Arbeitsanleitung/Manual

MutaPLEX® PRE/RU/LA

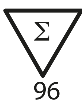
real time PCR kit

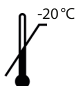
Test für den quantitativen In-vitro-Nachweis der DNA von Prevotella spec. und Ruminococcus spec. aus den Familien Ruminococcaceae und Lachnospiraceae

Test for the quantitative in vitro detection of DNA of Prevotella species and Ruminococcus species from the families Ruminococcaceae and Lachnospiraceae

Gültig ab / Valid from 2019-01-09

KG1913-96





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1 INTENDED USE

The MutaPLEX® PRE/RU/LA real time PCR kit is an assay for the quantitative detection of DNA of *Prevotella species* and *Ruminococcus species* from the families *Ruminococcaceae* and *Lachnospiraceae* using real time PCR microplate systems. Using the quantitative MutaPLEX® PRE/RU/LA real time PCR, the DNA content of the respective bacteria can be determined as copy number per reaction.

2 PATHOGEN INFORMATION

Prevotella species are members of the oral, vaginal, and gut microbiota. In a study of gut bacteria of children in Burkina Faso, *Prevotella* made up 53 % of the gut bacteria, but were absent in age-matched European children. Studies also indicate that long-term diet is strongly associated with the gut microbiome composition. Those who eat plenty of protein and animal fats typical of Western diet have predominantly *Bacteroides* bacteria, while for those who consume more carbohydrates, especially fibre, the *Prevotella* species dominate. An overgrowth of *Prevotella* and a reduction of *Lactobacillus* have been correlated with the onset of osteomyelitis in mice. The reduction of *Prevotella* in model mice led to an increase of *Lactobacillus* showing a protection effect against osteomyelitis. Thus, changes in the microbiota *Prevotella* may be related to the development of osteomyelitis.

Ruminococci are a polyphyletic group of bacteria with members in two families: *Ruminococcaceae* and *Lachnospiraceae*.

Ruminococcaceae in gut microbiomes play a major role in helping digest resistant starches - the complex carbohydrates found in high fiber foods such as lentils, beans, and unprocessed whole grains. The slow digestion of these special carbs by *Ruminococci* has been associated with numerous health benefits such as reversing infectious diarrhea and reducing risk of diabetes and colon cancer. One species of *Ruminococcus* has been associated with increased severity of irritable bowel symptoms, but most species are important and necessary for our digestive function.

The ***Lachnospiraceae*** family is formed by 24 named genera, including *Ruminococcus*, *Blautia*, *Dorea*, and *Lachnoanaerobaculum* as well as a number of *incertae sedis* strains sharing a high degree of similarity among their 16S rDNA sequences. Some members of this family are non-spore-forming, but all of them are strictly anaerobes. In human adults, members of this family have been associated with protection against *C. difficile* infections and obesity. They are also known as potent short fatty acid producers. However, despite their apparent importance, little is known about their presence and possible roles played by these bacteria in the early life of humans.

3 PRINCIPLE OF THE TEST

The MutaPLEX® PRE/RU/LA real time PCR kit contains specific primers and dual-labelled probes for the amplification and detection of DNA of *Prevotella species* and *Ruminococcus species* from the families *Ruminococcaceae* and *Lachnospiraceae*. The presence of nucleic acid is detected by an increase in fluorescence due to hydrolysis of the probes during amplification. The emitted fluorescence is measured in the FAM channel (*Ruminococcus species* from the family of *Ruminococcaceae*), the ROX channel (*Ruminococcus species* from the family *Lachnospiraceae*) and the Cy5 channel (*Prevotella species*). For simplicity purposes, the channels will be designated *Ruminococcaceae* (FAM) and *Lachnospiraceae* (ROX).

Furthermore, MutaPLEX® PRE/RU/LA real time PCR kit contains a control DNA, which is added during DNA extraction and detected in the same reaction by a differently labelled probe.

The control DNA allows the detection of PCR inhibition and acts as control for the isolation of the nucleic acid from the biological specimen.

The fluorescence of the control DNA is measured in the VIC®/HEX/JOE/TET channel.

4 PACKAGE CONTENTS

The reagents supplied are sufficient for 96 (KG1913-96) reactions.

Table 1: Components of the MutaPLEX® PRE/RU/LA real time PCR kit .

Label	Lid Colour	Content
Reaction Mix	yellow	1 x 1536 µl
Positive Control	red	1 x 100 µl
Negative Control	green	1 x 100 µl
Control DNA	colourless	1 x 480 µl
Standard 1	black	1 x 100 µl
Standard 2	purple	1 x 100 µl
Standard 3	orange	1 x 100 µl

5 EQUIPMENT AND REAGENTS TO BE SUPPLIED BY USER

- DNA isolation kit (e. g. MutaCLEAN® Universal RNA/DNA, KG1038, or the magnet particle based system NukEx® Complete Mag RNA/DNA, KG1020)
- PCR grade water
- Sterile microtubes

- Pipets (adjustable volume)
- Sterile pipet tips with filter
- Table centrifuge
- Vortex
- Real time PCR instrument
- Optical PCR reaction tubes with lid or reaction plates with foil
- Optional: Liquid handling system for automation

6 TRANSPORT, STORAGE AND STABILITY

The MutaPLEX® PRE/RU/LA real time PCR-Kit is shipped on dry ice. All components must be stored at maximum -20 °C in the dark immediately after receipt. Do not use reagents after the date of expiry printed on the package.

Up to 20 freeze and thaw cycles are possible.

For convenience, opened reagents can be stored at 2–8 °C for up to 6 months.

Protect kit components from direct sunlight during the complete test run.

7 IMPORTANT NOTES

- The MutaPLEX® PRE/RU/LA real time PCR must be performed by qualified personnel only.
- Good Laboratory Practice (GLP) has to be applied.
- Biological samples must always be regarded as potentially infectious material and all equipment used has to be treated as potentially contaminated.

8 GENERAL PRECAUTIONS

- Avoid microbial and nuclease (DNase/RNase) contamination of the eluates and the components of the kit.
- Always use DNase/RNase-free disposable pipette tips with aerosol barriers.
- Always wear protective disposable powder-free gloves when handling kit components.
- Use separated and segregated working areas for (1) sample preparation, (2) reaction setup and (3) amplification/detection activities. The workflow in the laboratory should proceed in unidirectional manner. Always wear disposable gloves in each area and change them before entering a different area. Dedi-

cate supplies and equipment to the separate working areas and do not move them from one area to another.

- Store positive and/or potentially positive material separated from all other components of the kit.
- Do not open the reaction tubes/plates post amplification, to avoid contamination with amplicons.
- Additional controls may be tested according to guidelines or requirements of local, state and/or federal regulations or accrediting organizations.
- Do not autoclave reaction tubes after the PCR, since this will not degrade the amplified nucleic acid and will bear the risk to contaminate the laboratory area.
- Discard sample and assay waste according to your local safety regulations.
- Do not combine MutaPLEX® PRE/RU/LA real time PCR kit components of different lot numbers.

9 SAMPLE PREPARATION

The MutaPLEX® PRE/RU/LA real time PCR is suitable for the detection of the DNA of *Prevotella species* and *Ruminococcus species* from the families *Ruminococcaceae* and *Lachnospiraceae* isolated from biological specimens with appropriate isolation methods.

Commercial kits for DNA isolation such as MutaCLEAN® Universal RNA/DNA (KG1038) or the magnet particle based system NukEx® Complete Mag RNA/DNA (KG1020) are recommended.

Important: In addition to the samples, always run a water control in your extraction. Treat this water control analogous to a sample.

Comparing the amplification of the control DNA in the samples to the amplification of the internal control in the water control will give insights on possible inhibitions of the real time PCR. Furthermore, possible contaminations during DNA extraction will be detectable.

If the real time PCR is not performed immediately, store extracted DNA according to the instructions given by the DNA extraction kit’s manufacturer.

10 CONTROL DNA

A control DNA is supplied and can be used as extraction control. This allows the user to control the DNA isolation procedure and to check for possible real time PCR inhibition.

MutaPLEX® PRE/RU/LA control DNA is added to the DNA extraction.

Add 5 µl control DNA per extraction (5 µl x (N+1)). Mix well. Perform the DNA isolation according to the manufacturer's instructions.

The control DNA must be added to the lysis buffer of the extraction kit.

11 REAL TIME PCR

11.1 Important points before starting

- Please pay attention to chapter 7 "Important Notes".
- Before setting up the real time PCR, familiarise yourself with the real time PCR instrument and read the user manual supplied with the instrument.
- The programming of the thermal profile should take place before the PCR set up.
- In every PCR run, one positive control and one negative control should be included.
- Before each use, all reagents should be thawed completely at room temperature, thoroughly mixed, and centrifuged very briefly.
- For quantification of DNA of *Prevotella species, Ruminococcus species* from the family of *Ruminococcaceae* and *Ruminococcus species* from the family of *Lachnospiraceae* in biological samples, a standard curve using standards 1, 2 and 3 must be applied. The standard curve needs to be saved separately on the real time PCR instrument. It can be imported and used in subsequent runs with kits of the same lot.

  Note: the application of the standard curve is needed once per lot.
- We recommend to keep reagents and samples at 2–8 °C (e. g. on ice or a cooling block) at all times.

11.2 Procedure

The control DNA was added during DNA extraction (see chapter 10 "Control DNA"). Prepare the master mix according to table 2.

The master mix contains all of the components needed for PCR except the sample. Prepare a volume of master mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 2: Preparation of the master mix (control DNA was added during DNA extraction)

Volume per reaction	Volume master mix
16 µl Reaction Mix	16 µl x (N+1)

Real time PCR set up

- Place the number of optical PCR reaction tubes needed into the respective tray of the real time PCR instrument.
- Pipet 16 µl of the master mix into each optical PCR reaction tube.
- Add 4 µl of the eluates from the DNA isolation (including the eluate of the water control), the positive control and the negative control to the corresponding optical PCR reaction tube (table 3).
- Add 4 µl of standard 1, 2 and 3, respectively, to the corresponding optical PCR reaction tube or the respective well of the optical reaction plate (table 3)
- Close the optical PCR reaction tubes immediately after filling in order to reduce the risk of contamination.

Table 3: Preparation of the real time PCR

Component	Volume
Master mix	16.0 µl
Sample	4.0 µl
Total volume	20.0 µl

11.3 Instrument settings

For the real time PCR use the thermal profile shown in table 4.

Table 4: real time PCR thermal profile

<table>
<tr><th>Description</th><th>Time</th><th>Temperature</th><th>No of cycles</th></tr>
<tr><td>Initial Denaturation</td><td>5 min</td><td>95 °C</td><td>1</td></tr>
<tr><td colspan="3">Amplification of DNA</td><td rowspan="4">45</td></tr>
<tr><td>Denaturation</td><td>10 s</td><td>95 °C</td></tr>
<tr><td rowspan="2">Annealing and Extension</td><td>40 s</td><td>60 °C</td></tr>
<tr><td colspan="2">Aquisition at the end of this step</td></tr>
</table>

The MutaPLEX® PRE/RU/LA real time PCR is designated for the LightCycler® 480 II real time PCR instrument. Instrument settings have to be adjusted according to table 5.

Table 5: Overview of the instrument settings required for the MutaPLEX® PRE/RU/LA real time PCR.

Real time PCR Instrument	Parameter	Detection channel	Notes		
			Colour Compensation Kit required		
			Melt Factor	**Quant Factor**	**Max Integration Time [s]**
LightCycler 480II	*Ruminococcaceae*	FAM (465–510)	1	10	1
	Lachnospiraceae	ROX (533–610)	1	10	2
	Control DNA	HEX (533–580)	1	10	2
	Prevotella species	CY5 (618–660)	1	10	3

Note: For standards 1, 2 and 3 the total copy number per reaction needs to be entered in the setup file of the LightCycler® 480 II instrument. 4 µl of each standard DNA are used, resulting in the following concentrations:

- Standard 1: 5 x 10^6 copies/reaction
- Standard 2: 5 x 10^4 copies/reaction
- Standard 3: 5 x 10^2 copies/reaction

12 ASSAY VALIDATION

Negative controls

All negative controls should be below the threshold. If there is a potential contamination (appearance of a curve in the negative control or a cluster of curves in specimens at high CT – for example above 36), results obtained are not interpretable and the whole run (including extraction) has to be repeated.

Positive controls

The positive control must show a positive (i. e. exponential) amplification curve. The positive control must fall below a CT of 30.

Extraction controls

All extraction controls must show a positive (i. e. exponential) amplification curve. The extraction control must fall below a CT of 35. If the extraction control is above CT

35, this points to a purification problem or a strong positive sample that can inhibit the EC. In the latter case, the assay is valid. If a water control run is performed, the EC must fall below a CT of 33.

Postive Control and the Negative Control do not contain Control DNA. Therefore, no amplification signal is detected.

Standards 1, 2 and 3

All standards must show a positive (i. e. exponential) amplification curve.

Standards 1, 2, 3 must show a CT: see certificate of analysis.

13 DATA ANALYSIS

The amplification specific for *Ruminococcus species* from the family of *Ruminococcaceae* is measured in the FAM channel (designated *Ruminococcaceae*), the amplification specific for *Ruminococcus species* from the family *Lachnospiraceae* in the ROX channel (designated *Lachnospiraceae*) and the *Prevotella species*-specific amplification in the Cy5 channel. For simplicity purposes, the channels will be designated *Ruminococcaceae* (FAM) and Lachnospiraceae (ROX).

The amplification of the control DNA is measured in the VIC®/HEX/JOE/TET channel.

The following results can occur:

- **A signal in the FAM, ROX and/or Cy5 channel is detected:**

  The result is positive, the sample contains bacterial DNA.

  In this case, detection of a signal of the control DNA in the VIC®/HEX/JOE/TET channel is inessential, as high concentrations of bacterial DNA may reduce or completely inhibit amplification of the control DNA .

- **No signal in the FAM, ROX and/or Cy5 channel, but a signal in the VIC®/HEX/JOE/TET channel is detected:**

  The result is negative, the sample does not contain bacterial DNA.

  The signal of the control DNA excludes the possibilities of DNA isolation failure and/or real time PCR inhibition. If the CT value of a sample differs significantly from the CT value of the water control, a partial inhibition occured, which can lead to negative results in weak positive samples (see „Troubleshooting“).

- **Neither in the FAM, ROX and/or Cy5 nor in the VIC®/HEX/JOE/TET channel a signal is detected:**

  A diagnostic statement cannot be made.

  The DNA isolation was not successful or an inhibition of the PCR has occurred.

Figure 1 and figure 2 show examples for positive and negative real time PCR results.

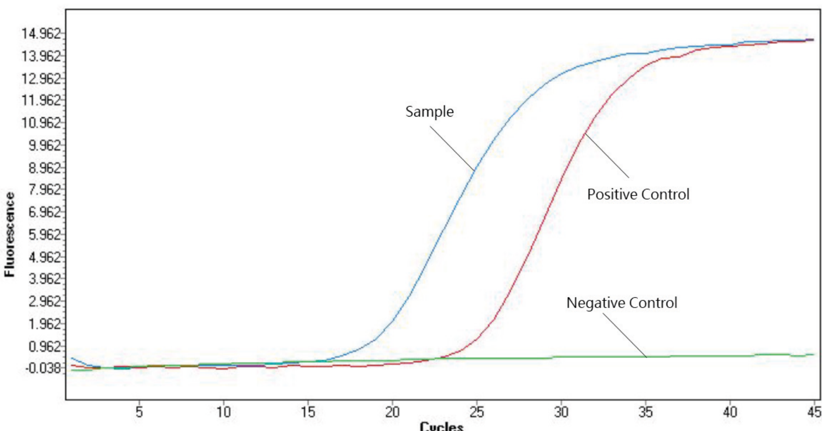

Figure 1: The positive sample shows bacteria-specific amplification in the FAM-/ROX-/Cy5 channel, whereas no fluorescence signal is detected in the negative control.

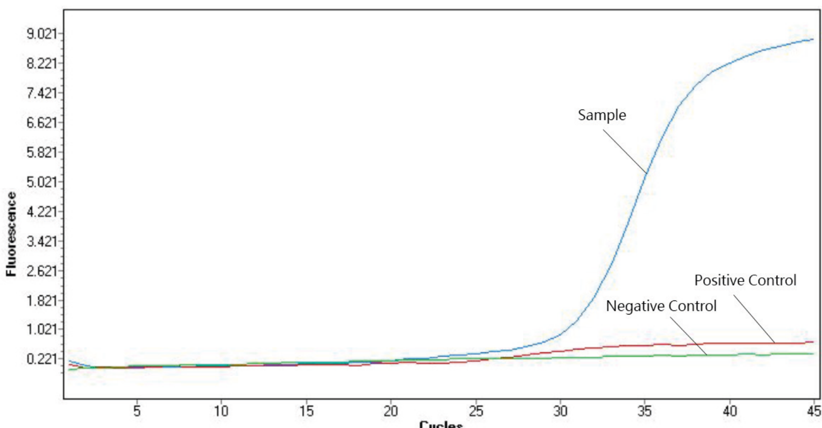

Figure 2: The sample shows a signal in the control DNA-specific VIC®/HEX/JOE/TET channel. The postive control and the negative control do not contain control DNA. Therefore, no amplification signal is detected.

14 QUANTIFICATION OF SAMPLES

For quantification of DNA positive for *Prevotella species*, *Ruminococcus species* from the family of *Ruminococcaceae* and the *Ruminococcus species* from the family of *Lachnospiraceae* in biological samples, a standard curve using standards 1, 2 and 3 must be applied. The standard curve needs to be saved separately on the real time PCR instrument. It can be imported and used in subsequent runs with kits of the same lot.

Note: the application of the standard curve needed once per each lot.

Using the quantitative MutaPLEX® PRE/RU/LA real time PCR, the DNA content of the respective bacteria are determined in copy number per reaction. Using correction factor K, the translation of copies/reaction to bacteria/g feces is made. The correction factor considers the dilution of the DNA extraction (dependent on the respective DNA extraction kit), the dilution of the PCR and the number of target sequences in the whole genome of *Prevotella species* and *Ruminococcus species* from the family of *Ruminococcaceae* and the *Ruminococcus species* from the family of *Lachnospiraceae*. For translation of the result of the PCR into bacterial load of the sample, the following formula is used:

$n_{Prevotella}$ (cells/g_{feces}) = n_{LC480} (copies/reaction) * $K_{Prevotella}$ (1/g)
$n_{Ruminococcaceae}$ (cells/g_{feces}) = n_{LC480} (copies/reaction) *$K_{Ruminococcaceae}$ (1/g)
$n_{Lachnospiraceae}$ (cells/g_{feces}) = n_{LC480} (copies/reaction) *$K_{Lachnospiraceae}$ (1/g)

n_{LC480} (copies/reaction) Calculated copy number per reaction by PCR Instrument, based on the CT value and the standard curve.

The calculation of the correction factor K requires the following parameters:

m_{Sample} [g]	Mass of the stool sample
b_{Buffer}	Dilution factor of the transport and storage buffer
$c_{Extraction}$	Correction factor for the extraction
V_{Eluate} [µl]	Total Eluate volume
a_{Eluate}	Dilution factor of the eluate
1/(4[µl])	Amount of µl used in the PCR reaction
1/(target copies)	Copies of target gene per genome

The target genes are 4 times present in *Prevotella species*, 4 times in *Ruminococcaceae* and 5 times in *Lachnospiraceae*.

$K_{Prevotella}$ (1/g) = 1/(m_{Sample} [g]) * b_{Buffer} * $c_{Extraction}$ * V_{Eluat} [µl] * a_{Eluate} * 1/(4[µl]) * 1/(target copies)

$K_{Ruminococcaceae}$ (1/g) = 1/(m_{Sample} [g]) * b_{Buffer} * $c_{Extraction}$ * V_{Eluat} [µl] * a_{Eluate} * 1/(4[µl]) * 1/(target copies)

$K_{Lachnospiraceae}$ (1/g) = 1/(m_{Sample} [g]) * b_{Buffer} * $c_{Extraction}$ * V_{Eluat} [µl] * a_{Eluate} * 1/(4[µl]) * 1/(target copies)

Table 6: Example for the calculation of correction factor K for *Prevotella species*

	Description	Factor
1/(m_{Sample} [g])	200 mg stool sample in 1 ml buffer	x 5
b_{Buffer}	250 µl stool buffer (incl. factor for density)	x 2.552
$c_{Extraction}$	Correction factor for extraction	x 50
V_{Eluat} [µl]	Volume of the eluate	x 100
a_{Eluat}	Dilution of the eluate	x 10
1/(4[µl])	4 µl sample volume for PCR	x 1/4
1/(target copies)	4 copies of target gene per genome	x 1/4
$K_{Prevotella}$ (1/g)	Correction factor for quantification	3.99×10^4

Table 7: Example for the calculation of correction factor K for *Ruminococcaceae*

	Description	Factor
1/(m_{Sample} [g])	200 mg stool sample in 1 ml buffer	x 5
b_{Buffer}	250 µl stool buffer (incl. factor for density)	x 2.552
$c_{Extraction}$	Correction factor for extraction	x 50
V_{Eluat} [µl]	Volume of the eluate	x 100
a_{Eluat}	Dilution of the eluate	x 10
1/(4[µl])	4 µl sample volume for PCR	x 1/4
1/(target copies)	4 copies of target gene per genome	x 1/4
$K_{Ruminococcaceae}$ (1/g)	Correction factor for quantification	3.99×10^4

Table 8: Example for the calculation of correction factor K for *Lachnospiraceae*

	Description	Factor
$1/(m_{Sample}\ [g])$	200 mg stool sample in 1 ml buffer	x 5
b_{Buffer}	250 µl stool buffer (incl. factor for density)	x 2.552
$c_{Extraction}$	Correction factor for extraction	x 50
$V_{Eluat}\ [\mu l]$	Volume of the eluate	x 100
a_{Eluat}	Dilution of the eluate	x 10
1/(4[µl])	4 µl sample volume for PCR	x 1/4
1/(target copies)	5 copies of target gene per genome	x 1/5
$K_{Lachnospiraceae}\ (1/g)$	Correction factor for quantification	3.19×10^4

15 LIMITATIONS

- Strict compliance with the instructions for use is required for optimal results.
- Use of this product is limited to personnel specially instructed and trained in the techniques of real-time PCR and *in vitro* diagnostic procedures.
- Good laboratory practice is essential for proper performance of this assay.
- All reagents should be closely monitored for impurity and contamination.
- Any suspicious reagents should be discarded.
- This assay must not be used on a biological specimen directly. Appropriate nucleic acid extraction methods have to be conducted prior to using this assay.
- The presence of PCR inhibitors may cause false negative or invalid results.
- Potential mutations within the target regions of the genomes of *Prevotella species*, *Ruminococcus species* from the family of *Ruminococcaceae* and the *Ruminococcus species* from the family of *Lachnospiraceae* covered by the primers and/or probes used in the kit may result in failure to detect the respective DNA.
- As with any diagnostic test, results of the MutaPLEX® PRE/RU/LA real time PCR kit need to be interpreted in consideration of all clinical and laboratory findings.

16 TROUBLESHOOTING

The following troubleshooting guide is included to help you with possible problems that may arise when performing a real time PCR.

No fluorescence signal in the FAM, ROX or Cy5 channel of the positive controls

The selected channel for analysis does not comply with the protocol

Select the Cy5 channel for analysis of the *Prevotella species*-specific amplification, the ROX channel for the *Lachnospiraceae*-specific amplification, the FAM channel for analysis of the *Ruminococcaceae*-specific amplification and the VIC®/HEX/JOE/TET channel for the amplification of the control DNA.

Incorrect configuration of the real time PCR

Check your work steps and compare with chapter "Procedure".

The programming of the thermal profile is incorrect

Compare the thermal profile with the protocol (table 4).

Incorrect storage conditions for one or more kit components or kit expired

Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in chapter "Transport, Storage and Stability".

Weak or no signal of the control DNA and simultaneous absence of a signal in the bacteria-specific FAM, ROX or Cy5 channel

real time PCR conditions do not comply with the protocol

Check the real time PCR conditions (table 4).

real time PCR inhibited

Make sure that you use an appropriate isolation method (see "Sample preparation") and follow the manufacturer's instructions. Make sure that the ethanol-containing washing buffer of the isolation kit has been completely removed. An additional centrifugation step at high speed is recommended before elution of the DNA.

DNA loss during isolation process

In case the control DNA was added before extraction, the lack of an amplification signal can indicate that the DNA isolation was not successful. Make sure that you use an appropriate isolation method (commercial kits are recommended) and stick to the manufacturer's protocol.

Incorrect storage conditions for one or more components or kit expired

Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in chapter "Transport, Storage and Stability".

Detection of a fluorescence signal in the FAM, ROX or Cy5 channel of the negative control

Contamination during preparation of the PCR

Repeat the real time PCR in replicates. If the result is negative in the repetition, the contamination occured when the samples were pipetted into the optical PCR reaction tubes. Make sure to pipet the positive control last and close the optical PCR reaction tube immediately after adding the sample. If the same result occurs, one or more of the kit components might be contaminated. Make sure that work space and instruments are decontaminated regularly. Use a new kit and repeat the real time PCR.

17 ABBREVIATIONS AND SYMBOLS

DNA	Deoxyribonucleid Acid	→REF	To be used with
CT	Cycle threshold	REF	Catalog number
PCR	Polymerase chain reaction	Σ	Contains sufficient for <n> test
REACTION MIX	Reaction Mix		Upper limit of temperature
CONTROL +	Positive control		Manufacturer
CONTROL –	Negative control		Use by
CONTROL DNA IC	Control DNA	i	Consult instructions for use
STANDARD 1	Standard 1	LOT	Lot number
STANDARD 2	Standard 2	CONT	Content
STANDARD 3	Standard 3		

18. LITERATURE

1. Tanaka S, Yoshida M, Murakami Y, et al. (2008). “The relationship of Prevotella intermedia, Prevotella nigrescens and Prevotella melaninogenica in the supragingival plaque of children, caries and oral malodor”. J Clin Pediatr Dent. 32 (3): 195–200.
2. De Filippo, C.; Cavalieri, D.; Di Paola, M.; Ramazzotti, M.; Poullet, J. B.; Massart, S.; Collini, S.; Pieraccini, G.; Lionetti, P. (2010). “The impact of diet in shaping gut microbiota is revealed by a comparative study in children from Europe and rural Africa” (PDF). Proceedings of the National Academy of Sciences. 107 (33): 14691–6.
3. Petrof EO, Gloor GB, Vanner SJ, Weese SJ, Carter D, Daigneault MC, et al. Stool substitute transplant therapy for the eradication of Clostridium difficile infection: “RePOOPulating” the gut. Microbiome (2013) 1:3.10.1186/2049-2618-1-3.
4. Cho I, Yamanishi S, Cox L, Methé BA, Zavadil J, Li K, et al. Antibiotics in early life alter the murine colonic microbiome and adiposity. Nature (2012) 488:621–6.10.1038/nature11400.