FetoGnost® Kit Control

Kit Version 1.2

Instructions for Use

REF **HUFG050**

Σ **50 reactions (up to 15 plasma samples)**

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Explanation of symbols

LOT	Batch code		Use by date
REF	Catalogue number		Manufacturer
Σ	Contains sufficient for <n> tests		Temperature limit (Store at)
	Consult instructions for use	UDI	Unique device identifier
	Keep away from sunlight	QTY	Contents

1 Intended Purpose

FetoGnost® Kit Control is a non-automated real-time PCR test for the qualitative detection of cell-free fetal DNA (cffDNA) in maternal plasma samples of pregnant women.
This test is suitable for women of all ages from the 12th week of pregnancy (gestational age ≥ 11+0) with singleton or multiple pregnancies. The test can be used both in a first pregnancy and in subsequent pregnancies.

The test is for research-use only and is exclusively intended for the validation of suitable extraction methods for the purification of cffDNA from maternal plasma.
The test is thus suitable for validating the extraction and for verification of the amount of cffDNA from human maternal plasma.
FetoGnost® Kit Control can be used with FetoGnost® Kit RHD (kit for qualitative detection of fetal RHD DNA) in one PCR run.

Contraindications:

- Not suitable for samples taken before gestational age ≥ 11+0.
- Not suitable for validation of extraction of cffDNA performed with extraction kits using carrier RNA at a concentration of > 0.5 µg/sample.

2 Product description

Fragmented DNA (< 150 bp, Qiao et al, 2019) of fetal origin (cffDNA, cell-free fetal DNA) represents only a small fraction of the circulating cell-free DNA (ccfDNA) present in maternal plasma and increases during pregnancy. In plasma samples from the second trimester of pregnant women, the concentration of cffDNA can reach 2-151 genome equivalents of cffDNA/ml of blood (Birch et al., 2005).

FetoGnost® Kit Control allows the non-invasive detection of cffDNA in samples purified from maternal plasma of pregnant women, based on real-time PCR technology. It serves as a control to ensure that enough cffDNA can be detected in extracted maternal plasma.

The detection of cffDNA by FetoGnost® Kit Control depends on a restriction enzyme digest of an aliquot of sample eluate to remove maternal DNA. The digested DNA sample is subsequently tested with real-time PCR. Probe-specific amplification curves in fluorescence channels for VIC, FAM and NED detect the amplification of two fetal markers, a maternal marker serves as an additional control.
When testing plasma samples in triplicates and controls in duplicates, the content of one kit allows the analysis of 15 plasma samples.

FetoGnost® Kit Control detects three genes:

- **Maternal Marker:** detection with VIC-labelled probe
  The Maternal Marker detects a region in maternal DNA which is digested with the restriction enzymes included in the kit. Therefore, it is not detected in digested plasma samples. The absence of this marker in the digested plasma sample guarantees that no amplifiable maternal DNA is present.
- **Fetal Marker 1:** detection with FAM-labelled probe
- **Fetal Marker 2:** detection with NED-labelled probe
  The Fetal Marker 1 and 2 detect two genes allowing a conclusion with respect to the presence of fetal DNA in the sample eluate. In fetal DNA these two genes are not digested. The Fetal Marker 1 must be detectable in the digested sample. The Fetal Marker 2 is detected in approximately 50 % of the fetuses.

FetoGnost® Kit Control has been developed for the ABI® 7500 instrument and QuantStudio™ 7 Pro (Thermo Fisher Scientific) - fast cycle parameters are not supported by this test. It is, however, also compatible with

other real-time PCR instruments which detect and differentiate fluorescence in FAM, VIC, NED, and ROX channels.
When using PCR-platforms not tested by ingenetix, an evaluation of the multiplex-PCR shall be done. Keep in mind that some PCR-platforms first must be calibrated with the corresponding dye before performing multiplex-PCR.

3 Principle of real-time PCR

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

4 Contents of the kit, stability, and storage

Table 1

Labelling	Content	Amount	Storage
FetoGnost® Kit Control Assay Mix (green cap)	Primers and probes for detection of - Maternal Marker (VIC) - Fetal Marker 1 (FAM) - Fetal Marker 2 (NED)	1 x 250 µl	-25 to -15 °C
FetoGnost® Kit Control Reaction Mix (white cap)	PCR reaction mix for DNA amplification	1 x 750 µl	**-25 to -15°C, after first opening 2 to 8 °C**
Nuclease-free water (blue cap)	Nuclease-free water	1 x 1000 µl	-25 to -15 °C
FetoGnost® Kit Control Restr. Enzymes (amber cap)	Enzymes for restriction digest	1 x 30 µl	-25 to -15 °C
FetoGnost® Kit Control Restr. Buffer (transparent cap)	Buffer for restriction digest	1 x 80 µl	-25 to -15 °C
FetoGnost® Kit Control Positive Control (red cap)	DNA Positive Control	1 x 300 µl	-25 to -15 °C

DNA Reaction Mix
The Master 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.

Delivery and Storage
Shipment is with coolpacks or on dry ice. When stored properly, the kit components are stable until the specified expiration date. This also applies after opening. Store kit protected from light.

Quality Control Release Testing
In accordance with the ISO 13485 certified Quality Management System of ingenetix, each lot is tested against predetermined specifications to ensure consistent product quality. Quality control is performed with a plasmid containing parts of the target DNA.

5 Additionally required materials and devices

- Reagents and devices for DNA-extraction which are appropriate for the extraction of cffDNA from maternal plasma (see 9. Preparation of the samples)
- Nuclease-free water
- Powder-free disposable gloves
- Pipettes (adjustable)
- Filter pipette tips
- Vortex-Mixer
- Benchtop centrifuge with rotor for 2 ml reaction tubes
- Real-time PCR instrument detecting and differentiating fluorescence in FAM, VIC, NED and ROX channel (see 2. Product description)
- Appropriate 96 well optical reaction plates or reaction tubes with corresponding (optical) closing material
- Optional: Laminar flow box
- Optional: PCR workstation

6 Precautions and safety information

6.1 General information

- The use of this kit is limited to qualified personnel instructed in real-time PCR.
- Transportation of clinical specimens must comply with local regulations for the transport of etiologic agents.
- Specimens should be handled as if infectious in accordance with safe laboratory procedures. Wear protective powder-free disposable gloves when handling kit reagents and specimens.
- Use established laboratory practices according to your local safety regulations for discarding specimens, reagents and waste disposal.
- Improper collection, transport or storage of specimens may hinder the ability of the assay to detect the target sequences.
- Quality of DNA has a profound impact on the test performance. Ensure that the used DNA extraction system is compatible with real-time PCR technology.
- Protect kit components from light.
- The real-time PCR instrument should be serviced and cleaned regularly.
- Do not mix reagents of different kits and lots and check expiry date of the kit.

6.2 Specific information

A workflow must be followed to avoid false positive results due to detection of contaminating DNA.

Recommended measures to avoid DNA contamination:

- Use separated working areas for specimen preparation, reagent preparation and amplification. Supplies and equipment must be dedicated to each of these separate working areas and ensure workflow in the laboratory from pre- to post-PCR.
- Laboratory benches and supplies must be cleaned regularly.
- Sample preparation should be performed in a laminar flow box. Clean laminar flow box regularly in all areas.
- Preparation of real-time PCR should be done in a PCR workstation.
- The use of sterile aerosol-resistant pipette tips is required.
- Use only DNA-free consumables.

- If possible, leave consumables and pipettes in the laminar flow box and PCR workstation.
- Wear lab coat.
- Work only with powder-free disposable gloves, do not touch the palm and fingers of the gloves on the outside when putting them on. Change gloves frequently. To avoid skin contact, wear gloves over the sleeves of the lab coat. Use disposable sleeve protectors if possible.
- Do not touch the rim or threads open vials.
- Care must be taken when handling specimens and positive control to avoid cross-contamination.
- Storage of positive and potentially positive material should be separate from all other reagents.
- For a valid interpretation of results, a negative control shall be included during DNA-extraction (e.g., extraction of water instead of sample material), to exclude false-positive results due to contamination with human DNA during extraction.

7 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 gestational 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).

8 Preparation of samples

FetoGnost® Kit Control has been validated for the detection of cell-free fetal DNA obtained from maternal plasma. The primary clinical specimen is whole blood from which plasma is separated according to standard procedures (see below).

8.1 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. Transport and store samples at approximately 10-25 °C, do not freeze the sample prior to plasma collection to prevent cell lysis.

Blood samples of gestational age ≥ 11+0 can be collected with different collection systems:

- EDTA blood collection tubes (recommended) with or without separating gel.
  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.

8.2 Plasma collection

Plasma must be separated within 6 days after sample collection (Clausen et al., 2013; Legler 2020; Müller et al., 2011).

<u>Conventional EDTA blood collection tubes</u>
Centrifuge the samples using the protocol established in your laboratory for plasma collection.

8.3 DNA extraction from plasma

Store plasma samples at approximately 10-25 °C. Plasma samples that are not extracted on the same day should be stored frozen (Londero et al., 2019; clinical trial validation data). After thawing, plasma must be extracted immediately.

DNA extraction can be performed manually or with automated extraction procedures suitable for the isolation of short DNA fragments (Legler et al., 2007, Yang et al., 2019). Please note that an **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.

Plasma from a haemolyzed blood sample must not be used for the analysis. Before nucleic acid extraction, a visual inspection for haemolysis must take place after centrifugation of the sample. In case of a haemolyzed sample, a new sample must be collected.

It is recommended to perform an evaluation of cffDNA extraction from maternal plasma with FetoGnost® Kit Control (HUFG050) to ensure that cffDNA can be obtained using the chosen extraction method.

Recommended extraction methods:

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 the 16th week of pregnancy, extraction of at least 1 ml of plasma is recommended. From ≥ 16 gestational age, a sample volume of 0.5 ml of plasma eluted in up to 100 µl is sufficient.

Elution

The elution volume should be selected so that 10 µl of eluate corresponds to at least 100 µl of plasma.

- Per ml plasma: elution in ≤ 100 µl.

DNA samples can be refrigerated for one day or stored long-term at -15 to -25 °C until analysis.

For manual extraction recommended

- QIAamp® DSP Virus Kit (QIAGEN) for 500 µl plasma. Add 1 µl FetoGnost® Kit IPC Target (orange cap)* to the lysis buffer. Elute in 60 µl.
- QIAamp Circulating Nucleic Acid Kit (QIAGEN) for 1-2 ml plasma. Add 1 µl FetoGnost® Kit IPC Target (orange cap)* to the lysis buffer. Elute in up to 100 µl.

For automated extraction recommended

- QIAsymphony DSP Circulating DNA Kit (QIAGEN) with the QIAsymphony® SP instrument using a QIAGEN adapted protocol for 1-2 ml plasma and elution in 80-100 µl. Per sample add 1 µl of FetoGnost® Kit IPC Target (orange cap)* to the required Proteinase K solution.
- QIAsymphony DSP Virus/Pathogen Midi Kit (QIAGEN) for 1 ml plasma with the QIAsymphony® SP instrument using a QIAGEN adapted protocol for elution in 80-100 µl. Add 3 µl of FetoGnost® Kit IPC Target (orange cap)* to the Carrier RNA per sample. Use 0.5 µg Carrier RNA per sample.
- chemagic cfDNA 2k Kit H24 (Perkin Elmer®) for 1-2 ml plasma with the Chemagic™ 360 instrument (Perkin Elmer®). To 1- 2 ml plasma add first Proteinase K, then Poly(A) RNA and the Lysis Buffer 1 followed by 1 µl FetoGnost® Kit IPC Target (orange cap)*. Elution in 80-100 µl.
- Mag-Bind® cfDNA Kit (Omega) for 1 ml plasma with the KingFisher instrument (Thermo Fisher Scientific) with 24-pin magnetic head using the protocol for cfDNA. Addition of 67 µl DSP, and 15 µl Proteinase K. After Proteinase K digestion, add 1 µl of FetoGnost® Kit IPC Target (orange cap)* to each sample. Elution in 80-100 µl.

*Component of FetoGnost® Kit RHD.

When using extraction methods not recommended by ingenetix, an evaluation of the extraction method must be performed.

8.4 Preparation of restriction digest

The restriction digest is performed using 35 µl of the respective eluate, the FetoGnost® Kit Control Positive Control (red cap) and the extraction negative control.

Pipetting scheme restriction digest

		Per sample
Buffer + Enzyme Mix	FetoGnost® Kit Control Restr. Buffer (transparent cap)	4.0 µl
	FetoGnost® Kit Control Restr. Enzymes (amber cap)	1.5 µl
	Total volume	**5.5 µl**
Preparation of restriction digest	Buffer + Enzyme Mix	5.5 µl
	Sample	35.0 µl
	Total volume	**40.5 µl**

Digest: Mix well by pipetting the restriction digest up and down several times (IMPORTANT!) and incubate at 37 °C for 1 hour in a thermoblock with heating lid (e. g., PCR instrument).

Restriction Enzmyes and Buffer last for 20 restriction digests. The content of one kit allows the analysis of 15 plasma samples in triplicates and controls in duplicates.

9 Preparation of real-time PCR

Make sure that one RE-digested Positive Control (FetoGnost® Kit Control Positive Control, red cap) and one RE-digested extraction negative control are included per PCR-run.

- **Digested samples** should be analyzed in triplicates.
  Use 10 µl of digested sample.
- **Negative control from DNA extraction** should be analyzed in duplicates.
  Use 10 µl of digested negative control from the DNA extraction.
- **Positive Control** should be analyzed in duplicates.
  Use 10 µl of digested FetoGnost® Kit Control Positive Control.

9.1 Pipetting scheme

		Per sample
Preparation of Master Mix (mix well)	FetoGnost® Kit Control Reaction Mix (white cap)	15.0 µl
	FetoGnost® Kit Control Assay Mix (green cap)	5.0 µl
	Total volume Master Mix	20.0 µl
Preparation of PCR assay	Master mix Sample	20.0 µl 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 digested samples 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.

9.2 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: Maternal Marker VIC-NONE
Fetal Marker 1 FAM-NONE
Fetal Marker 2 NED-NONE

Passive reference dye: ROX (e. g., for ABI® 7500, QuantStudio™ 5/6/7)

Reaction volume: 30 µl

Ramp speed: without "fast cycling" parameter for ABI® 7500 Instrument or QuantStudio™ 5/6/7 (Thermo Fisher Scientific)

Program 1 Cycles: 1 Analysis: None **UNG Incubation***	**Program 2** Cycles: 1 Analysis: None **Polymerase Activation**	**Program 3** Cycles: 45 Analysis: Quantification Acquisition at 60°
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 Control Reaction Mix as are dNTPs with dUTP to eliminate future amplicon carryover.

10 Interpretation of PCR-data

Reactions with positive Cq values are considered positive, those with negative or Cq ≥ 45 (cut-off) as negative.
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.

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 sample results cannot be interpreted.

For a valid interpretation, the following criteria must be fulfilled:

Target (Detection channel)	Extraction negative control digested (Duplicates)	Positive Control digested (Duplicates)	Plasma sample digested (Triplicates)
VIC (Maternal Marker)	Negative	Negative/weak positive	Negative
FAM (Fetal Marker 1)	Negative	Negative/weak positive	Positive (Cq < 38)
NED (Fetal Marker 2)	Negative	Positive	Positive (approx. 50 % of fetuses) (Cq < 38) or Negative

In case of invalid data the analysis has to be repeated with the remaining or a freshly extracted DNA sample (see 11. Troubleshooting).

Maternal Marker (VIC channel)
Digested plasma sample: The Maternal Marker detects a section of maternal DNA that is cut by the restriction enzymes included in the kit. Thus, the Maternal Marker should not - or at least barely - be detected in digested plasma samples. The absence of this marker in the digested plasma sample validates the restriction digest and guarantees that no amplifiable maternal DNA is present.
Digested FetoGnost® Kit Control Positive Control: The Maternal Marker in the Positive Control is digested with the restriction enzymes included in the kit and should not be detected (or barely detected). The detection of a small amount is valid, the Maternal Marker should, however, show a shift of Cq-values > 5 with respect to the Fetal Marker 2.
For information, the Maternal Marker must be detectable in the undigested positive control.

Fetal Marker 1 (FAM channel) and **Fetal Marker 2** (NED channel)
Digested plasma sample: Fetal markers 1 and 2 are two genes whose detection allows a statement with respect to the presence of fetal DNA in the sample eluate. These markers are digested in DNA of maternal origin with the restriction enzymes included in the kit – maternal DNA should thereafter not be detected. In fetal DNA these genes are not digested. Thus, the Fetal Marker 1 must be detected in the digested sample. In addition, the Fetal Marker 2 is detected in approximately 50 % of the fetuses. Depending on the progress of the pregnancy, Cq values between 32-40 can be expected. At Cq values > 38, it is no longer guaranteed that sufficient cffDNA has been purified from maternal plasma.
Digested FetoGnost® Kit Control Positive Control: The Fetal Marker 1 is cut by the restriction enzymes included in the kit. Thus, the Fetal Marker 1 should not - or at least barely - be detected in digested plasma samples. The detection of a small amount is valid, the Maternal Marker should, however, show a shift of Cq-values > 5 with respect to the Fetal Marker 2.
The Fetal Marker 2 must be detected, since they are not digested with the restriction enzymes included in the kit. See also Figure 1.

11 1Troubleshooting

11.1 No specific signal in FAM, VIC and NED 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

- Incorrect configuration of the PCR reaction.

→ Check your work steps (see 9. Preparation of real-time PCR) and repeat the PCR, if necessary.

11.2 Valid results with controls and no signal in FAM, VIC and NED channels with sample

- Incorrect assignment of detection channels in sample.

→ Please verify the correct assignment of detection channels.

- Inhibition of PCR may have occurred.
- DNA extraction was unsuccessful.
- Extracted DNA was not added to the PCR-reaction.
- Not enough fetal DNA in the sample.

→ No statement is possible. Make sure that you use a recommended method for DNA isolation and stick closely to the manufacturer's instructions.

11.3 Signal with negative control of extraction or PCR negative control

- A contamination occurred during extraction or preparation of the PCR.

→ 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 Specifications and performance evaluation

12.1 Test performance on different real-time PCR instruments

Figure 1 shows the performance of FetoGnost® Kit Control with the ABI® 7500 instrument (Thermo Fisher Scientific).

Figure 2 shows an example of analysis of maternal plasma with the QuantStudio™ 7 Pro (Thermo Fisher Scientific). A sample positive with fetal RHD cffDNA was tested using FetoGnost® Kit Control (digested DNA sample) and FetoGnost® Kit RHD (undigested DNA sample).

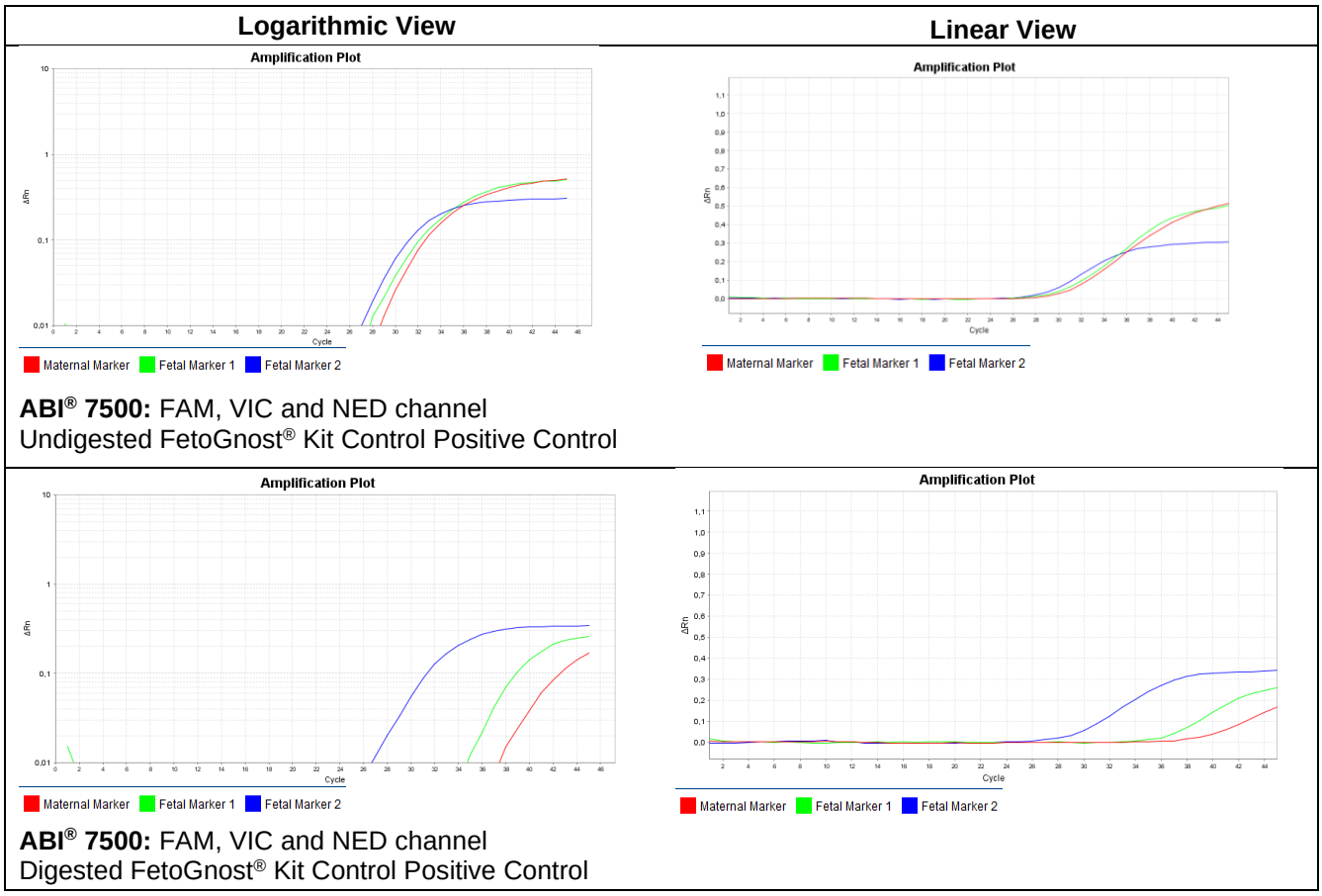

Figure 1

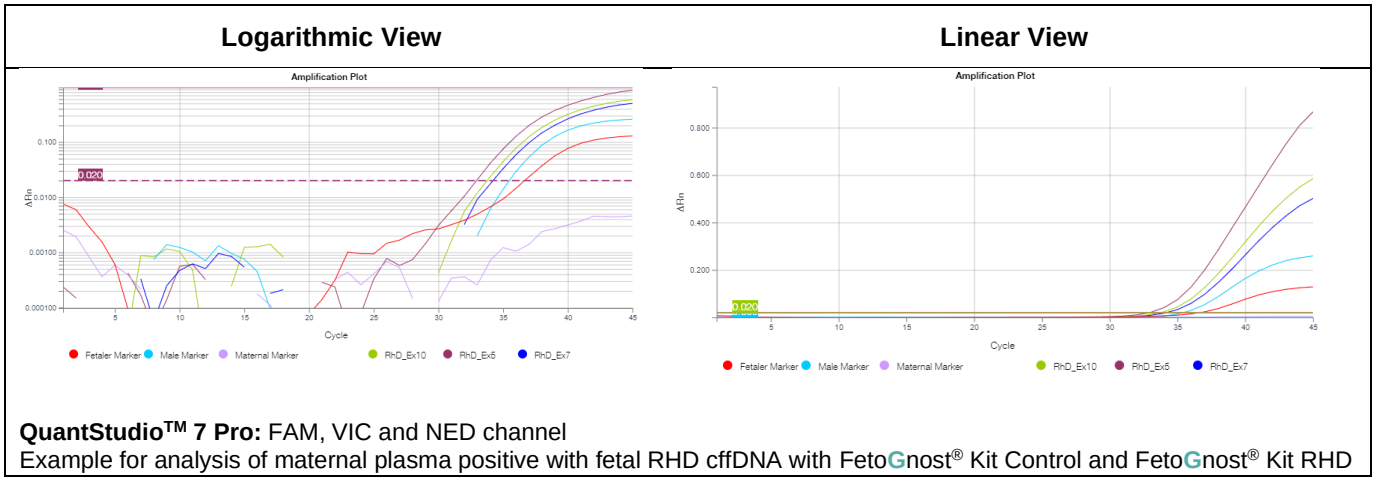

Figure 2

12.2 Analytical sensitivity – limit of detection

FetoGnost® Kit Control has been tested with human genomic DNA and 10-fold dilution series of plasmids containing fragments of the three marker DNAs. The limit of detection (LoD95% = smallest number of copies of target DNA, which can be detected in 95 % of cases) has been determined:

Maternal Marker: In 50 % a minimum of 2 target copies/reaction could be detected.
The limit of detection LoD95% is 15 copies/reaction.

Fetal Marker 1: In 50 % a minimum of 3 target copies/reaction could be detected.
The limit of detection LoD95% is 20 copies/reaction.

Fetal Marker 2: In 50 % a minimum of 8 target copies/reaction could be detected.
The limit of detection LoD95% is 8 copies/reaction.

12.3 Linearity

The linearity has been determined with a 10-fold dilution series of plasmids.

Maternal Marker: The assay shows linearity over the range of 100 – 1,000,000 target copies/reaction with a slope of – 3.4 and a R^2 of > 0.99.

Fetal Marker 1: The assay shows linearity over the range of 100 – 1,000,000 target copies/reaction with a slope of – 3.3 and a R^2 of > 0.99.

Fetal Marker 2: The assay shows linearity over the range of 100 – 1,000,000 target copies/reaction with a slope of – 3.3 and a R^2 of > 0.99.

12.4 Analytical specificity

The specificity is ensured by the selection of highly specific primers and probes. Primers and probes have been checked for possible homologies to currently published sequences by sequence comparison analyses. This also validated the detection of the three markers.

13 Literature

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- ISO 20186-3: 2019

14 Revision history

Revision	Date	Description
V1.1en	06 June 2021	See 10. Interpretation of PCR data and Figure 1. The positive control was changed: The Fetal Marker 1 is cut by the restriction enzymes included in the kit and should show a shift of Cq-values > 5 with respect to the Fetal Marker 2.
V1.2en	xxxx	**Change of the company address** **1 Intended purpose** Update Information on incompatibility of the test with samples extracted by extraction methods using carrier RNA at a concentration of >0.5 µg/sample. **5 Contents, stability, and storage** Kit version 1.1: - Filling volume of Positive Control was changed from 200 µl to 300 µl - Cap color of FetoGnost® Kit Control Restr. Enzymes was changed from blue to amber - Cap color of FetoGnost® Kit Control Restr. Buffer was changed from blue to transparent. - **8.3 DNA extraction from plasma** Update