Manual

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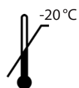
# MutaPLEX® AKM/FAEP

## real time PCR kit

***Test for the quantitative in vitro detection of DNA of Akkermansia muciniphila and Faecalibacterium prausnitzii in clinical specimens***

Valid from 2018-04-03



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# 1 INTENDED USE

The MutaPLEX® AKM/FAEP real time PCR kit is an assay for the quantitative detection of DNA of *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* in clinical specimens (stool samples) using real time PCR microplate systems. Using the quantitative MutaPLEX® AKM/FAEP real time PCR, the DNA content of the respective bacteria can be determined as copy number per reaction.

# 2 PATHOGEN INFORMATION

*Akkermansia muciniphila* is a gram-negative, strictly anaerobic, non-motile, non-spore-forming, oval-shaped bacterium. *A. muciniphila* is able to use mucin as its sole source of carbon and nitrogen, is culturable under anaerobic conditions on medium containing gastric mucin, and is able to colonise the gastrointestinal tracts of a number of animal species. *A. muciniphila* is believed to have anti-inflammatory effects in humans, and studies have shown inverse relationships between *A. muciniphila* colonisation and inflammatory conditions such as appendicitis or irritable bowel syndrome. Researchers have discovered that *A. muciniphila* may be able to be used to combat obesity and type 2 diabetes. The bacterium is naturally present in the human digestive tract at 3–5 %, but has been seen to fall with obesity. It is thought that eating the bacterium increases the gut wall thickness, with the addition of mucin, which will block food from being absorbed by the body. *A. muciniphila* corresponded to a reduction in inflammation, indicating a link between dietary fats, gut flora composition, and inflammation levels.

*Faecalibacterium* is a genus of gram-negative bacteria. Its sole known species, *Faecalibacterium prausnitzii*, is one of the most abundant and important commensal bacteria of the human gut microbiota. These bacteria produce butyrate and other short-chain fatty acids through the fermentation of dietary fiber. In healthy adults, *Faecalibacterium prausnitzii* represent more than 5 % of the bacteria in the intestine, making it one of the most common gut bacteria. It boosts our immune system, and many other things. Lower than usual levels of *F. prausnitzii* in the intestines have been associated with Crohn's Disease, obesity, asthma and Major Depressive Disorder.

# 3 PRINCIPLE OF THE TEST

The MutaPLEX® AKM/FAEP real time PCR kit contains specific primers and dual-labelled probes for the amplification and detection of DNA of *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* in clinical specimens. 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 (*Faecalibacterium prausnitzii*) and Cy5 channel (*Akkermansia muciniphila*).

Furthermore, MutaPLEX® AKM/FAEP 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 clinical 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 (KG1911-96) or 384 (KG1911-384) reactions, respectively.

Table 1: Components of the MutaPLEX® AKM/FAEP real time PCR kit .

| Label | Lid Colour | Content | |
|---|---|---|---|
| | | 96 | 384 |
| Reaction Mix | yellow | 1 x 1536 µl | 4 x 1536 µl |
| Positive Control | red | 1 x 100 µl | |
| Negative Control | green | 1 x 100 µl | |
| Control DNA | colourless | 1 x 480 µl | 4 x 480 µl |
| Standard 1 | black | 1 x 100 µl | once per lot* |
| Standard 2 | purple | 1 x 100 µl | |
| Standard 3 | orange | 1 x 100 µl | |

* Standards (sufficient for 25 standard curves) can be ordered separately using the article no KG1911-STD

# 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® AKM/FAEP 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® AKM/FAEP real time PCR must be performed by qualified personnel only.
- Good Laboratory Practice (GLP) has to be applied.
- Clinical samples must always be regarded as potentially infectious material and all equipment used has to be treated as potentially contaminated.

## 8 GENERAL PRECAUTIONS

- Stick to the protocol described in the instructions for use.
- Set up different laboratory areas for the preparation of samples and for the set up of the PCR in order to avoid contaminations.
- Pipettes, tubes and other materials must not circulate between those different laboratory areas.
- Always use filter tips.
- Regulary decontaminate equipment and benches with ethanol-free decontaminant.
- Do not combine MutaPLEX® AKM/FAEP real time PCR kit components of different lot numbers.

## 9 SAMPLE MATERIAL

Starting material for the MutaPLEX® AKM/FAEP real time PCR is nucleic acid isolated from clinical specimens (stool samples).

# 10 SAMPLE PREPARATION

The MutaPLEX® AKM/FAEP real time PCR is suitable for the detection of the DNA of *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* isolated from clinical 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.

**Please note chapter 11 "Control DNA".**

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

# 11 CONTROL DNA

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

## *DNA isolation from clinical specimens*

### Control DNA used as extraction control

MutaPLEX® AKM/FAEP 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.**

# 12 REAL TIME PCR

## *12.1 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 *Akkermansia muciniphila*- und *Faecalibacterium prausnitzii*-positive DNA in clinical 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.

## *12.2 Procedure*

The control DNA was added during DNA extraction (see chapter 11 "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 |

## *12.3 Instrument settings*

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

Table 4: real time PCR thermal profile

| Description | Time | Temperature | No of cycles |
|---|---|---|---|
| Initial Denaturation | 5 min | 95 °C | 1 |
| Amplification of DNA | | | 45 |
| Denaturation | 10 s | 95 °C | |
| Annealing and Extension | 40 s<br>Aquisition at the end of this step | 60 °C | |

The MutaPLEX® AKM/FAEP 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® AKM/FAEP real time PCR.

| Real time PCR Instrument | Parameter | Detection channel | Notes | | |
|---|---|---|---|---|---|
| | | | Colour Compensation Kit not required | | |
| | | | **Melt Factor** | **Quant Factor** | **Max Integration Time [s]** |
| LightCycler 480II | *F. prausnitzii* | FAM (465–510) | 1 | 10 | 1 |
| | - | ROX (533–610) | 1 | 10 | 2 |
| | *Control DNA* | HEX (533–580) | 1 | 10 | 2 |
| | *A. muciniphila* | 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: 1 x $10^6$ copies/reaction
- Standard 2: 1 x $10^4$ copies/reaction
- Standard 3: 1 x $10^2$ copies/reaction

# 13 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.

## 14 DATA ANALYSIS

The *Faecalibacterium prausnitzii*-specific amplification is measured in the FAM channel, and the *Akkermansia muciniphila*-specific amplification in the Cy5 channel.

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 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 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 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.

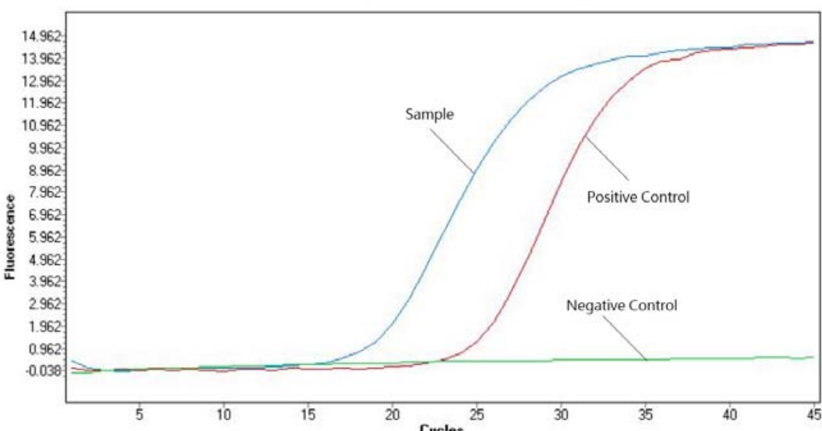


Figure 1: The positive sample shows bacteria-specific amplification in the FAM/Cy5 channel, whereas no fluorescence signal is detected in the negative control.

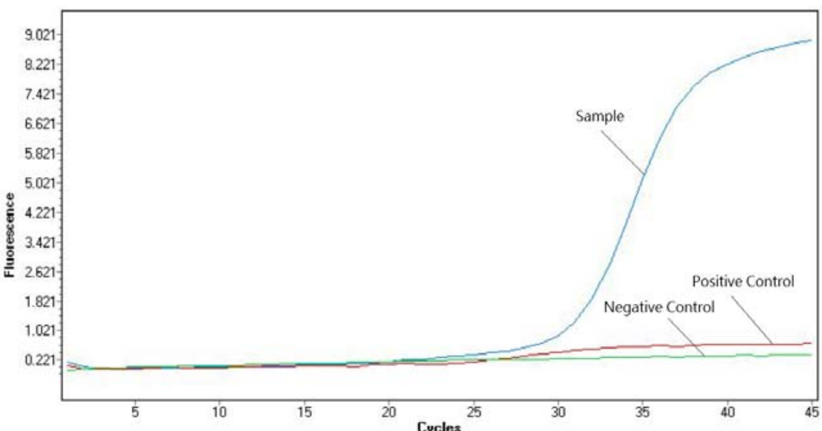


Figure 2: The sample shows a signal in the control DNA-specific VIC®/HEX/JOE/TET channel. The positive control and the negative control do not contain control DNA. Therefore, no amplification signal is detected.

# 15 QUANTIFICATION OF SAMPLES

For quantification of *Akkermansia muciniphila*- and *Faecalibacterium prausnitzii*-positive DNA in clinical 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® AKM/FAEP 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 *Akkermansia muciniphila* and *Faecalibacterium prausnitzii*. For translation of the result of the PCR into bacterial load of the sample, the following formula is used:

$n_{A.\ muciniphila}$ (cells/$g_{feces}$) = $n_{LC480}$ (copies/reaction) * $K_{A.\ muciniphila}$ (1/g)

$n_{F.\ prausnitzii}$ (cells/$g_{feces}$) = $n_{LC480}$ (copies/reaction) *$K_{F.\ prausnitzii}$ (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 3 times present in *A. muciniphila* and 6 times in *F. prausnitzii*. |

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

$K_{F.\ prausnitzii}$ (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 *Akkermansia muciniphila*

| | 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) | 3 copies of target gene per genome | x 1/3 |
| $K_{A.\ muciniphila}$ (1/g) | Correction factor for quantification | $5.32 \times 10^4$ |

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

| | 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) | 6 copies of target gene per genome | x 1/6 |
| $K_{F.\ prausnitzii}$ (1/g) | Correction factor for quantification | $2.66 \times 10^4$ |

## 16 LIMITATIONS OF THE METHOD

The results must always be considered in relation to the clinical symptoms. Therapeutical consequences should be made in consideration of clinical data.

## 17 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 or Cy5 channel of the positive controls**

***The selected channel for analysis does not comply with the protocol***

Select the FAM channel for analysis of the *Faecalibacterium prausnitzii*-specific amplification, the Cy5 channel for analysis of the *Akkermansia muciniphila*-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 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 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.

## 18 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 | IVD | In vitro diagnostic medical device |

## 19. LITERATURE

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