Virus), HCV (Hepatitis C Virus), and HIV 1 and 2 (Human Immunodeficiency Virus 1 and 2).

All biological and blood samples should be treated as possibly infectious.

Handling, use, storage and disposal of biological samples and kit compounds must be made according to local regulations.

The wearing of personal protective equipment (PPE) is mandatory during laboratory work.

Material safety data sheets (MSDSs) are available from www.bediagenomics.com.

5. Intended use

This kit is intended for specialized medical biology laboratories holding an approval for prenatal biological diagnosis. Therefore, it can only be used by qualified medical professionals. It allows the determination of the *RHD* fetal genotype from a maternal plasma. Its use is part of the monitoring of pregnant women in the following indications:

- to target RhD-negative (RH:-1) pregnant women that should benefit from anti-RhD immunoglobulins.
- In case of immunisation, to select RhD-negative women who should benefit from a specialized follow-up.

6. Limitations of use

- The assay must be performed on cell-free DNA extracted from fresh or frozen human plasma prepared from EDTA-blood.
- Samples should be taken from 11 weeks of amenorrhea.
- Do not used plasma from a hemolyzed blood sample.
- A negative RHD genotype, observed on a sample coming from a 18th gw blood sample must be considered as provisional. Result must be validated with a second genotyping performed on blood sampled at least 2 weeks after the first sample, and after 16th gw, to avoid false negative results.
- Do not use the reagents after their expiry date.
- After opening, the kit can be stored up to 6 months ≤ -20°C.
- Do not mix reagents from different batches.
- The user must validate any modification of the present procedure.
- The equipment must be validated according to the regulation of molecular biology laboratories.
- The qPCR multiplexes are not designed, optimized, or validated for a quantitative analysis of *RHD* gene.

7. Principle

The presence of a small amount of cell free fetal DNA (cffDNA) in maternal plasma was reported in the late nineties (Lo et al. 1997) opening up many new perspectives for Non-Invasive Prenatal Diagnosis (NIPT). Pre-analytical steps were described as critical (Clausen et al. 2013, Repiska et al. 2013, Legler et al. 2007). The use of K2-EDTA tubes with plasma separation within 48 hours is today the subject of a broad consensus, even if a delay of up to three or even five days is proposed by some studies (Wilkman et al. 2012, Müller et al. 2011).

It is possible to identify the fetal *RHD* gene in the DNA extracted from the plasma of a RhD-negative mother. Indeed, in the Caucasian population the RhD-negative phenotype is almost exclusively linked to a homozygous deletion of the *RHD* gene. Thus, the detection of *RHD* gene in maternal plasma reveals a *RHD* positive fetal genotype allowing the implementation of targeted antenatal prophylaxis (Kent et al. 2014). Indeed, to know the fetal *RHD* status make possible to avoid unnecessary administration of anti-RhD immunoglobulin and to adapt the monitoring of the pregnancies of RhD-negative allo-immunized women.

Our test uses real time PCR (qPCR) to detect in a duplex reaction both exons 5 and 7 of *RHD* gene. A triplicates amplification for each plasma sample produced six results for *RHD* gene (three for each exon).

Primers and probes are specific of *RHD* gene and were shown not to amplify the *DIIIa-CE(4-7)-D* and *D-CE(4-7)-D* silent alleles found in *(C)ces type 1* and *(C)ces type 2* haplotypes respectively. In the presence of *RHD*RHDpsi* allele, only exon 7 is amplified. The probe specific of *RHD* exon 5 is labelled with 6-FAM (6-Carboxyfluorescein) while the probe targeting exon 7 is labelled with Yakima Yellow®.

The kit provides several controls:

- Water: to check the lack of contamination during handling procedure
- Negative control: DNA negative for exons 5 and 7 of *RHD* gene used to verify the specificity of primers and probes
- Extraction control: to validate the extraction process and check the lack of PCR inhibitor. This small size DNA is spiked into plasma sample and is measure by qPCR after DNA extraction
- Positive control: DNA positive for exons 5 and 7 of *RHD* gene used to verify PCR efficiency

Expected results for fetal genotyping in function of the maternal and fetal RHD genotypes

***RHD* genotype**		**Expected result [$]**			**Interpretation[§]**
Mother	**Fetus**	**Extraction Control**	**Exon 5**	**Exon 7**	
RHD deletion* *(C)ces type 1* *(C)ces type 1*	*RHD*	++	+	+	Positive fetus
	Deletion	++	-	-	Negative fetus[£]
	RHDpsi	++	-	+	Positive fetus as a precaution
	(C)ces type 1 ou 2	++	-	-	Negative fetus[£]
	Other RHD variant	++	+ -	- +	Positive fetus as a precaution
RHDpsi	*RHD*	++	+	++	Maternal *RHD* suspected
	Deletion	++	-	++	Maternal *RHD* suspected
Other *RHD* variant		++	++	++	Maternal *RHD* suspected

[§] See details in paragraph 10.d

[$] Negative result (-), positive result (+), positive result with early Ct/Cp (++)

* Most frequent mechanism (>99% in Caucasian)

[£] If sample was taken <18wa, this result must be verified on a second maternal blood sample taken two weeks later

8. Equipment

a. Equipment required but not supplied

General equipment:

- Calibrated micropipettes and tips with hydrophobic filters
- Micro centrifuge
- Vortex
- Microtiter plate centrifuge

Specific equipment:

- Extraction system for cell-free fetal DNA (cffDNA). The approved equipment is listed in paragraph 8.b.

- qPCR machine with filters for 6-FAM (abs. 495nm / em. 517nm) et du Yakima Yellow® (abs. 530,5nm / em. 549nm), optical reaction plates and optical adhesive seals. Devices must be calibrated according to the supplier's recommendations.

b. Validated devices

Cell-free fetal DNA extraction:

The extraction has been validated on automated systems using either column or magnetic beads techniques. For each system, the extraction was carried out on a 1 ml of plasma with an elution volume of 50µl.

- Column procedure: LabTubo-24 together with 24C-LVX480-1000 extraction kit (Taigen, LabTurbo Biotech Corp.)
- Magnetic beads procedure:

    MagPurix together with CFC DNA extraction Kit (Zinexts)

    MagNA Pure 24 and MagNA Pure 96 together with MagNA Pure 24 total NA isolation kit and MagNA Pure 96 DNA and viral NA large volume kit respectively (Roche Life Science)

    Chemagic 360d together with NextPrep-Mag cfDNA Automated Isolation kit (Perkin Elmer)

Amplification:

The validated qPCR devices are

QuantStudio 7 (Life technologies)	software version 1.3
qTOWER^3 (Analytic Jena AG)	software version 3.4
LC480 (Roche Life Science)	software version 1.5.1.62
M2000rt (Abbott)	software version 8.1.9.0

9. Sample collection and preparation

a. Plasma samples

The assay is validated for the analysis of fresh or frozen human plasma. Blood samples collected on K2-EDTA were stored at room temperature and processed within 72 hours. Plasma samples were obtained by centrifugation of the whole blood at room temperature for 10min at 1,700g. The supernatants were aliquoted and frozen to -20°C in polypropylene tubes.

b. cffDNA extraction

Thaw an aliquot of plasma sample to be tested.

Thaw the **Extraction Control** (red cap) mix thoroughly and centrifuge briefly.

Add 5µl of extraction control in each aliquot of 1ml of plasma, mix thoroughly, and centrifuge briefly.

ⓘ *A dilution of the EC can be needed for its distribution by an automated system. In such a case, it can be extemporally diluted in molecular biology grade water or in 1X PBS without Ca & Mg.*

Process to cell free fetal DNA extraction according to the validated procedure.

ⓘ *DNA extraction was validated on automated systems using either column or magnetic beads (see §8). DNA was extracted from 1ml of plasma with an elution volume of 50µl*

10. Protocol

a. Sample setup sheet

Prepare a sample setup sheet following the example on the next page.

b. PCR preparation

Set up all reactions in a pre-PCR lab under ambient conditions.

Thaw reagents under ambient temperature and protected from light. Briefly vortex and then shortly centrifuge all tubes before opening to ensure that the solution is well mixed and at the bottom of the tube.

ⓘ *Limit the exposure of reagents to light and the time they are kept at room temperature. Store the kit ≤ -20°C and in the dark immediately after use.*

Dispense 15µl of the appropriate PCR mix into the wells as indicated by sample setup sheet.

- The ***Reaction Mix Extraction Control*** (blue cap) is dispatched to the wells of columns 1, 5 and 9.
- The ***Reaction Mix RHD*** (green cap) is dispatched to the wells of columns 2 to 4, 6 to 8 and 10 to 12.

Then dispense 5µl of DNA in accordance with the sample setup sheet shown below:

- Controls included in the kit are dispatch to wells A1 to A4 for Water (colorless cap), to wells B1 to B4 for **Positive Control** (**PC**, purple cap) and to wells C1 to C4 for **Negative Control** (**NC**, black cap). These controls must be included in all experiments
- The plasma samples are dispatch as follow: wells D1 to D4 for the first sample, E1 to E4 for the second, and so on.

Cover and seal the plate with optical adhesive film, making certain that all wells are completely sealed. Centrifuge the plate briefly to collect the contents at the bottom of the wells (500g for 30 secondes).

Example of sample setup sheet

	1	2	3	4	5	6	7	8	9	10	11	12
A	W	W	W	W								
B	PC	PC	PC	PC								
C	NC	NC	NC	NC								
D	♀1	♀1	♀1	♀1								
E	♀2	♀2	♀2	♀2								
F	♀3	♀3	♀3	♀3								
G	♀4	♀4	♀4	♀4								
H												

Reaction mixes (15µl/well)

Extraction Control

RHD

DNA (5µl/well)

W Water (*colorless* cap)

PC Positive Control (**purlpe** cap)

NC Negative Control (**black** cap)

♀1 plasma 1

♀1 plasma 2…

c. Thermal cycler configuration and data analysis

Record the position and ID of the controls and samples in the qPCR software.

Load the plate into your qPCR machine and run conditions as described below.

Step	Number of cycles	Temperature (°C)	Time (min:s)
Initial denaturation	1	95	03:00
Cycles	46	95	00:10
		60	00:30*

* Fluorescences are recorded at the end of this step

QuantStudio 7 (Life technologies)

Ramping 1,6°C/second (default value)

Acquisition in 6-FAM and YY channels

Set ROX as passive reference

qTOWER³ (Analytic Jena AG)

Ramping 1,6°C/second

Acquisition in 6-FAM, YY and ROX channels

Set ROX as passive reference

Set the Gain to 3 for each of the fluorophores

LC480 (Roche Life Science)

Ramping 1,6°C/second and cooling 2,2°C/second

Acquisition in 6-FAM and YY channels

M2000rt (Abbott)

Acquisition in 6-FAM and YY channels

Set ROX as passive reference

Fluorescence is collected at the end of the hybridization/elongation step (step scored with * in the above table). The amplification of extraction control is monitored with Yakima Yellow®. The amplifications of *RHD* exons 5 and 7 are revealed with 6-FAM et Yakima Yellow® fluorophore respectively. ROX can be use as passive reference if required (master mixes contain low ROX).

After the qPCR run is finished, set the threshold. This can be done automatically on most qPCR software packages. For qTOWER³ device set the threshold to 10 for 6-FAM and 5 for YY. For QS5/7 and LC480 devices automatic threshold can be used.

Perform a visual check of the amplification curves to ensure that none of them have an aberrant profile and, if necessary, exclude the affected wells.

d. Results interpretation

Analysis and interpretation of the results is based on Ct values (Cycle threshold, also called Cp for Crossing point). A validation step of the experiment is carried out. Each plasma sample is thenafter validated thanks to the extraction control then the interpretation of the RH genotype is achieved.

Amplification of exons 5 and 7 of the *RHD* gene is performed in triplicate. The match of two results of a triplicate is sufficient to determine a result.

The Ct values above 41 are regarded as negative

Run Validation - Results of control samples (Water, Positive Control, Negative Control)

Expected results for each control well are reported in the table below. If the result of one of the control samples is inconsistent, the experiment is invalid and must be repeated.

Sample	Target*		Expected results£	
			Ct_{FAM}	Ct_{YY}
Water	EC	(well A1)	/	No Ct
	RHD	(wells A2/A3/A4)	No Ct	No Ct
Positive Control	EC	(well B1)	/	No Ct
	RHD	(wells B2/B3/B4)	$28 \leq Ct \geq 33$	$28 \leq Ct \geq 33$
Negative Control	EC	(well C1)	/	No Ct
	RHD	(wells C2/C3/C4)	No Ct	No Ct

*EC: Extraction Control; RHD: exons 5 (6-FAM) and 7 (YY) of *RHD* gene

£For at least two out of the three determinations

Sample validation - Results of Extraction Control (EC)

The extraction control is used to validate the extraction step and ensure the absence of PCR inhibitors in the extracted DNA. For each plasma sample, its Ct/Cp must be between 28 and 33. If this criterion is not fulfilled for a sample, the sample is rejected, and no interpretation can be made. The analysis of that sample should be repeated starting from a new plasma aliquot.

RHD interpretation - Results of RHD gene exons 5 and 7 amplification and comparison of Ct/Cp to those of the extraction control (EC).

The four representative results are:

- ✓ "RHD negative fetus": no amplification of exons 5 and 7; in the case of a first determination on a sample taken before 18wa, the result must be confirmed on a new sample of maternal blood in two weeks.
- ✓ "RHD-positive fetus": amplification of exons 5 and 7.
- ✓ "Probable *RHD* variant to be considered as positive as a precaution": discrepancy between the results of exons 5 and 7 which may indicate the presence of a fetal RHD variant allele or a small amount of fetal DNA, a technical issue cannot be rule out. In compliance with the recommendations of the *Haute Autorité de Santé* (HAS, 2011), a verification on a second sample must be performed prior to a definitive conclusion.
- ✓ "Maternal allele": early amplification of at least one *RHD* exon which usually indicates the detection of a maternal allele or may be associated with a significant amount of fetal DNA. The presence of a maternal allele is not inconsistent with an RHhD-negative phenotype (silent allele). It can also be observed in the context of an improper indication for fetal genotyping (allele encoding a weak, partial or Del RhD phenotype). The result "Maternal allele" is given independently of the fetal genotype. As the fetal genotype cannot be determined, the fetus should be considered positive as a precautionary measure. Verification of the patient's phenotype and determination of her RHD genotype are both recommended. Confirmation of the fetal genotype on a second sample must be performed before a definitive conclusion is reached (HAS, 2011).

Various results might be encountered during the second fetal genotype determination (confirmation of the results "Probable *RHD* variant to be considered as positive as a precaution" and "Maternal allele")

- detection of both exons of the RHD gene and the result is "RHD-positive fetus”

- result remains "Probable RHD variant to be considered positive as a caution" or "Maternal allele" and the patient should be considered at risk of maternal-fetal alloimmunization

- the absence of amplification of both exons of the *RHD* gene should not be seen. This would suggest contamination of the sample at the time of the first determination. However, if this is the case, we recommend that the patient be considered at risk for maternal-fetal alloimmunization

The table below collates the results of exons 5 and 7 of the RHD gene and associated results

***RHD* exon 5 (6-FAM)**		***RHD* exon 7 (YY)**		**Result**
Ct	**Δ Ct ($Ct_{exon\ 5}$ - Ct_{EC})**	**Ct**	**Δ Ct ($Ct_{exon\ 7}$ - Ct_{EC})**	
No Ct	-	No Ct	-	*RHD* negative fetus*
		26 ≤ Ct ≤ 34	<2	Maternal allele
		30 ≤ Ct ≤ 41	≥ 2	Probable *RHD* variant to be considered as positive as a precaution
26 ≤ Ct ≤ 34	<2	No Ct	-	Maternal allele
		26 ≤ Ct ≤ 34	<2	Maternal allele
		30 ≤ Ct ≤ 41	≥ 2	Maternal allele
30 ≤ Ct ≤ 41	≥ 2	No Ct	-	Probable *RHD* variant to be considered as positive as a precaution
		26 ≤ Ct ≤ 34	<2	Maternal allele
		30 ≤ Ct ≤ 41	≥ 2	*RHD* positive fetus

* Result to be confirmed on a new maternal blood sample to be taken in two weeks' time

For other configurations, the results are not interpretable, the analysis must be repeated from a new plasma aliquot

An example of interpretation is given in the appendix

11. Test performances

a. Limit of detection (LOD)

Tests were performed on RhD-positive plasma diluted in RhD-negative plasma. The detection limit is set at 1/64 with 100% of samples detected (vs. 42% at 1/100 dilution). The mean Ct for exon 5 and exon 7 amplifications are 36 and 37 respectively.

b. Fidelity

Assay fidelity is assessed by measuring the repeatability, intermediate fidelity, and reproducibility of the extraction control, *RHD* exon 5, and *RHD* exon 7 amplifications. The coefficients of variation calculated for each target are reported in the table below.

	Coefficient of variation (CV)		
	Répétabilité	**Fidélité intermédiaire**	**Reproductibilité**
EC	2,03%	3,86%	4,95%
RHD exon 5	2,75%	4,47%	8,38%
RHD exon 7	1,96%	3,70%	8,17%

c. Diagnostic sensibility and specificity

In a first study, the test was evaluated on 23 patient samples collected between 11 and 38 weeks of amenorrhea. DNAs were extracted with MagPurix® (Zynexts) using the MagPurix® CFC DNA Extraction Kit and amplification was performed on QuantStudioTM 7 (QS7, Thermo fisher) for all samples or on qTOWER3 (Analytik Jena) for 21 of the samples. Fifteen of these samples were also run on a QS7 after automated extraction with the LabTurbo column system (Taigen, LabTurbo Biotech Corp.) using the Viral DNA:RNA mini kit and amplification. This study was performed without confirmation of results. The results were compared to the reference test with a concordance of 96.6%. One sample negative with the reference test is identified as a "Probable *RHD* variant to be considered as positive as a precaution" due to the amplification of exon 7 in the absence of detection of exon 5.

No false negative results, defined as the absence of amplification of both exons 5 and 7 of the RHD gene, were found in the samples assigned positive by the reference test. The sensitivity of the test was 100% and its specificity 94.69%.

A second multicenter study was performed on three sites. Two hundred samples collected between 8 and 34 weeks of amenorrhea (WA) were processed as follow:

- Extraction on MagNA Pure 24 (Roche) with the MagNA Pure 24 total NA isolation kit (Roche) and amplification on LC480 (Roche), n= 66

- Extraction on Chemagic 360d (Perkin Elmer) with NextPrep-Mag cfDNA Automated Isolation Kit and amplification on M2000rt (Abbott), n=65

- Extraction on MagNA Pure 96 (Roche) with MagNA Pure 96 DNA and viral NA large volume kit (Roche) and amplification on LC480 (Roche), n=69

Results were compared with those of the reference test. No false positive results were observed among the 33 negative samples.

One hundred and sixty-seven samples are positive with the reference test. One hundred and sixty-five of them are identified as positive with the NIMoTest®Fetal RHD qPCR kit. The two remaining, collected at 18 and 21 WA were discordant. For discordant samples, an amplification of the RHD exon 5 is present in one of the three wells, the same applies to the amplification of exon 7.

These results were used for calculating the diagnostic sensitivity and specificity of the test, which are 98.80% and 100% respectively.

Samples of two patients with an RHD gene were intentionally included in this evaluation:

- One sample from a patient carrying a *RHD*08N.01* (*RHD*ψ*) allele was analyzed. The reference test indicates an indeterminate fetal RHD genotype due early amplification of exons 7 and 10 and no amplification of exon 5. The NIMoTest®Fetal RHD qPCR kit reports "Maternal allele" owing to amplification of exon 7 with a Ct of about 31 (Ct of extraction control is 32) in the absence of amplification of exon 5.
- A sample from an Afro-Caribbean woman was analyzed. The reference test indicates a positive fetal *RHD* genotype with amplification of both exons 5 and 7 of *RHD* in the presence of early

amplification of exon 10. The NIMoTest®Fetal RHD qPCR kit report "RHD positive fetus" as a result of amplification of *RHD* exons 5 and 7.

These results lead to the implementation of prophylactic procedures according to the clinical context.

12. References

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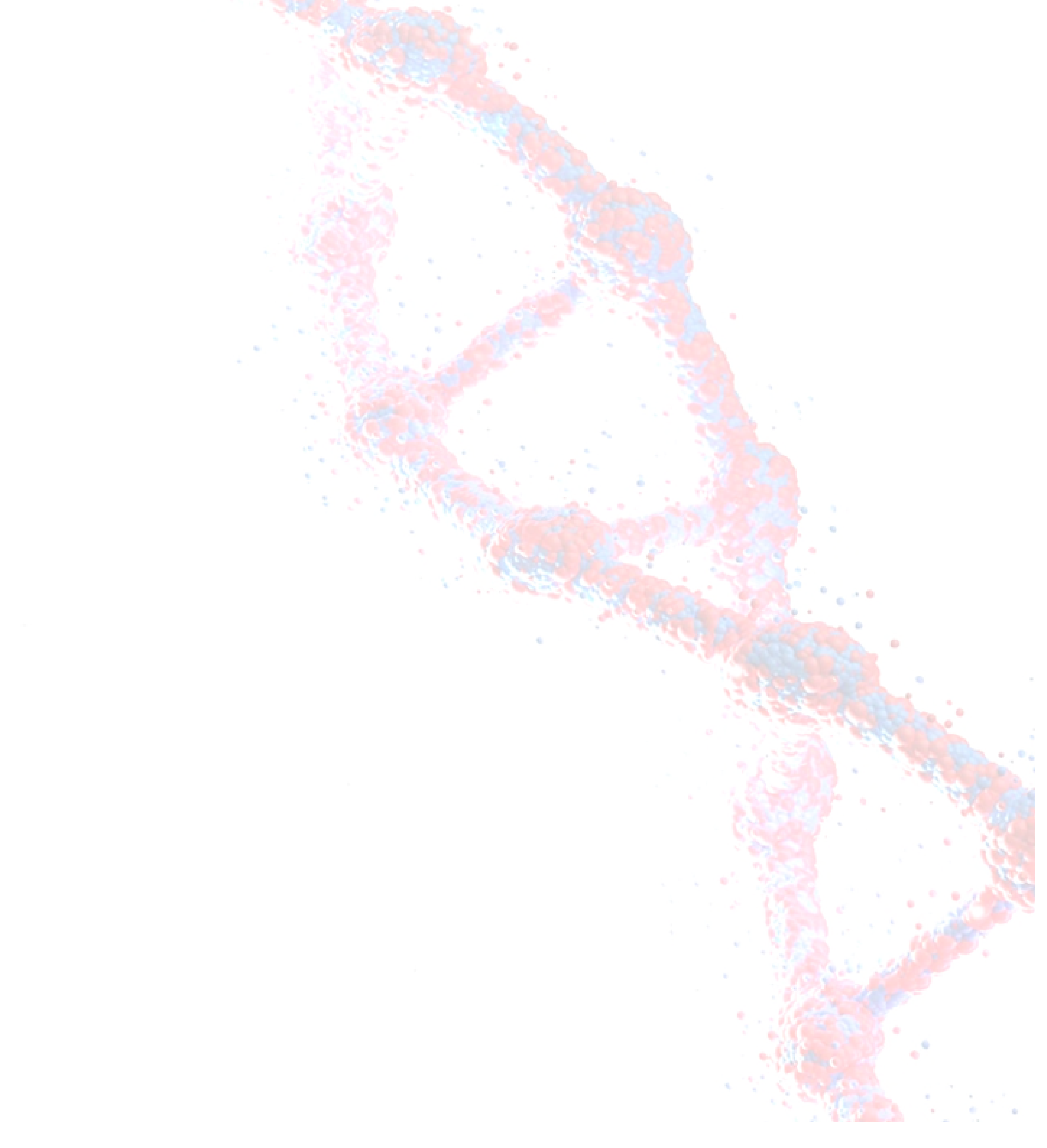
13. Troubleshooting guide

No fluorescence signal	
Incorrect thermal cycler program	Check the time and temperature set
	Check the selected reading channels
	Verify that fluorescence acquisition is performed at the end of the hybridization/extension step
Reagent degradation	Check expiration date and discard expired kits

	Check the kit correct storage
Supplies not suited for the qPCR device	Check that qPCR plates and optical seals are suitable for the device
Water control is positive	
Reagent degradation	Repeat the experiment with new reagent aliquots
	Check storage and handling conditions
	Discard contaminated reagents
Reagent contamination	Check storage and handling conditions
	Discard contaminated reagents
Pre-PCR area contamination	Check that good laboratory practices are followed in the pre-PCR area
	Check for possible contamination problems in other PCR techniques
Pipetting error	Always check that the added sample matches the sample setup sheet
Negative control (NC) is positive	
Cross contamination	Handling the kit compounds following good laboratory practices
Pipetting error	Always check that the added sample matches the sample setup sheet
Positive control (PC) is negative	
Pipetting error	Always check that the added sample matches the sample setup sheet
Degradation of positive control	Check the control DNAs correct storage (≤ -20°C)
Low or no signal for extraction control amplification in all samples while control samples are OK	
Issue with the DNA extraction run	Perform DNA extractions again using new plasma aliquots
Pipetting error of the extraction control (EC)	Check that the right volume of EC was added to plasma samples
Degradation of the extraction control (EC)	Check the extraction control correct storage (≤ -20°C)
Low or no signal for extraction control amplification in one sample while control samples are OK	
Issue with DNA extraction of one sample	Perform a new DNA extraction of the sample with another plasma aliquot

Pipetting error of the extraction control	Check that the right volume of EC was added to plasma sample
Signal strength is heterogeneous	
Poorly sealed plate	Check compatibility of qPCR plates and optical adhesive seals
	Ensure that the optical seal adheres securely to the qPCR plate
Presence of dirt on supplies (plates, seals)	Wear powder-free gloves when handling materials
Presence of air bubbles in the wells or reaction mix on the well side	Centrifuge the plate to ensure that the sample is at the bottom of the well and that there are no air bubbles.



14. General information

Manufacturer: BeDia Genomics
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78100 Saint Germain en Laye
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Phone : +33 (0) 4 42 12 38 82

For orders and quote request: contact your local distributor or info@bediagenomics.com.

For technical assistance and information: fill in the claim form available on our website (www.bediagenomics.com) and contact your local distributor.

Illustration photo ©[denisismagilov] /Adobe Stock

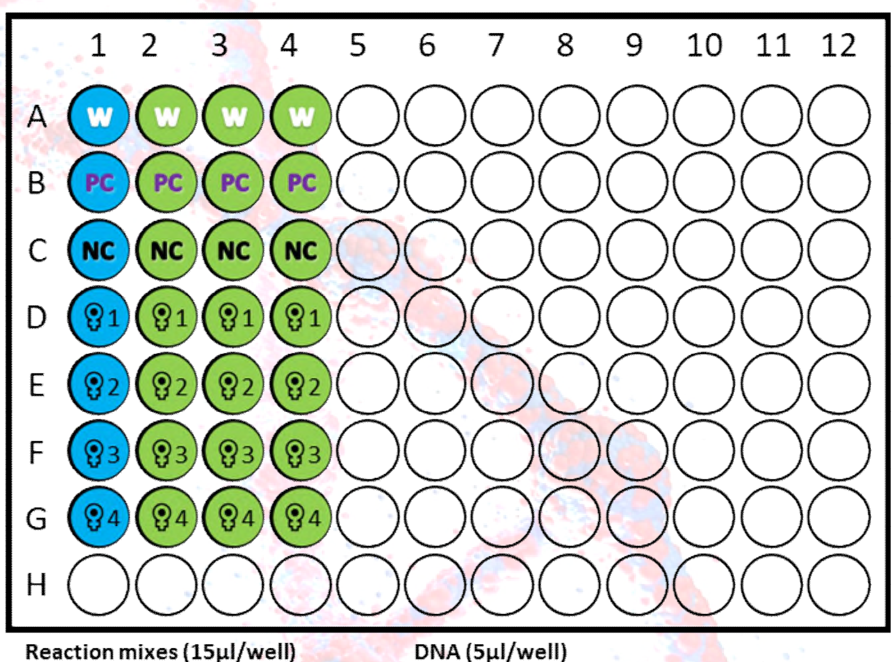
15. Appendices

a. Overview of an experiment

Preparation of cell free fetal DNA

- 1ml plasma + 5µl ***Extraction Control (EC)***
- Process to cell free fetal DNA extraction according to the validated procedure (elution in 50µl)

Plat setup according to the setup sheet



qPCR

- Protocol : 95°C, 3min – [95°C, 10 seconds ; 60°C, 30 seconds] x 46
  Ramping 1,6°C/second; cooling à 2,2°C/second (LC480); gain 3 (qTOWER³)
- Acquisition of 6-FAM (exon 5) and YY (exon 7 and EC) and ROX if required (QS5/7; qTOWER³)

Analysis and results interpretation

- Set threshold)
  Automatic (QS5/7; LC480) or 10 for 6-FAM and 5 for YY (qTOWER3)
- Visual check of amplification curves
- Validate the experiment and interpret the results by referring to § 10.d

b. Interpretation examples

Run validation based on the results of the controls (water, **Positive Control**, **Negative Control**) is not shown in this example.

Only the steps of *sample validation* (results of the extraction control, EC) and *interpretation of RHD results* (amplification of *RHD* exon 5 and 7 and comparison of Ct/Cp with those of EC) are presented (see tables in §10d).

VALIDATION: $28 < Ct_{EC} < 33$

INTERPRETATION: According to Table on page 13

Well	Sample ID	Target	Ct/Cp
D1	Sample 1	EC (YY)	30,18
D2	Sample 1	RHD5 (6-FAM)	36,76
D2	Sample 1	RHD7 (YY)	34,89
D3	Sample 1	RHD5 (6-FAM)	35,56
D3	Sample 1	RHD7 (YY)	35,24
D4	Sample 1	RHD5 (6-FAM)	35,98
D4	Sample 1	RHD7 (YY)	34,57

✓

Δ Ct (Ct_{RHD}-Ct_{EC})	Well Result	Sample Result
=36,76-30,18 = 6,58 =34,89-30,18 = 4,71	→ positive	
=35,56-30,18 = 5,38 =35,24-30,18 = 5,05	→ positive	**RHD positive fetus**
=35,98-30,18 = 5,80 =34,57-30,18 = 4,39	→ positive	

Well	Sample ID	Target	Ct/Cp
E1	Sample 2	EC (YY)	30,27
E2	Sample 2	RHD5 (6-FAM)	no Ct/Cp
E2	Sample 2	RHD7 (YY)	no Ct/Cp
E3	Sample 2	RHD5 (6-FAM)	no Ct/Cp
E3	Sample 2	RHD7 (YY)	no Ct/Cp
E4	Sample 2	RHD5 (6-FAM)	38,37
E4	Sample 2	RHD7 (YY)	no Ct/Cp

✓

Δ Ct (Ct_{RHD}-Ct_{EC})	Well Result	Sample Result
	→ negative	
	→ negative	**RHD negative fetus**
=38,37-30,27 = 8,10	→ probable RH	

Well	Sample ID	Target	Ct/Cp
F1	Sample 3	EC (YY)	33,20
F2	Sample 3	RHD5 (6-FAM)	no Ct/Cp
F2	Sample 3	RHD7 (YY)	no Ct/Cp
F3	Sample 3	RHD5 (6-FAM)	no Ct/Cp
F3	Sample 3	RHD7 (YY)	37,37
F4	Sample 3	RHD5 (6-FAM)	no Ct/Cp
F4	Sample 3	RHD7 (YY)	no Ct/Cp

✗

Sample 3 result : Result to be confirmed on a new maternal blood sample

Well	Sample ID	Target	Ct/Cp
G1	Sample 4	EC (YY)	29,44
G2	Sample 4	RHD5 (6-FAM)	31,17
G2	Sample 4	RHD7 (YY)	31,67
G3	Sample 4	RHD5 (6-FAM)	31,07
G3	Sample 4	RHD7 (YY)	31,63
G4	Sample 4	RHD5 (6-FAM)	31,11
G4	Sample 4	RHD7 (YY)	31,38

✓

Δ Ct (Ct_{RHD}-Ct_{EC})	Well Result	Sample Result
=31,17-29,44 = 1,74 =31,67-29,44 = 2,23	→ Maternal allele	
=31,01-29,44 = 1,64 =31,63-29,44 = 2,19	→ Maternal allele	**Maternal allele**
=31,11-29,44 = 1,67 =31,38-29,44 = 1,95	→ Maternal allele	