Manual

MutaPLEX®
BV-1 quant. (LAC/AV/GV)

real-time-PCR Kit

For in vitro detection and quantification of the DNA of Gardnerella vaginalis, Atopobium vaginae and Lactobacillus species extracted from biological specimens.

Valid from 2022-08-10

REF **KG197596**

Σ 96

-20 °C

IVD

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1 INTENDED PURPOSE

The MutaPLEX® BV-1 quant. (LAC/AV/GV) real time PCR Kit is designed for the quantitative detection of the nucleic acid of *Gardnerella vaginalis*, *Atopobium vaginae* and *Lactobacillus species* in eluates from biological specimens. The assay is an in vitro diagnostic medical device and intended to be used by professional users in a laboratory environment. It can be performed manually or using an automated platform. The assay serves as an aid in the diagnosis, screening and monitoring of bacterial vaginosis.

2 PATHOGEN INFORMATION

Gardnerella vaginalis is a facultative anaerobic, coccoid bacterium of the family of Bifidobacteriaceae. In low copy numbers it is part of the common vaginal flora. When the equilibrium of the vaginal microbiome is disturbed, the number of *G. vaginalis* can increase a lot. *G. vaginalis* is not considered to be the cause of bacterial vaginoses [1] [2] but it can be used as a reporter for the altered vaginal microbiome.

Atopobium vaginae is a facultative anaerobic, rod-shaped bacterium of the family of *Atopobiaceae*. It is also known as *Fannyhessea vaginae* and isolated in a lot of cases of bacterial vaginosis [3]. *A. vaginae* also seems to be implicated in treatment failures and birth risks.

Lactobacilli, namely *L. crispatus, L. gasseri, L. jenseni* and *L. iners* are rod-shaped aerotolerant anaerobes and represent the dominant bacteria clade in the human vaginal microbiome. In cases of bacterial vaginosis, the number of *lactobacilli* is often declined and the equilibrium in the vaginal microbiome disturbed. This results usually in a loss of acidity.

3 PRINCIPLE OF THE TEST

The MutaPLEX® BV-1 quant. (LAC/AV/GV) real time PCR Kit contains specific primers and dual-labeled probes for the amplification of the DNA of *Gardnerella vaginalis*, *Atopobium vaginae* and *Lactobacillus species* extracted from biological specimens.

The presence of nucleic acid is detected by an increase in fluorescence due to hydrolysis of the probes during amplification. The fluorescence of the *Lactobacillus species* specific probes is measured in the FAM channel. The fluorescence of the *Gardnerella vaginalis* specific probes is measured in the ROX channel. The fluorescence of the *Atopobium vaginae* specific probes is measured in the Cy5 channel.

The simple presence of the *Gardnerella vaginalis* or *Atopobium vaginae* does not compulsory imply a bacterial vaginosis. The diagnosis is dependent on the proportions of the different bacteria in relation to each other.

For the quantification of the different bacteria, the MutaPLEX® BV-1 quant. (LAC/AV/GV) real time PCR Kit contains three quantification standards. Each quantification standard includes synthetic DNA of all of the three individual target sequences.

Furthermore, the MutaPLEX® BV-1 quant. (LAC/AV/GV) real time PCR Kit contains a Control DNA (Internal Process Control, IPC), which is added during DNA extraction and detected in the same reaction by a HEX-labeled probe.

The Control DNA allows the detection of PCR inhibition and acts as control, that the nucleic acid was isolated from the biological specimen.

4 PACKAGE CONTENTS

The reagents supplied are sufficient for 96 reactions.

Table 1: Components of the MutaPLEX® BV-1 quant. (LAC/AV/GV) real time PCR Kit

Label	Lid Colour	Content	Concentration copies/µl
Reaction Mix	yellow	1 x 1 344 µl	-
Positive Control	red	1 x 150 µl	-
Negative Control	green	1 x 150 µl	-
Control DNA	colourless	1 x 480 µl	-
Standard 1	black	1 x 100 µl	100,000
Standard 2	violett	1 x 100 µl	10,000
Standard 3	orange	1 x 100 µl	1,000

5 EQUIPMENT AND REAGENTS TO BE SUPPLIED BY USER

- DNA isolation kit (e. g. MutaCLEAN® Mag RNA/DNA, KG1023 or KG1024).
- PCR grade water
- Sterile microtubes
- Calibrated precision pipets (adjustable volume) and sterile single-use tips with filter
- Table centrifuge
- Vortex
- Real time PCR instrument
- Optical PCR reaction tubes with lid or optical PCR reaction plate with optical foil
- Optional: Liquid handling system for automation

6 TRANSPORT, STORAGE AND STABILITY

The MutaPLEX® BV-1 quant. (LAC/AV/GV) 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. Protect kit components from direct sunlight during the complete test run.

7 WARNINGS AND PRECAUTIONS

Read the Instruction for Use carefully before using the product.

Before first use check the product and its components for:

- Use of this product is limited to personnel specially instructed and trained in the techniques of Real-Time PCR procedures.
- Specimens should always be treated as infectious and/or biohazardous in accordance with safe laboratory procedures.
- 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.
- Dedicate 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 organisations.
- 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® BV-1 quant. (LAC/AV/GV) real time PCR Kit components of different lot numbers.

8 SAMPLE MATERIAL

Starting material for MutaPLEX® BV-1 quant. (LAC/AV/GV) real time PCR Kit is DNA isolated from biological specimens. Common samples for the DNA extraction for the analysis of the vaginal microbiome are vaginal swabs or urine.

9 SAMPLE PREPARATION

Commercial kits for DNA isolation such as the following are recommended:

- MutaCLEAN® Mag RNA/DNA, Immundiagnostik Cat. No. KG1023 or KG1024

Please follow the Instructions for Use of the respective extraction kit.

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 the chapter ‚Control DNA'.

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

10 CONTROL DNA

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

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 the chapter 7 ‚Warnings and Precautions'.
- 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.

11.2 Quantification Standards

The MutaPLEX® BV-1 quant. (LAC/AV/GV) real time PCR Kit includes three Quantification Standards. Use 6 µl of each Quantification Standard to evaluate the target concentrations of the PCR targets. The concentrations are calculated according to chapter 'Data Analysis'.

The Quantification Standards can be used in every single experiment or saved in a standard curve file on the qPCR instrument. It is recommended to produce a new Quantification Standard file for each new lot.

11.3 Procedure

The Master Mix contains all of the components needed for the real time 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

Volume per reaction	Volume master mix
14.0 µl Reaction Mix	14.0 µl x (N+1)

11.4 Real time PCR set-up

- Place the number of optical PCR reaction tubes needed into the respective tray of the real time PCR instrument / take an optical PCR reaction plate.
- Pipet **14 µl** of the Master Mix into each optical PCR reaction tube / the optical PCR reaction plate.

- Add **6 µl** of the eluates from the DNA isolation (including the eluate of the water control), the Positive Control, the Negative Control and the Standards (optional) to the corresponding optical PCR reaction tube / the optical PCR reaction plate (Table 3).
- Close the optical PCR reaction tubes / the optical PCR reaction plate immediately after filling in order to reduce the risk of contamination.

Table 3 Preparation of the real-time-PCR

Component	Volume
Master mix	14.0 µl
Sample	6.0 µl
Total volume	20.0 µl

11.5 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	Number of Cycles	Aquisition
Initial Denaturation	5 min	95 °C	1	no
Amplification of DNA				
Denaturation	10 sec	95 °C	45	no
Annealing and Extension	40 sec	60 °C		end of step

If in the same run samples should be tested for pathogens with RNA genome, use the thermal profile shown in Table 5.

Table 5: real-time-RT-PCR thermal profile

Description	Time	Temperature	Number of Cycles	Aquisition
Reverse Transcription	10 min	45 °C	1	no
Initial Denaturation	5 min	95 °C	1	no
Amplification of DNA				
Denaturation	10 sec	95 °C	45	no
Annealing and Extension	40 sec	60 °C		end of step

Dependent on the real time instrument used, further instrument settings have to be adjusted according to Table 6.

Table 6 Overview of the instrument settings required for the MutaPLEX® BV-1 quant. (LAC/AV/GV) real time PCR.

<table>
<tr><th>Real time PCR instrument</th><th>Parameter</th><th>Detection channel</th><th colspan="2">Notes</th></tr>
<tr><td>LightCycler 480II</td><td>Lactobacillus spp.
Control DNA (IPC)
Gardnerella vaginalis
Atopobium vaginae</td><td>465–510
533–580
533–610
618–660</td><td colspan="2">Colour Compensation Kit MutaPLEX® CC-1 (KG19-5-CC) required
<table><tr><th>Melt factor</th><th>Quant factor</th><th>Max integration time (s)</th></tr><tr><td>1</td><td>10</td><td>1</td></tr><tr><td>1</td><td>10</td><td>2</td></tr><tr><td>1</td><td>10</td><td>2</td></tr><tr><td>1</td><td>10</td><td>3</td></tr></table></td></tr>
<tr><td>Stratagene Mx3000P/ Mx3005P</td><td>Lactobacillus spp.
Control DNA (IPC)
Gardnerella vaginalis
Atopobium vaginae</td><td>FAM
HEX
ROX
Cy5</td><td>Gain 8
Gain 1
Gain 1
Gain 4</td><td>Reference Dye: None</td></tr>
<tr><td>QuantStudio 5
CFX96
CFX Opus96
Aria Mx
qTower^3 G</td><td>Lactobacillus spp.
Control DNA (IPC)
Gardnerella vaginalis
Atopobium vaginae</td><td>FAM
HEX
ROX
Cy5</td><td colspan="2">Option Reference Dye ROX: NO</td></tr>
<tr><td>Mic qPCR Cycler</td><td>Lactobacillus spp.
Control DNA (IPC)
Gardnerella vaginalis
Atopobium vaginae</td><td>Green
Yellow
Orange
Red</td><td colspan="2">Gain 8
Gain 10
Gain 10
Gain 10</td></tr>
</table>

12 DATA ANALYSIS

Following results can occur:

Signal / C_T Values				Interpretation
FAM Channel	**Cy5 Channel**	**ROX Channel**	**HEX Channel**	
Lactobacillus spp.	***Atopobium vaginae***	***Gardnerella vaginalis***	**Control DNA (IPC)**	
positive (one or more parameters)			positive or negative [1]	**For Interpretation, comparison of the calculated quantities is needed.**
negative	negative	negative	**≤ 34** [2]	**Negative result.** The sample contains no *Lactobacillus spp.*, *A. vaginae* or *G. vaginalis* DNA.
negative	negative	negative	negative or > 34 [2]	**No diagnostic statement can be made.** The real time PCR is either inhibited or errors occurred while RNA/DNA extraction.

[1] A strong positive signal in the FAM, ROX or the Cy5 can inhibit the IPC. In such cases the result for the Control DNA can be neglected.

[2] In case of high C_T values, the IPC should be compared to the water extraction control as described in the chapter 'Assay validation'.

12.1 Quantitative Analysis

The MutaPLEX® BV-1 quant. (LAC/AV/GV) real time PCR Kit includes three Quantification Standards. The calculation of the bacterial load can be done manually or, in most PCR cyclers, directly in the instrument software by the definition of quantification standards. The result will show the concentration of the specific bacterial load in the eluate.

$$C_T = a * \log(N) + b$$

$$N = 10^{\frac{(C_T - b)}{a}}$$

C_T	Threshold Cycle	a	Slope
N	copy number [copies/µl]	b	Intercept

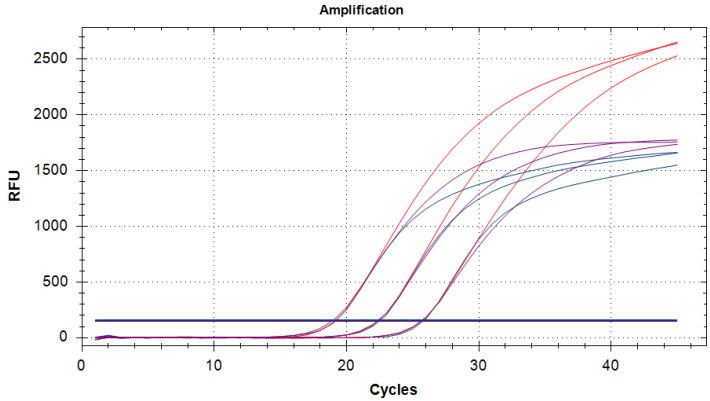

Figure 1: The amplification curves of the three standards for *Lactobacillus species* (FAM channel, blue), *Gardnerella vaginalis* (ROX channel, red) and *Atopobium vaginae* (Cy5 channel, purple) with 100,000 copies per µl (Standard 1), 10,000 copies per µl (Standard 2) and 1,000 copies per µl (Standard 3).

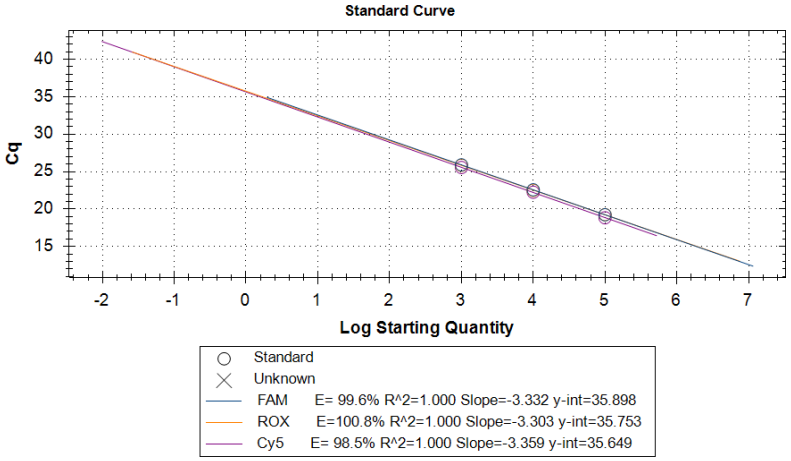

Figure 2: The C_T values of the three standards for *Lactobacillus species* (FAM channel), *Gardnerella vaginalis* (ROX channel) and *Atopobium vaginae* (Cy5 channel) plotted against the target copies per µl.

12.2 Interpretation of the Quantitative Analysis

The diagnosis of bacterial vaginosis is dependent on the proportions of the different bacteria in relation to each other. This is usually addressed by the Amsel criteria and the Nugent score, both microscopic methods. Up to now, there is no strict rule how qPCR matches those methods, but recent publications indicate *Atopobium vaginae* and *Gardnerella vaginalis* in comparison to *Lactobacilli* as useful to identify bacterial vaginosis [4].

To show potential outcomes of the MutaPLEX® BV-1 quant. (LAC/AV/GV) real time PCR Kit, 4 samples are listed in Table 6. The corresponding amplification curves are presented in the following figures.

	Lactobacillus species	***Gardnerella vaginals***	***Atopobium vaginae***	Percentage *Lactobacillus species*
	copies/µl	**copies/µl**	**copies/µl**	
Sample A	1.4×10^6	-	-	100 %
Sample B	1.025×10^6	1.003×10^5	-	90.8 %
Sample C	9.719×10^3	1.090×10^3	5.236×10^5	1.7 %
Sample D	1.66×10^3	8.331×10^4	-	2.3 %

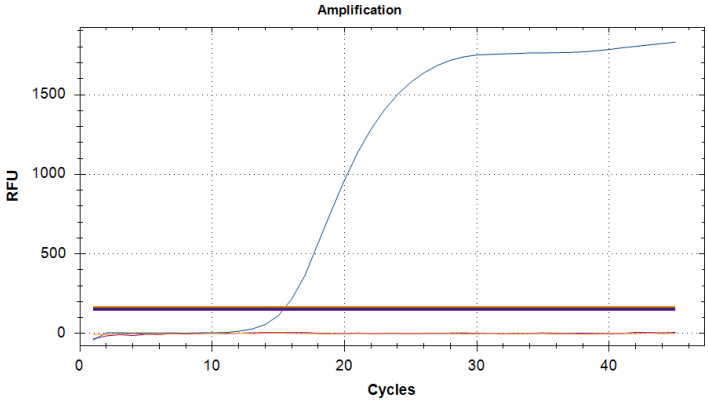

Figure 3: Sample A, only *Lactobacillus species* (blue) are present, the equilibrium of the vaginal microbiome is not affected by *Atopobium vaginae* or *Gardnerella vaginalis*.

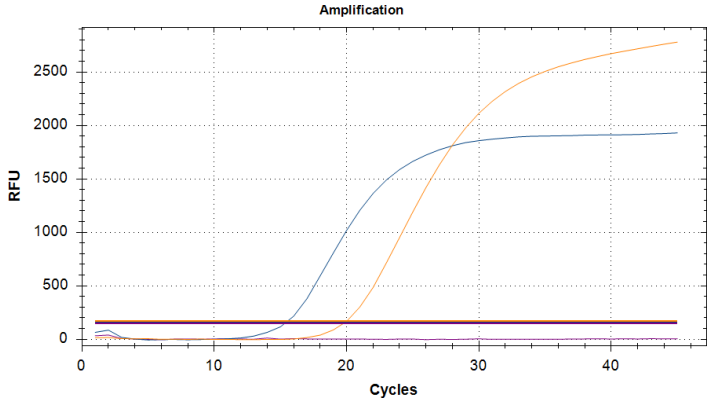

Figure 4: Sample B, high copy numbers for *Lactobacillus species* (blue) and *Gardnerella vaginalis* (orange). *Lactobacillus species* represent still 90.8% of the detected bacteria. The equilibrium of the vaginal microbiome seems to be unaffected or only slightly affected by the presence of *Gardnerella vaginalis.*

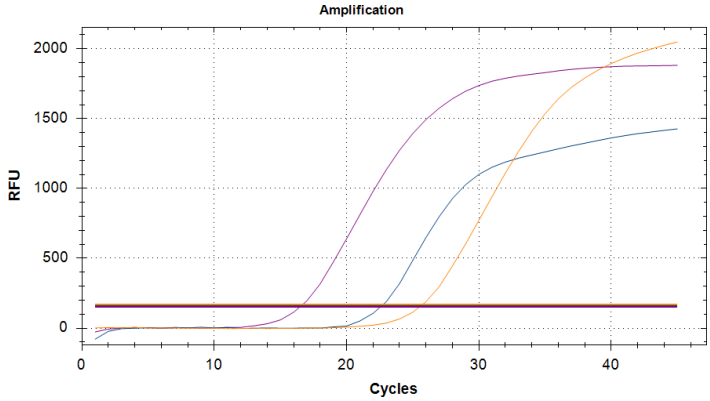

Figure 5: Sample C, very high copy numbers for *Atopobium vaginae* (purple). Medium copy numbers for *Lactobacillus species* (blue) and *Gardnerella vaginalis* (orange). *Lactobacillus species* represent 1.7 % of the detected bacteria. The equilibrium of the vaginal microbiome seems to be affected, especially by *Atopobium vaginae.*

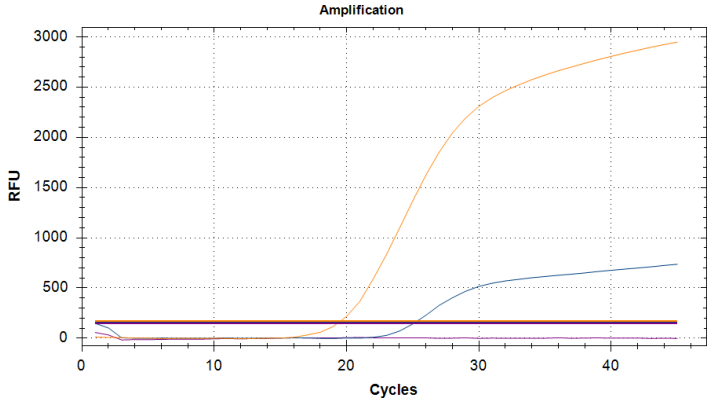

Figure 6: Sample D, high copy numbers for *Gardnerella vaginalis* (orange). Medium copy numbers for *Lactobacillus species* (blue). *Lactobacillus species* represent 2.3% of the detected bacteria. The equilibrium of the vaginal microbiome seems to be intermediate affected by *Gardnerella vaginalis*.

13 ASSAY VALIDATION

Negative Control

The Negative Control must show no C_T in the FAM, Cy5, ROX and HEX channel.

Positive control

The Positive Control must show a positive (i.e. exponential) amplification curve in the different channels FAM, Cy5 and ROX. The Positive Control must fall below C_T 30.

Internal Controls

The following values for the amplification of the internal controls are valid using Immundiagnostik nucleic acid extraction kit MutaCLEAN® Mag RNA/DNA. The Control DNA (IPC) must show a positive (i.e. exponential) amplification curve.

The IPC must fall below a C_T of 34. If the IPC is above C_T 34 this points to a purification problem or a strong positive sample that can inhibit the IPC. In the latter case, the assay is valid. It is recommended to perform the extraction of a water control in each run. The IPC in the water control must fall below a C_T of 34.

If other nucleic acid extraction kits are used, the customer must define own cutoffs. In this case the C_T value of the Control DNA (IPC) in an eluate from a sample should not be delayed for more than 4 C_T in comparison to an eluate from an extracted water control.

14 LIMITATIONS OF THE METHOD

- 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.
- As with any diagnostic test, results of the MutaPLEX® BV-1 quant. (LAC/AV/GV) real time PCR Kit need to be interpreted in consideration of all clinical and laboratory findings.

15 TROUBLESHOOTING

The following troubleshooting guide is included to help you with possible problems that may arise when performing a real-time PCR. If you have further questions, please do not hesitate to contact our scientists on info@immundiagnostik.com.

No fluorescence signal in the FAM, Cy5, ROX channel of the Positive Control.

The selected channel for analysis does not comply with the protocol

Select the FAM channel for analysis of *Lactobacillus specific* amplification, the Cy5 channel for the *A. vaginae* specific amplification, the ROX channel for the *G. vaginalis* specific amplification and the HEX channel for the amplification of the Control DNA (IPC).

Incorrect configuration of the real-time-PCR

Check your work steps and compare with chapter 11.

The programming of the thermal profile is incorrect

Compare the thermal profile with the protocol ‘in chapter 11.4.

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 'Transport, storage and stability' (chapter 6).

Weak or no signal of the Control DNA (IPC) and simultaneous absence of a signal in the specific FAM and/or Cy5 and/or ROX channel.

real time PCR conditions do not comply with the protocol

Check the real time PCR conditions in Table 4.

real time PCR inhibited

Make sure that you use an appropriate isolation method (see chapter 'Sample Preparation') and follow the manufacturer's instructions. Make sure that the ethanol-containing washing buffers have been completely removed.

sample material not sufficient

Make sure that enough sample material has been applied to the extraction. Use an appropriate isolation method (see chapter 9 - 'Sample Preparation') and follow the manufacturer's instructions.

DNA loss during isolation process

Lack of an amplification signal in the HEX channel 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 'Transport, Storage and Stability (chapter 6).

Detection of a fluorescence signal in the FAM and/or ROX and/or Cy5 channel of the Negative Control.

Contamination during preparation of the real-time PCR

Repeat the real time PCR in replicates. If the result is negative in the repetition, the contamination occurred 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 re-

sult 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.

16 KIT PERFORMANCE

16.1 Analytical Sensitivity

For the FAM, ROX and Cy5 channels, the limits of detection (LoD) of MutaPLEX® BV-1 quant. (LAC/AV/GV) real time PCR Kit were determined using serial dilutions of the of synthetic DNA-fragments containing the specific gene target sequence. The determination of the LoD was done on a Biorad CFX Opus 96 Instrument (Bio-Rad).

The LoD of MutaPLEX® BV-1 quant. (LAC/AV/GV) real time PCR Kit is ≤ 2.5 genome copies per µl for the FAM, Cy5 and ROX channel.

16.2 Analytical Specificity

The specificity of the MutaPLEX® BV-1 quant. (LAC/AV/GV) real time PCR Kit was evaluated with different ring trial samples of known status and different other relevant viruses and bacteria found in biological samples and basing on in silico analyses.

All ring trial samples and other eluates with known status were detected correctly. Results are shown in Table 8 and Table 9.

Table 8: Ring trial samples tested for the validation of the sensitivity and specificity of the MutaPLEX® BV-1 quant. (LAC/AV/GV) real time PCR Kit.

sample	***Lactobacillus species***	***Atopobium vaginae***	***Gardnerella vaginalis***
	FAM channel	**Cy5 channel**	**ROX channel**
QCMD 2020 Sexually Transmitted Infections I			
STI_I101S-01 Trichomonas vaginalis	negative	negative	negative
STI_I101S-02 Mycoplasma hominis	negative	negative	negative
STI_I101S-03 G. vaginalis + T. vaginalis	negative	negative	***positive***
STI_I101S-04 M. genitalium (drug resistant)	negative	negative	negative
STI_I101S-05 M. genitalium (wild type)	negative	negative	negative
STI_I101S-06 Negative	negative	negative	negative
STI_I101S-07 Gardnerella vaginalis	negative	negative	***positive***
STI_I101S-08 Trichomonas vaginalis	negative	negative	negative
STI_I101S-09 M. hominis + C. trachomatis	negative	negative	negative

sample	*Lactobacillus species*	*Atopobium vaginae*	*Gardnerella vaginalis*
	FAM channel	**Cy5 channel**	**ROX channel**
STI_I101S-10 Trichomonas vaginalis	negative	negative	negative

Table 9: Eluted DNA/RNA from bacterial and viral pathogens tested for the determination of the analytical specificity of MutaPLEX® BV-1 quant. (LAC/AV/GV) real time PCR Kit.

Eluates with known status	MutaPLEX® BV-1 quant. (LAC/AV/GV)		
	Lactobacillus species	*Atopobium vaginae*	*Gardnerella vaginalis*
	FAM channel	**Cy5 channel**	**ROX channel**
Chlamydia pneumoniae	negative	negative	negative
Chlamydia trachomatis	negative	negative	negative
Cytomegalievirus	negative	negative	negative
Gardnerella vaginalis	negative	negative	***positive***
Herpes Simplex Virus Type 1	negative	negative	negative
Herpes Simplex Virus Type 2	negative	negative	negative
Mycoplasma genitalium	negative	negative	negative
Mycoplasma hominis	negative	negative	negative
Mycoplasma pneumoniae	negative	negative	negative
Neisseria gonorrhoeae	negative	negative	negative
Trichomonas vaginalis	negative	negative	negative
Ureaplasma parvum	negative	negative	negative
Ureaplasma urealyticum	negative	negative	negative
Varicella zoster virus Genotype 3	negative	negative	negative
Varicella zoster virus Genotype 5	negative	negative	negative

16.3 Linear Range

The linear range of the MutaPLEX® BV-1 quant. (LAC/AV/GV) real time PCR Kit was evaluated by analysing logarithmic dilution series of quantified synthetic DNAs of the target sequences.

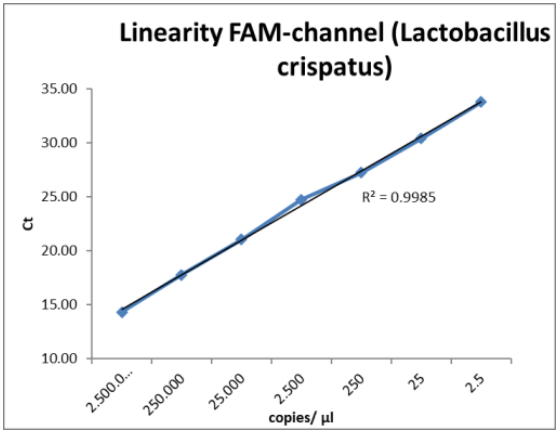

Figure 7: Determination of the linear range of MutaPLEX® BV-1 quant. (LAC/AV/GV) real time PCR Kit for *Lactobacillus crispatus* in the FAM channel.

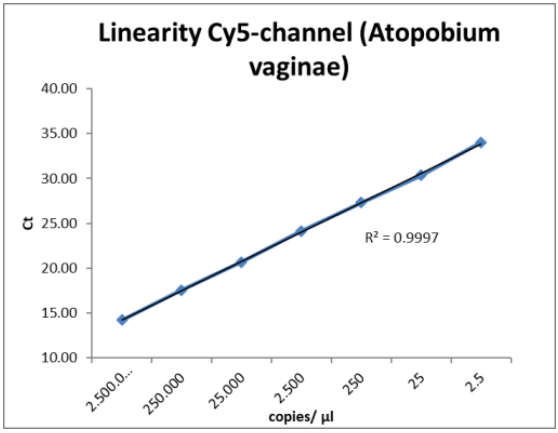

Figure 8: Determination of the linear range of MutaPLEX® BV-1 quant. (LAC/AV/GV) real time PCR Kit for *Atopobium vaginae* in the Cy5 channel

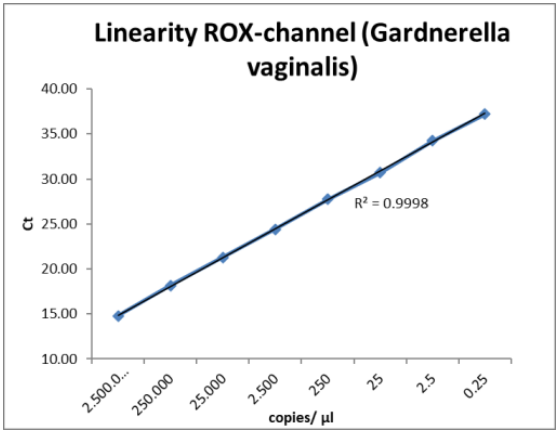

Figure 9: Determination of the linear range of MutaPLEX® BV-1 quant. (LAC/AV/GV) real time PCR Kit for *Gardnerella vaginalis* in the ROX channel.

16.4 Precision

The precision of the MutaPLEX® BV-1 quant. (LAC/AV/GV) real time PCR Kit was determined as intra-assay variability, inter-assay variability and inter-lot variability.

Variability data are expressed by standard deviation and coefficient of variation. The data are based on quantification analyses of defined concentrations of *Lactobacillus specific* synthetic DNA, *A. vaginae* specific synthetic DNA and *G. vaginalis* specific synthetic DNA and additionally on the threshold cycle of the Control DNA (IPC). The results are shown in Table 10.

Table 10: Precision of the MutaPLEX® BV-1 quant. (LAC/AV/GV) real time PCR Kit.

***Lactobacillus crispatus* (FAM)**	**copies/µl**	**Standard Deviation**	**Coefficient of Variation [%]**
Intra-Assay Variability	2.5	0.36	1.05
Inter-Assay-Variability	2.5	0.41	1.22
Inter-Lot-Variability	2.5	0.09	0.27
***Atopobium vaginae* (Cy5)**	**copies/µl**	**Standard Deviation**	**Coefficient of Variation [%]**
Intra-Assay Variability	2.5	0.37	1.07
Inter-Assay-Variability	2.5	0.32	0.96
Inter-Lot-Variability	2.5	0.44	1.28
***Gardnerella vaginalis* (ROX)**	**copies/µl**	**Standard Deviation**	**Coefficient of Variation [%]**
Intra-Assay Variability	2.5	0.43	1.25
Inter-Assay-Variability	2.5	0.20	0.59
Inter-Lot-Variability	2.5	0.03	0.10
IPC (HEX)	**copies/µl**	**Standard Deviation**	**Coefficient of Variation [%]**
Intra-Assay Variability	2500	0.09	0.33
Inter-Assay-Variability	2500	0.20	0.70
Inter-Lot-Variability	2500	0.07	0.24

16.5 Diagnostic Sensitivity

The diagnostic sensitivity of real time (RT-)PCR assays is mainly dependent on the DNA/RNA extraction method used to isolate DNA and RNA from various biological specimens. DNA/RNA extraction reagents are not part of the Immundiagnostik real time (RT-)PCR kits. Immundiagnostik real time (RT-)PCR kits include an extraction control and guidelines for the validation criteria of the extraction control in each reaction. The extraction control indicates inhibition of the real time (RT-)PCR and/or inefficient nucleic acid extraction. It cannot be used as a calibrator.

Therefore, Immundiagnostik guarantees the analytical sensitivities and specificities of the real time (RT-)PCR kits, performed with eluted DNA and RNA from reference materials and ring trial samples and with synthetic nucleic acid fragments. Immun-

diagnostik does not guarantee diagnostic sensitivities. If diagnostic sensitivities are mentioned in manuals of Immundiagnostik real time (RT-)PCR kits, the data are strictly correlated to a specific nucleic acid extraction method that has been used during the validation of the respective kits and cannot be transferred to other extraction methods. It is the responsibility of the user to qualify the extraction methods used for DNA/RNA isolation from biological samples.

17 LITERATURE

[1] Schwebke et al. (2014). Role of Gardnerella vaginalis in the Pathogenesis of Bacterial Vaginosis: A Conceptual Model. JID 2014:210 (1. August)

[2] Hickey et al. (2014). Gardnerella vaginalis Does Not Always Cause Bacterial Vaginosis. JID 2014:210 (15. November)

[3] Polatti (2012). Bacterial Vaginosis, Atopobium vaginae and Nifuratel. CCP 2012, 7, 36-40

[4] Loquet et al. (2021). Classification and Regression Trees for Bacterial Vaginosis Diagnosis in Pregnant Women Based on High-Throughput Quantitative PCR. JMD Vol.23 No.2 (February)

18 ABBREVIATIONS AND SYMBOLS

DNA	(complementary) Deoxyribonucleic acid	REF	Catalog number
PCR	Polymerase chain reaction	→REF	To be used with
REACTION MIX	Reaction mix		Contains sufficient for <n> test
CONTROL +	Positive Control		Upper limit of temperature
CONTROL –	Negative Control		Manufacturer
CONTROL DNA IPC	Control DNA (IPC)		Use by
STANDARD 1	Standard 1	LOT	Lot number
STANDARD 2	Standard 2	CONTENT	Content
STANDARD 3	Standard 3		Consult instructions for use
UDI	Unique Device Identification	IVD	*In vitro* diagnostic medical device
		CE	European conformity

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