RIDA®GENE Faecalibacterium prausnitzii

real-time PCR

Art. Nr.: PG0155

100 reactions

For *in vitro* diagnostic use.

-20 °C

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1. Intended use

For *in vitro* diagnostic use. RIDA®GENE Faecalibacterium prausnitzii is a real-time PCR for the direct qualitative and quantitative detection of *Faecalibacterium prausnitzii* DNA in human stool samples.

2. Summary and Explanation of the test

Faecalibacterium prausnitzii is a gram-positive, strictly anaerobic, commensal bacterium and belongs to the family of *Clostridiacea*. *F. prausnitzii* is one of the most important bacteria in the human gut flora and makes up to 5-10 % of the total number of bacteria detected in stool samples from healthy humans.[1] F. prausnitzii localization seemed to depend upon anaerobic conditions only found in the lower gut.[2] In early infancy the number of *F. prausnitzii* is very low and increases after the establishment of primocolonizing bacteria.[2] They are strictly anaerobic and thus difficult to grow in culture. Consequently, this explains why the mechanisms of its protection potential are still poorly understood. *F. prausnitzii* produces butyric acid derivates e.g. butyrate, which are essential for the intestinal activity. Butyrate plays an important role for the metabolism in the colon. They serve as energy sources for the colonocytes and have anti-inflammatory effects and maintain the activity of bacterial enzymes and protects the digestive system against intestinal pathogens.[3] Changes in the number of *F. prausnitzii* are an indication for dysbiosis in the human gut flora. A significant reduction of *F. prausnitzii* is detected by patients with diabetes and chronic inflammatory intestinal disorders (Morbus Crohn, Colitis ulcerosa, IBS). For the latter case, the intestinal barrier is disrupted or rather the intestinal wall gets more permeable. This leads to an unregulated mass exchange and causes diarrheal diseases and inflammatory reactions.

3. Test principle

RIDA®GENE Faecalibacterium prausnitzii is a real-time PCR for the direct, qualitative and quantitative detection *Faecalibacterium prausnitzii DNA* in human stool samples. After DNA isolation, amplification of the gene fragment (if present) specific for *Faecalibacterium prausnitzii* (16S-rRNA) occurs. The amplified targets are detected with hydrolysis probes, which are labeled at one end with a quencher and at the other end with a fluorescent reporter dye (fluorophoree). In the presence of a target the probes hybridize to the amplicons. During the extension step, the Taq-polymerase breaks the reporter-quencher proximity. The reporter emits a fluorescent signal, which is detected by the optical unit of a real-time PCR instrument. The fluorescence signal increases with the amount of formed amplicons. With the

Standard DNA A, B and C included in the kit, it is possible to quantify the results. The RIDA®GENE Faecalibacterium prausnitzii real-time PCR kit contains an internal control (ICD) that detects PCR inhibition, monitors reagent integrity and confirms that nucleic acid extraction was sufficient.

4. Reagents provided

Tab. 1: Reagents provided (Reagents provided in the kit are sufficient for 100 determinations)

Kit Code	Reagent	Amount	Lid Color
1	Reaction Mix	2x 1100 µl	yellow
2	Taq-Polymerase	1x 11 µl	red
D	Internal Control DNA	2x 1800 µl	orange
N	PCR Water	1x 500 µl	white
P	Positive Control	1x 100 µl	blue
A	Standard DNA A	1x 100 µl	dark blue
B	Standard DNA B	1x 100 µl	dark blue
C	Standard DNA C	1x 100 µl	dark blue

5. Storage instructions

- Protect all reagents from light and store at -20 °C. All reagents can be used until the expiration date. After expiry the quality guarantee is no longer valid.
- Carefully thaw reagents before using (e.g. in a refrigerator at 2 - 8 °C).
- Reagents can sustain up to 5 freeze/thaw cycles without influencing the assay performance (e.g. after the first thawing separate it in aliquots and freeze immediately).
- During PCR preparation all the reagents should be stored cold in an appropriate way (2 - 8 °C).

6. Additional necessary reagents and necessary equipment

- The RIDA®GENE Faecalibacterium prausnitzii real-time PCR Assay is suitable for use with following extraction platforms and real-time PCR instruments:
  - Extraction platforms:
    RIDA® Xtract
    Maxwell® 16, Maxwell® RSC (Promega)

- Real-time PCR instrument:

Roche:	LightCycler® 480II
Agilent Technologies:	Mx3005P
Applied Biosystems:	ABI 7500
Abbott:	m2000rt
Bio-Rad:	CFX96™
Cepheid:	SmartCycler®
QIAGEN:	Rotor-Gene Q

  Note: Only use 0.1 ml tubes on the Rotor-Gene Q (QIAGEN).

  If you want to use other extraction platforms or real-time PCR instruments please contact R-Biopharm at mdx@r-biopharm.de.

- RIDA®GENE Color Compensation Kit II (PG0002) for use with the LightCycler® 2.0
- Real-time PCR consumables (plates, tubes, foil)
- Centrifuge with a rotor for the reaction vials
- Vortexer
- Pipettes (0.5 – 20 µl, 20 – 200 µl, 100 – 1000 µl)
- Filter tips
- Powder-free disposal gloves

7. Precautions for users

For *in-vitro* diagnostic use.
This test must only be carried out by trained laboratory personnel. The guidelines for working in medical laboratories have to be followed. The instruction manual for the test procedure has to be followed. Do not pipet samples or reagents by mouth. Avoid contact with bruised skin or mucosal membranes. During handling reagents or samples, wear appropriate safety clothing (appropriate gloves, lab coat, safety goggles) and wash your hands after finishing the test procedure. Do not smoke, eat or drink in areas where samples or reagents are being used.

- Extraction, PCR preparation and the PCR run should be separated in different rooms to avoid cross-contaminations.
- Samples must be treated as potentially infectious as well as all reagents and materials being exposed to the samples and have to be handled according to the national safety regulations.
- Do not use the kit after the expiration date.

All reagents and materials used have to be disposed properly after use. Please refer to the relevant national regulations for disposal.

For more details see Material Safety Data Sheets (MSDS) at www.r-biopharm.com

8. Collection and Storage of Samples

8.1 Sample Preparation

For DNA isolation of human stool samples, use a commercially available DNA isolation kit (e.g. RIDA® Xtract) or DNA extraction system (e.g. Maxwell® 16 (Promega)). Extract DNA according to the manufacturer's instructions.

We recommend to dilute the stool samples before extraction 1:3 with water. Vortex the diluted stool sample intensely and centrifuge at 3,000 rpm for 30 sec. From the supernatant, use the appropriate volume according to manufacturer's instructions.

The RIDA®GENE Faecalibacterium prausnitzii real-time PCR kit contains an internal control (ICD) that detects PCR inhibition, monitors reagent integrity and confirms that nucleic acid extraction was sufficient.

If the Internal Control DNA is used only as a PCR inhibition control, 1 µl of the Internal Control DNA should be added to the Master-Mix (see Tab. 3).

If the Internal Control DNA is used as an extraction control for the sample preparation procedure **and** as PCR inhibition control, 20 µl of the Internal Control DNA has to be added during extraction procedure. The Internal Control DNA should always be added to the specimen-lysis buffer mixture and must **not** be added directly to the specimen.

9. Test procedure

9.1 Master-Mix preparation

Calculate the total number of PCR reactions (sample and control reactions) needed. One positive control and negative control must be included in each assay run.

We recommend calculating an additional volume of 10% to compensate imprecise pipetting (see Tab. 2, Tab. 3). Thaw, mix gently and briefly centrifuge the Reaction Mix, the Taq-Polymerase, the Positive Control, the PCR Water and the Internal Control DNA before using. Keep reagents appropriately cold during working step (2 - 8 °C).

Tab. 2: Calculation and pipetting example for 10 reactions of the Master-Mix (ICD as extraction and PCR inhibition control)

Kit code	Master-Mix components	Volume per reaction	10 reactions (10 % extra)
1	Reaction Mix	19.9 µl	218.9 µl
2	Taq-Polymerase	0.1 µl	1.1 µl
	Total	**20.0 µl**	**220 µl**

Mix the components of the Master-Mix gently and briefly spin down.

Tab. 3: Calculation and pipetting example for 10 reactions of the Master-Mix (ICD only as PCR inhibition control)

Kit Code	Master-Mix components	Volume per reaction	10 reactions (10 % extra)
1	Reaction Mix	19.9 µl	218.9 µl
2	Taq-Polymerase	0.1 µl	1.1 µl
D	Internal Control DNA	1.0 µl	11 µl
	Total	**21.0 µl**	**231.0 µl**

Mix the components of the Master-Mix gently and briefly spin down.

9.2 Preparation of the PCR-Mix

Pipette 20 µl of the Master-Mix in each reaction vial (tube or plate).

Negative control: Add 5 µl PCR Water as negative control to the pre-pipetted Master-Mix.

Note: If the ICD is used as extraction control for the sample preparation procedure and as PCR inhibition control, we recommend to add 1 µl of the ICD to the negative control PCR-Mix.

Sample: Add 5 µl DNA-Extract to the pre-pipetted Master-Mix.

Positive control: Add 5 µl Positive Control to the pre-pipetted Master-Mix.

Note: If the* Internal Control DNA *is used as extraction control for the sample preparation procedure and as PCR inhibition control, we recommend to add 1 µl of the* Internal Control DNA *to the positive control PCR-Mix.

Standard DNA (A, B, C): Add 5 µl Standard DNA (A, B, C) to the pre-pipetted Master-Mix in the designated reaction tubes.

Note: For all other cyclers, only one sample of the standard curve has to be included in the experimental set-up as calibrator for each new real-time PCR run. Here, the application of a standard curve is only required to run once per lot number.

Cover tubes or plate. Spin down and place in the real-time PCR instrument. The PCR reaction should be started according to the PCR instrument Set-up (see Tab. 4, Tab. 5).

9.3 PCR Instrument Set-up

Tab. 4: Real-time PCR Profil for LightCycler® 480II, SmartCycler® and Rotor-Gene Q

Initial Denaturation	1 min, 95 °C
Cycles	45 Cycles
PCR Denaturation	10 sec, 95 °C
Annealing/Extension	15 sec, 60 °C
Temperature Transition Rate / Ramp Rate	Maximum

Note: *Annealing and Extension occur in the same step.*

Note: Check that the "Manual Thres. Fluor Units" for Channel 1 is set to 10.0 and for Channel 2 is set to 5.0 on the SmartCycler® (Cepheid). Due to variations between different cyclers, it may be required to individually adapt the "Manual Thres. Fluor Units" for channel 1.

Tab. 5: Real-time PCR profile for Mx3005P, ABI7500, m2000rt and CFX96™

Initial Denaturation	1 min, 95 °C
Cycles	45 Cycles
PCR Denaturation	15 sec, 95 °C
Annealing/Extension	30 sec, 60 °C
Temperature Transition Rate / Ramp Rate	Maximum

Note: *Annealing and Extension occur in the same step.*

Note: A minimum annealing/extension time of 35 sec. is required for the m2000rt

Note: The total copy number per reaction of Standard DNA A, B and C has to be typed in into the Setup File of the software programme of the respective real-time PCR cycler. A total volume of 5 µl DNA is used resulting in following concentrations:

Standard DNA A: 5×10^2 *copies/reaction*

Standard DNA B: 5×10^4 *copies/reaction*

Standard DNA C: 5×10^6 *copies/reaction*

Note: ***The standard curve can be saved on the real-time PCR cycler for each parameter. Apart from the SmartCycler® (Cepheid), the ABI 7500 (Applied Biosystems), the m2000rt (Abbott) and the CFX96™ (Bio-Rad) cycler, the standard curve is only required to run once per lot number. Using the SmartCycler® (Cepheid, closed system), the ABI 7500 (Applied Biosystems), the m2000rt (Abbott) and the CFX96™ (Bio-Rad) cycler requires to include a standard curve in each run. For all other cyclers, only one sample of the standard curve has to be included in the experimental set-up as calibrator for each new real-time PCR run.***

9.4 Detection channel Set-up

Tab. 6: Selection of appropriate detection channels

Real-time PCR Instrument	Detection	Detection Channel	Note
Roche LightCycler® 2.0	*Faecalibacterium prausnitzii*	530	**RIDA®GENE Color Compensation Kit II(PG0002) is required**
	ICD		
Roche LightCycler® 480II	*Faecalibacterium prausnitzii*	465/510	**RIDA®GENE Color Compensation is not required**
	ICD	533/580	
Cepheid SmartCycler®	*Faecalibacterium prausnitzii*	Kanal 1	**Check that the "Manual Thres. Fluor Units" for Channel 1 is set to 10.0 and for Channel 2 is set to 5.0***
	ICD	Kanal 2	
ABI 7500	*Faecalibacterium prausnitzii*	FAM	**Check that passive reference option ROX is none**
	ICD	VIC	
Abbott m2000rt	*Faecalibacterium prausnitzii*	FAM	**-**
	ICD	VIC	
Agilent Techn. Mx3005P	*Faecalibacterium prausnitzii*	FAM	**Check that reference dye is none**
	ICD	HEX	
Qiagen Rotor-Gene Q	*Faecalibacterium prausnitzii*	Green	**The gain settings have to be set to 5**
	ICD	Yellow	
Bio-Rad CFX96™	*Faecalibacterium prausnitzii*	FAM	**-**
	ICD	VIC	

*** Due to variations between different cyclers, it may be required to individually adapt the Manual Thres. Fluor Units" for channel 1.**

10. Quality Control

The analysis of the samples is done by the software of the used real-time PCR instrument according to the manufacturer` s instructions. Positive and negative controls have to show correct results (see Table 7, Fig. 1) in order to determine a VALID run.

The positive control has a concentration of 10^3 copies/µl. In each PCR run it is used in a total amount of 5 x 10^3 copies.

Tab.7: For a VALID run, the following conditions must be met:

Sample	Assay result	ICD Ct	Target Ct
PTC	Positive	NA *[1]	See Quality Assurance Certificate
NTC	Negative	Ct > 20	0

*[1] *No Ct value is required for the ICD to make a positive call for the positive control.*

If the Positive Control (PTC) is not positive within the specified Ct range but the Negative Control is valid, prepare all new reactions using remaining purified nucleic acids and a new Positive Control.

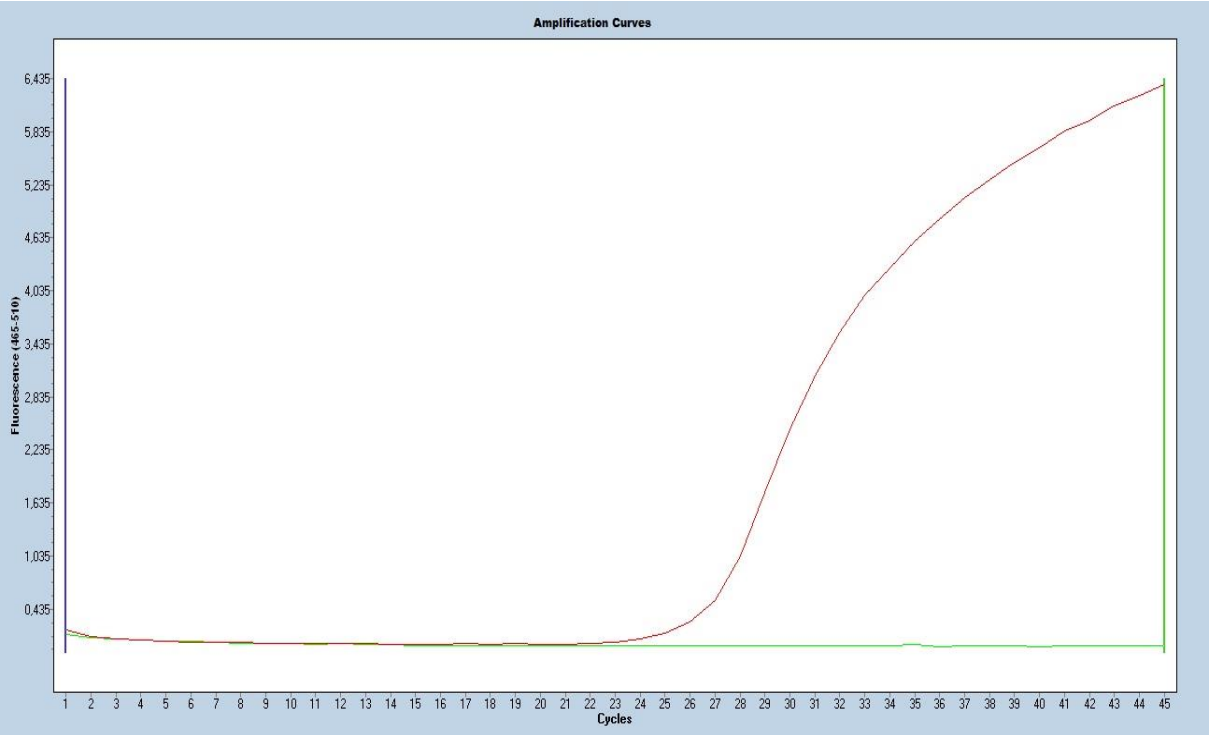
If the Negative Control (NTC) is not negative but the Positive control is valid prepare all new reactions using remaining purified nucleic acids and a new Negative Control.

If the required criteria are not met, following items have to be checked before repeating the test:

- Expiry of the used reagents
- Functionality of the used instrumentation
- Correct performance of the test procedure

The analysis of the samples is done by the software of the used real-time PCR instrument according to the manufacturer` s instructions. Positive and negative controls have to show correct results (see Fig. 1).

Fig. 1: Correct run of the positive and negative control (*Faecalibacterium prausnitzii*) on the LightCycler® 480II



11. Result interpretation

The result interpretation is done according to Table 8.

Tab. 8: Sample interpretation

Faecalibacterium prausnitzii	**ICD**	**Result**
positive	**positive/negative**	***Faecalibacterium prausnitzii* detected**
negative*	**positive**	**Target gene is not detected***
negative	negative	**Invalid**

Faecalibacterium prausnitzii is detected, if the sample DNA and the Internal Control DNA (ICD) show an amplification signal in the detection system.

Faecalibacterium prausnitzii is also detected, if the sample DNA shows an amplification signal but none for the Internal Control DNA (ICD) in the detection system. The detection of the internal amplification control is not necessary, because high concentrations of the amplicon can cause a weak or absent signal of the Internal Control DNA (ICD).

Faecalibacterium prausnitzii is not detected, if the sample DNA shows no amplification signal, but an amplification signal for the Internal Control DNA (ICD) in the detection system. An inhibition of the PCR reaction can be excluded by the detection of the Internal Control DNA (ICD).

A sample is invalid, if the sample DNA and Internal Control DNA (ICD) show no amplification signal in the detection system. The sample contains a PCR inhibitor. The extracted sample needs to be further diluted with PCR water (1:10) and re-amplified, or the isolation and purification of the sample has to be improved.

* ***Note:*** ***A negative result for Faecalibacterium prausnitzii DNA is unlikely since it belongs to the human commensal bacteria. If a negative result occurs for Faecalibacterium prausnitzii DNA, it is likely that, upon use of the ICD as inhibition control, the sample extraction was not successful. If a negative result occurs for Faecalibacterium prausnitzii DNA, it is recommended to improve isolation and purification of the sample and repeat amplification of the sample.***

11.1 Quantification of samples

To quantify *Faecalibacterium prausnitzii*-positive samples, a standard curve with the Standard DNA A, B and C has to be performed separately. The standard curve measurement has to be saved separately. However, the same standard curve measurement can be used in all runs with products from the same lot number by importing the saved experiment.

Note: This is not valid for the following cyclers: SmartCycler® (Cepheid), ABI 7500 (Applied Biosystems), m2000rt (Abbott) and CFX96™ (Bio-Rad). Here, a standard curve has to be measured with each run.

For all other cyclers, one sample of the standard curve has to be included in the experimental set-up as calibrator for each new real-time PCR run.

To quantify *Faecalibacterium prausnitzii*-positive samples, all Standard DNA samples (A, B and C), the positive and negative control as well as the unknown samples to be quantified, have to be selected and analyzed according to the instructions of the cycler manufacturer.

Note: For further information on quantification of please contact mdx@r-biopharm.de

With the quantitative RIDA®GENE Faecalibacterium prausnitzii real-time PCR the amount of DNA in Copies/Reaction of the parameter is calculated. The conversion in

cell concentration/g stool sample is done with a correction factor K and takes into account the dilution of the extraction procedure (dependent on the extraction kit used) and the PCR Set-up as well as the number of target sequences in the whole genome.

The conversion of the result of the quantitative RIDA®GENE Faecalibacterium prausnitzii real-time PCR in cells/g stool is calculated with following formula:

C [cells/g stool] = c [copies/reaction] x K

C [cells/g stool]	- bacterial concentration of sample in cells/g stool
c [copies/reaction]	- DNA concentration in PCR reaction (result of quantitative PCR)
K	- correction factor

For the calculation of the correction factor, following information has to be considered:

- Sample dilution
- Usage of partial sample during extraction
- DNA extract from total eluate used for PCR reaction
- Number of target sequence in the whole genome

Tab. 9: Example calculation of correction factor using RIDA® Xtract for sample preparation of a 1:3 diluted sample

Description	**Factor**
Sample dilution 1:3 before extraction	x 3
200 µl sample for extraction*	x 5
5 µl DNA extract into PCR reaction ((total eluate 60 µl (= 1/12))	x 12
a. Target sequence contained 5x in total *Faecalibacterium*-genome	x 1/5 (*Faecalibacterium prausnitzii*)
Correction factor (K) for *Faecalibacterium prausnitzii***	**0.36 x 10^2**

* Result corresponds to 1 g stool

** This value can be saved in the real-time PCR instrument

Tab. 10: Example calculation of correction factor using Maxwell®16 LEV Blood DNA Kit AS1290 (Promega) for sample preparation of a 1:3 diluted sample

Description	**Factor**
Sample dilution 1:3 before extraction	x 3
300 µl sample for extraction*	x 3.33
5 µl DNA extract into PCR reaction ((total eluate 100 µl (= 1/20))	x 20
a. Target sequence contained 5x in total *Faecalibacterium*-genome	x 1/5 (*Faecalibacterium prausnitzii*)
Correction factor (K) for *Faecalibacterium prausnitzii***	**0.40 x 10²**

* Result corresponds to 1 g stool

** This value can be saved in the real-time PCR instrument

12. Limitations of the method

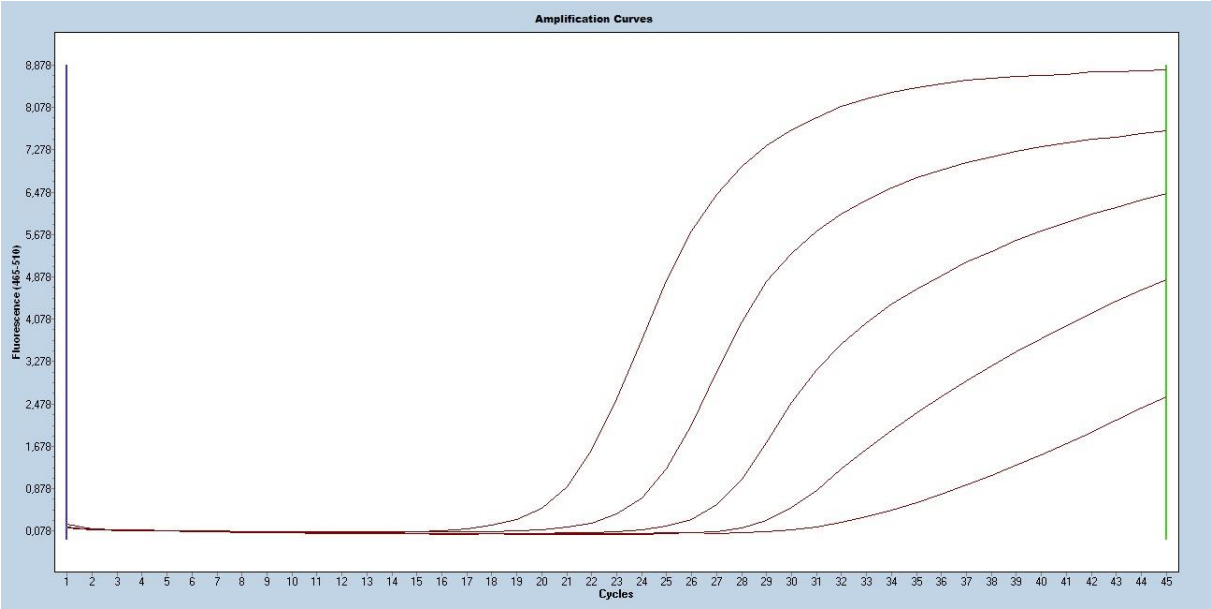
1. The result of molecular analysis should not lead to the diagnosis, but always be considered in the context of medical history and symptoms of the patient.
2. This assay is only validated for human stool samples.
3. Inappropriate specimen collection, transport, storage and processing or a pathogen load in the specimen below the analytical sensitivity can result in false negative results.
4. The presence of PCR inhibitors may cause invalid results.
5. Mutations or polymorphisms in primer or probe binding regions may affect detection of new variants resulting in a false negative result with the RIDA®GENE Faecalibacterium prausnitzii assay.
6. As with all PCR based *in vitro* diagnostic tests, extremely low levels of target below the limit of detection (LoD) may be detected, but results may not be reproducible.
7. A positive test result does not necessarily indicate the presence of viable organisms. However, a positive result is indicative for the presence of the target genes for *Faecalibacterium prausnitzii* (16s-rRNA).

13. Performance characteristics

13.1 Analytical sensitivity

The RIDA®GENE Faecalibacterium prausnitzii real-time PCR has a limit of detection limit of ≥ 10 DNA copies per reaction for *Faecalibacterium prausnitzii* (s. Fig. 2).

Fig. 2: Dilution series *Faecalibacterium prausnitzii* (10^6 – 10^2 DNA copies/µl) on the LightCycler® 480II



The detection limit of the whole procedure depends on the sample matrix, DNA extraction and DNA concentration.

13.2 Analytical specificity

The RIDA®GENE Faecalibacterium prausnitzii real-time PCR is specific for *Faecalibacterium prausnitzii*. No cross-reaction could be detected for the following species (see Tab. 11):

Tab. 11: Cross-reactivity testing

Adenovirus	-	Candida albicans	-	*E. coli* (O26:H-)	-	*Pseudomonas aeruginosa*	-
Adenovirus 41, Human, Strain Tak	-	*Citrobacter freundii*	-	*E. coli* (O157:H7)	-	Rotavirus	-
Arcobacter butzleri	-	*Clostridium difficile*	-	Entamoeba histolytica	-	*Salmonella enteritidis*	-
Aeromonas hydrophila	-	*Clostridium perfringens*	-	*Enterobacter cloacae*	-	*Salmonella typhimurium*	-
Bacillus cereus	-	*Clostridium bifermentans*	-	*Enterococcus faecalis*	-	*Serratia liquefaciens*	-
Campylobacter coli	-	*Clostridium novyi*	-	Giardia intestinalis WB Clone C6	-	*Shigella flexneri*	-
Campylobacter jejuni	-	*Clostridium sporogenes*	-	Giardia intestinalis Portland 1	-	*Staphylococcus aureus*	-
Campylobacter fetus subsp. Fetus	-	*Clostridium septicum*	-	Giardia lamblia	-	*Staphylococcus epidermidis*	-
Campylobacter lari subsp. Lari	-	*Clostridium sordellii*	-	Klebsiella oxytoca	-	*Vibrio parahaemolyticus*	-
Campylobacter upsaliensis	-	*E. coli* (O6)	-	*Proteus vulgaris*	-	*Yersinia enterocolitica*	-
Adenovirus 1, Human, strain Adenoid 71	-	Adenovirus 7, Human, Strain Gomen	-				

Explanation of Symbols

IVD	For *in vitro* diagnostic use
	Consult instructions for use
LOT	Lot number
	Expiry
	Store at
REF	Article number
	Number of test
	Date of manufacture
	Manufacturer

Literature

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