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For Professional Use Only

GUIDELINES

to **AmpliSens[®] Florocenosis / Bacterial vaginosis-FRT** PCR kit
for diagnosing bacterial vaginosis (quantitation of DNA of
Gardnerella vaginalis, *Atopobium vaginae*, *Lactobacillus* spp., and
total amount of bacteria) in the clinical material by using real-time
hybridization-fluorescence detection

AmpliSens[®]



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INTENDED USE

Guidelines describe the procedure of the use of **AmpliSens® Florocenosis / Bacterial vaginosis-FRT** PCR kit for diagnosing bacterial vaginosis. Bacterial vaginosis is the clinical syndrome. It is caused by a change in the vaginal ecosystem. Normally, the vaginal flora is represented by the *Lactobacillus* spp. In bacterial vaginosis, *Lactobacillus* bacteria are fully or partly replaced by mostly anaerobic flora. Today more than 200 BV-associated microorganisms are described (mostly anaerobic). The studies in the recent years show that *Gardnerella vaginalis* plays the key role in development of bacterial vaginosis because of several reasons:

- it is detected almost in all patients with bacterial vaginosis,
- it has the strongest pathogenic potential among all the described BV-associated bacteria,
- it has significant adhesiveness,
- it forms biofilm,
- it produces cytolysin (vaginolysin), sialidase.

Another marker of bacterial vaginosis is *Atopobium vaginae*. It is a highly specific microorganism for this syndrome. *Atopobium vaginae* is a highly metronidazole resistant microorganism. Thus, its detection can cause the change of treatment strategy.

Taking this into account **AmpliSens® Florocenosis / Bacterial vaginosis-FRT** PCR kit is based on quantitative detection and calculation of ratios between the loads of DNA of *Gardnerella vaginalis*, *Atopobium vaginae*, *Lactobacillus* spp., and total amount of bacteria in clinical material by real-time hybridization-fluorescence detection using the following instruments:

- Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia);
- Rotor-Gene Q (Qiagen, Germany);
- iCycler iQ, iQ5 (Bio-Rad, USA);
- CFX96, CFX384 (Bio-Rad, USA).

SPECIMEN TYPE

The material used for this test is vaginal swab (epithelial cells scrapes from the lateral walls of vagina, vaginal discharge). The material should be obtained by the universal or flocced swabs with plastic shafts into a 2-ml tube with **Transport Medium with Mucolytic Agent** [REF] 952-CE; [REF] 953-CE. Deep the swab into the discharge of the posterior vaginal vault. Turn the swab while rubbing it against the surface of epithelium. Collect as much of the material as possible by the swab.

Transfer the swab into a tube with the **Transport Medium with Mucolytic Agent**. Break off the lower part of the swab and leave it in the tube with transport medium. Tightly cap the tube avoiding an airspace formation and deformation of the interior part of the cap. Mark the tube.

The unreliable results can be obtained in the following cases:

- insufficient material quantity or improper sampling;
- the material was obtained from another locus (for example, from cervical canal);
- antibiotic use. In this case, it is recommended to perform the analysis in 2 weeks after the end of therapy;
- the material was obtained from prepubertal girls (before the menarche) or menopausal women;
- the male (urethral) material was provided for the analysis.

DNA EXTRACTION

DNA-sorb-AM nucleic acid extraction kit **REF** K1-11-100-CE is recommended for the DNA extraction.

Procedure:

NOTE: **Internal Control-FL (IC)** is not used in this PCR kit. It is added if the extracted DNA is used for PCR kits in which the Internal Control-FL (IC) is used as exogenous control.

1. Add **10 µl** of **Internal Control-FL (IC)** into each prepared sterile disposable polypropylene tube.
2. Resuspend the sorbent. Add **20 µl** of **Universal Sorbent** and **300 µl** of **Lysis Solution** into each tube using the filter tips.

NOTE: If the number of samples exceeds 50, it is recommended that the whole volume of sorbent and IC are transferred to the tube with Lysis Solution (2 ml of Universal Sorbent and 1 ml of IC per 30 ml of Lysis Solution). Thoroughly stir this suspension and transfer 330 µl of it into each tube. Prepared mix can be stored at room temperature for up to 2 days. Stir well before use.

3. Add **100 µl** of clinical sample into each tube using filter tip. To the tube intended for Negative Control of Extraction (C–) add **100 µl** of **Transport Medium with Mucolytic Agent**. To the tube intended for Positive Control of Extraction (BV–) add **10 µl** of **Positive Control BV–** and **90 µl** of **Transport Medium with Mucolytic Agent**. To the tube intended for Positive Control of Extraction (BV+) add **10 µl** of **Positive Control BV+** and **90 µl** of **Transport Medium with Mucolytic Agent**.

4. Tightly cap the tubes, carefully mix the tubes on vortex mixer, and incubate at 65 °C for 5 min in a heating block. Vortex once again and incubate at room temperature for 2 min.
5. Sediment the sorbent by centrifugation at 10,000 rpm for 30 sec and carefully remove supernatant from each tube without disturbing the pellet using a vacuum aspirator. Use a new tip (without filter) for every tube.
6. Add **1 ml of Washing Buffer** into each tube. Vortex vigorously until sorbent is fully resuspended.
7. Repeat step 5.
8. Incubate all tubes with open caps at 65 °C for 5-10 min (for sorbent predrying).
9. Add **100 µl of TE-buffer for DNA elution** using tip with aerosol filter. Vortex vigorously until sorbent is fully re-suspended. Incubate tubes at 65 °C for 5 min.
10. Centrifuge tubes at 12,000 rpm for 1 min. The supernatant contains purified DNA and is ready for PCR.

The purified DNA could be stored:

- at 2-8 °C for 1 week;
- at the temperature not more than minus 16 °C for 1 year.

To test the samples after storage it is necessary to vortex the tubes vigorously and repeat step 10.

AMPLIFICATION AND DATA ANALYSIS USING Rotor-Gene 3000/6000 (Corbett Research, Australia) and Rotor-Gene Q (Qiagen, Germany)

When working with Rotor-Gene 3000 one should use the Rotor-Gene Version 6.1 software or higher and the Rotor-Gene 6000 versions 1.7 (build 67) software or higher for Rotor-Gene 6000 and Rotor-Gene Q instruments.

Hereinafter, all the terms corresponding to different instruments and software are indicated in the following order: for Rotor-Gene 3000 / for Rotor-Gene 6000.

Carry out the sample pretreatment and reaction mixture preparation stages according to the PCR kit *Instruction Manual*. When carrying out the amplification it is recommended to use thin-walled PCR tubes (0.2 ml) with flat caps (e.g. Axygen, USA), or Rotor-Gene PCR tubes (0.1 ml) with caps from the four-pieces-strips (e.g. Corbett Research, Australia; QIAGEN, Germany) (detection through the bottom of the tube).

Programming the Rotor-Gene instrument

1. Turn on the instrument, run the Rotor-Gene software.
2. Insert the tubes or strips into the rotor of the Rotor-Gene 3000/6000/Q instrument

beginning from the first well, insert the rotor into the instrument, close the lid (the rotor wells are numbered, the numbers are used for the further programming of the samples' order in the thermocycler). The tubes should be inserted in the following order: samples first, then BV– and BV+ control samples, then negative controls of amplification, and then FC1 and FC2 DNA calibrators.

NOTE: Balance the rotor of the instrument if it is not loaded entirely. Fill the spare wells with empty tubes (don't use the tubes left after previous experiments). Well 1 must be filled with any analyzed tube.

3. Program the instrument according to the *Instruction Manual* given by the manufacturer of the instrument.

Creating the template for the run

1. Click the **New** button in the main program menu.
2. In the opened window, select the **Advanced** and **Dual Labeled Probe/Hydrolysis probes**. Click the **New** button.
3. Select **36-Well Rotor** (or **72-Well Rotor**) and tick the **No Domed 0,2ml Tubes / Locking Ring Attached** option. Click **Next**.
4. Select the operator and set the **Reaction volume** as **25 µl**. Tick the **15 µl oil layer volume** option. Click **Next**.
5. Select the **Edit profile** button and set the temperature profile of the experiment.

AmpliSens-1 amplification program for rotor-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Cycles
Hold	95	15 min	–	1
1 Cycling	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
2 Cycling	95	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red	
	72	15 s	–	

NOTE: **AmpliSens-1** program is a universal amplification program for carrying out tests with the help of AmpliSens kits to identify the DNA of STI pathogens and other reproductive organs infections. Any tests combinations can be carried out in one instrument simultaneously.

Note – The **Cy5.5/Crimson** channel is enabled when required if the “multiprime” format tests with the use of this channel are performed.

6. Click **OK**.

7. Select the **Calibrate/Gain Optimisation** button in the **New Run Wizard** window.

- perform the fluorescence measurement in FAM/Green, JOE/Yellow, ROX/Orange, and Cy5/Red channels (click the **Calibrate Acquiring/Optimize Acquiring** button);
- to set channels calibration, indicate **5** in the **Min Reading** box and **10** in the **Max Reading** box for all the channels. Tick the **Perform Calibration Before 1st Acquisition/Perform Optimisation Before 1st Acquisition**. Click the **Close** button.

8. Click **Next** and then the **Start run** button.

9. Name the experiment and save it to the disk (results of the run will be automatically saved in this file).

10. Enter the data into the table of samples (opens automatically after the amplification starts). Indicate the names/numbers of clinical and control samples in the **Name** column.

NOTE: Name the control samples as specified in the instruction for the template of results calculation (the software) in Microsoft Excel[®] format enclosed to the PCR kit.

11. Set the **Unknown** type next to all clinical samples. Set the **None** type for the wells loaded with empty tubes.

NOTE: Samples defined as **None** will not be analyzed.

Data analysis

Obtained data (the curves of accumulation of fluorescent signals for four channels) are analyzed by the Rotor-Gene software.

Data analysis for FAM/Green channel:

1. Make sure that calibrator names and their concentrations are set in the sample table.
2. Click the **Analysis** button in the menu, select **Quantitation**, and then **Cycling A. FAM/Cycling A.Green**. Click **Show**.
3. Cancel the automatic choice of the threshold line level **Threshold**.
4. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of main window (**Quantitation analysis**).
5. In **CT Calculation** menu (at the right part of the window), set **Threshold = 0.05** (see Table 1).

Table 1

Channel	Detected feature	NTC Threshold	Threshold	Slope Correct
FAM/Green	<i>Gardnerella vaginalis</i> DNA	10-20%	0.05	On
JOE/Yellow	<i>Atopobium vaginae</i> DNA	10-20%	0.05	On
ROX/Orange	<i>Lactobacillus</i> spp. DNA	5-10%	0.05	On
Cy5/Red	Bacteria DNA	10-30%	0.05	On

6. Select the **More settings/Outlier Removal** option and set **NTC Threshold = 10–20 %**

(see Table 1).

7. Ct values for *Gardnerella vaginalis* DNA will appear in the results grid (**Quant. Results** window).

Data analysis for JOE/Yellow, ROX/Orange and Cy5/Red channels:

Analyze the results in JOE/Yellow, ROX/Orange and Cy5/Red channels as described in paragraphs 1–6 of data analysis for the FAM/Green channel using the settings specified in Table 1.

Results interpretation

The result of the PCR analysis is considered reliable only if the results for the controls of the amplification and the extraction are correct in accordance with the table of assessment of results for controls (see the *Instruction manual*) and boundary values specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

The interpretation of the test samples is to be carried out in accordance with the *Instruction Manual* and the *Important Product Information Bulletin* enclosed to the PCR kit.

AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ and iQ5 (Bio-Rad, USA)

Carry out the sample pretreatment and reaction mixture preparation stages according to the PCR kit *Instruction Manual*. When carrying out the amplification it is recommended to use thin-walled PCR tubes (0.2 ml) with domed or flat optically transparent caps, or tubes (0.2 ml) with transparent caps from the eight-pieces-strips (e.g. Axygen, USA) (detection through the cap of the tube).

Programming the thermocycler

1. Turn on the instrument and the power supply unit of the optical block of the instrument.

NOTE: The lamp is to be warmed up during 15 min before starting the experiment.

2. Start the iCycler iQ5 / iQ program.
3. Insert the tubes or strips into the reaction module of the thermocycler and program the instrument according to the *Instruction Manual* given by the manufacturer of the instrument.

NOTE: Monitor the tubes. There must not be drops left on the walls of the tubes as falling drops during the amplification process may lead to the signal failure and complicate the results analysis. Don't turn the tubes (strips) upside down while inserting them into the instrument.

1. Click the **Create New** button in the **Workshop** module in order to create a new protocol.
2. Set the amplification parameters in the opened window. Name the new protocol and save it to the disk.

AmpliSens-1 amplification program for plate-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	95	15 min	–	1
2	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
3	95	5 s	–	40
	60	30 s	FAM, JOE/HEX, ROX, Cy5	
	72	15 s	–	

NOTE: **AmpliSens-1** program is a universal amplification program for carrying out tests with the help of AmpliSens kits to identify the DNA of STI pathogens and other reproductive organs infections. Any tests combinations can be carried out in one instrument simultaneously.

3. Create a new plate of samples (**Plate Setup**). Set the order of the tubes in the plate.
4. In the opened window mark all clinical samples as **Unknown**. For all the samples set the detection of fluorescence in four channels JOE/HEX, FAM, ROX and Cy5.
5. Set the reaction volume (**Sample Volume**) as **25 µl**.
6. Name and save the scheme of the plate.
7. Click the **Run** button. In the opened window, click the **Use Persistent Well Factors** button, then click the **Begin Run** button and save the experiment.

Data analysis:

The obtained results are analyzed by the software of the iCycler iQ5 instrument. The results are interpreted according to the crossing (or not-crossing) of the S-shaped (sigmoid) fluorescence curve with the threshold line set at the specific level, that corresponds to the presence (or absence) of the *Ct* (threshold cycle) value in the corresponding column of the results table. The calibration curve plotting and calculation of the *Gardnerella vaginalis*, *Atopobium vaginae*, *Lactobacillus* spp. and Bacteria DNA concentration are performed automatically in accordance with the *Ct* values.

1. Start the software and open the saved file: select **Data file** in the **Workshop** module and select the required data file. Proceed to the **Data Analysis** mode.
2. View data separately for each channel.
3. Check the correctness of threshold line automatic choice for each channel. The threshold line is to cross only with S-shaped (sigmoid) curves describing the accumulation of the signal detecting positive samples and controls. The threshold line is not to cross the base line. If it happens, it is necessary to set the threshold line level for each channel manually. To do this, click the **Log View** (logarithmic scale selection) and set (with the left mouse button) the threshold line at the level where the

fluorescence curves have a linear character and do not cross with the curves of the negative samples. As a rule, the threshold line is set at the level of 10-20 % of maximum fluorescence obtained for the FC2 sample in the last amplification cycle.

4. To analyze the results, click **PCR Quant** and **Results** button (under the buttons with the names of fluorophores).

Results interpretation

The result of the PCR analysis is considered reliable only if the results for the controls of the amplification and the extraction are correct in accordance with the table of assessment of results for controls (see the *Instruction manual*) and boundary values specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

The interpretation of the test samples is to be carried out in accordance with the *Instruction Manual* and the *Important Product Information Bulletin* enclosed to the PCR kit.

AMPLIFICATION AND DATA ANALYSIS USING CFX96 and CFX384 (Bio-Rad, USA)

Carry out the sample pretreatment and reaction mixture preparation stages according to the PCR kit *Instruction Manual*. When carrying out the amplification it is recommended to use thin-walled PCR tubes (0.2 ml) with domed or flat optically transparent caps, or tubes (0.2 ml) with transparent caps from the eight-pieces-strips (e.g. Axygen, USA) (detection through the cap of the tube).

Programming the thermocycler

1. Turn on the instrument and start the Bio-Rad CFX Manager software.
2. Program the instrument according to the *Instruction Manual* provided by the manufacturer.

Creating the template for the run

1. Select **Create a new Run** in a start window. Set the universal **AmpliSens-1** amplification program.

AmpliSens-1 amplification program for plate-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	95	15 min	–	1
2	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
3	95	5 s	–	40
	60	30 s	FAM, HEX, ROX, Cy5	
	72	15 s	–	

NOTE: **AmpliSens-1** program is a universal amplification program for carrying out tests with the help of AmpliSens kits to identify the DNA of STI pathogens and other reproductive organs infections. Any tests combinations can be carried out in one instrument simultaneously.

Note – The **Quasar705** channel is enabled when required if the “multiprime” format tests with the use of this channel are performed.

To do this select or create this program in the **Experiment Setup** module in the **Protocol Editor** window.

NOTE: Set **Ramp Rate 2,5 °C/s** by clicking the **Step Options** button for each step of cycling (see the figure bellow). Click **OK**.

1	95,0 C for 15:00
→ 2	95,0 C for 0:05 Slow Ramp Rate to 2,5 C per second
3	60,0 C for 0:20 Slow Ramp Rate to 2,5 C per second
4	72,0 C for 0:15 Slow Ramp Rate to 2,5 C per second
5	GOTO 2 , 4 more times
→ 6	95,0 C for 0:05 Slow Ramp Rate to 2,5 C per second
7	60,0 C for 0:30 + Plate Read Slow Ramp Rate to 2,5 C per second
8	72,0 C for 0:15 Slow Ramp Rate to 2,5 C per second
→ 9	GOTO 6 , 39 more times
	END

2. In the next window (**Plate** module) set the scheme of the plate – set the order of the samples in the reaction module and set the fluorescence detection in five channels **FAM**, **HEX**, **ROX** and **Cy5** for all the samples. To do this, tick the necessary fluorophores in the list using **Select Fluorophores...** button. Select **Unknown** in **Sample type** field for all the samples. Set the identifiers of the samples in **Sample name** field. Save the plate scheme file, click **OK**.
3. Open the instrument lid by **Open Lid** button. Insert the reaction tubes in the wells of the thermocycler according to the scheme of the plate. Close the lid of the instrument using **Close Lid** button.

NOTE: Monitor the tubes. There must not be drops left on the walls of the tubes as falling drops during the amplification process may lead to the signal failure and complicate the results analysis. Don't turn the tubes (strips) upside down while inserting them into the instrument.

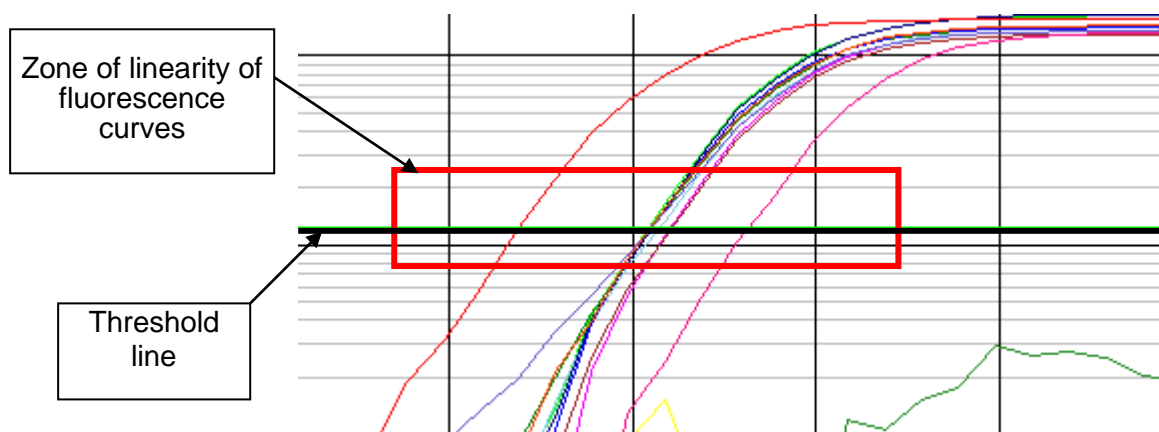
4. Start **AmpliSens-1** program according to the scheme of the plate clicking **Start Run**.
5. After the program has finished proceed to the results analysis.

Data analysis

The obtained results are analyzed by the software of CFX96 instrument. The results are interpreted according to the crossing (or not-crossing) of the S-shaped (sigmoid)

fluorescence curve with the threshold line set at the specific level, that corresponds to the presence (or absence) of the C_t (threshold cycle) value in the corresponding column of the results table. The calibration curve plotting and calculation of the *Gardnerella vaginalis*, *Atopobium vaginae*, *Lactobacillus* spp. and *Bacteria* DNA concentration are performed automatically in accordance with the C_t values.

1. Start the software and open the saved file. To do this, select **File** in the menu, then click **Open** and **Data file**, select the file.
2. Analyze data separately for each channel turning off other channels (delete the ticks in the channel box under the main window with the **Amplification** curves).
3. Check the correctness of threshold line automatic choice for each channel. The threshold line is to cross only with S-shaped (sigmoid) curves describing the accumulation of the signal detecting positive samples and controls. The threshold line is not to cross the base line. If it happens, it is necessary to set the threshold line level for each channel manually. To do this, click the **Log View** (logarithmic scale selection) and set (with the left mouse button) the threshold line at the level where the fluorescence curves have a linear character and do not cross with the curves of the negative samples. As a rule, the threshold line is set at the level of 10-20 % of maximum fluorescence obtained for the FC2 sample in the last amplification cycle.



4. C_t values detected in the analyzed channel will appear in the results grid.

Results interpretation

The result of the PCR analysis is considered reliable only if the results for the controls of the amplification and the extraction are correct in accordance with the table of assessment of results for controls (see the *Instruction manual*) and boundary values specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

The interpretation of the test samples is to be carried out in accordance with the *Instruction Manual* and the *Important Product Information Bulletin* enclosed to the PCR kit.

CALCULATION OF DNA CONCENTRATION

The concentrations of the DNA-calibrators needed for the calculation of the sample concentrations and the control samples characteristics are specified in the *Important Product Information Bulletin* enclosed to each PCR kit.

The results analysis is performed using the template of results calculation (the software) in Microsoft® Excel format according to the enclosed instruction. See the calculation algorithm of the software below.

For obtaining the results it is necessary to:

- Enter the data from the *Important Product Information Bulletin* to the calculation table;
- Complete the columns in the **Run Information** section;
- Copy the sample names and the *Ct* values for all the channels;
- Click the **Calculate** button. The following data will be displayed automatically in the corresponding cells:
 1. The values of ratio coefficients (RC1, RC2 and RC3),
 2. Status of the samples,
 3. The results for each sample and its interpretation.

The obtained concentrations of *Gardnerella vaginalis*, *Atopobium vaginae*, *Lactobacillus* spp., Bacteria are calculated in GE/ml.

Use the formula to recalculate the concentrations of *Gardnerella vaginalis*, *Atopobium vaginae*, *Lactobacillus* spp., Bacteria in copies/ml:

$$\text{[Number copies/ml] of microorganisms DNA} = \text{[Number GE/ml]} \times K$$

NOTE: The coefficient K for recalculation of the result in copies/ml is specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

Then obtained results and the interpretation should be copied to the laboratory form. The reference values should be specified for each test parameter. The reference value for total amount of Bacteria DNA is $\geq 10^6$ GE/ml. The less amount of Bacteria DNA in the sample can be obtained as a result of improper sampling, analysis of the material from girls before the menarche or menopausal women, antibiotic/antiseptic use or syringing during two weeks prior to sampling. Healthy woman has almost the same concentration of *Lactobacillus* spp. DNA and Bacteria DNA because the normal vaginal flora is dominated by *Lactobacillus* spp. The *Gardnerella vaginalis* and *Atopobium vaginae* can be present on the vaginal mucosa in low concentrations not exceeding the concentration of *Lactobacillus* spp. DNA.

Calculation algorithm

The template of results calculation (the software) in Microsoft® Excel format calculates ratio coefficients RC1, RC2 and RC3.

RC1 ratio coefficient represents the relative concentrations of the *Lactobacillus* spp. (Lac) and anaerobic microorganisms (*Gardnerella vaginalis*+*Atopobium vaginae* (Gv+Av)). It is calculated as the difference between logarithms of DNA concentrations of specified microorganisms:

$$RC1 = \lg(\text{Lac DNA}) - \lg(\text{Gv+Av DNA}),$$

RC2 ratio coefficient represents the relative concentrations of the total amount of bacteria (Bac) and *Lactobacillus* spp. (Lac). It is calculated as the difference between logarithms of DNA concentrations of specified microorganisms:

$$RC2 = \lg(\text{Bac DNA}) - \lg(\text{Lac DNA}),$$

RC3 ratio coefficient represents the relative concentrations of the total amount of bacteria (Bac) and anaerobic microorganisms (*Gardnerella vaginalis*+*Atopobium vaginae* (Gv+Av)). It is calculated as the difference between logarithms of DNA concentrations of specified microorganisms:

$$RC3 = \lg(\text{Bac DNA}) - \lg(\text{Gv+Av DNA}),$$

The following results are displayed automatically on the basis of RC1, RC2 and RC3:

- **Relative concentrations of microbial DNA correspond to bacterial vaginosis** (RC1<0.5);
- **Relative concentrations of microbial DNA do not correspond to bacterial vaginosis** (RC1>1);
- **Relative concentrations of microbial DNA correspond to intermediate flora** (0,5≤RC1≤1);
- **Relative concentrations of microbial DNA correspond to flora alteration, unspecified** (RC2>1, RC3>2, any RC1 value);
- **Bacterial load decreased** (RC1>1 and total amount of Bacteria DNA is less than 10⁶ GE/ml and greater than 10⁵ GE/ml);
- **Bacterial load insufficient for analysis** (total amount of Bacteria DNA is less than 10⁵ GE/ml).

Examples of results

1. The amount of *G.vaginalis* and/or *A.vaginae* is almost equal or exceeds the amount of *Lactobacillus* spp. – **Relative concentrations of microbial DNA correspond to**

bacterial vaginosis.

Parameters	Results	Reference value
Bacteria DNA – total amount of bacteria (GE/ml)	3*10⁷	≥ 10 ⁶
<i>Lactobacillus</i> spp. DNA (GE/ml)	3*10⁴	no less than Bacteria DNA
<i>Gardnerella vaginalis</i> DNA (GE/ml)	2*10⁷	no greater than <i>Lactobacillus</i> DNA concentration
<i>Atopobium vaginae</i> DNA (GE/ml)	3*10⁶	no greater than <i>Lactobacillus</i> DNA concentration
Result	<i>Relative concentrations of microbial DNA correspond to bacterial vaginosis</i>	
Note – It should be taken into account for therapy that <i>Atopobium vaginae</i> is highly metronidazole resistant microorganism *		
* This note is important for giving the results because the detection of <i>Atopobium vaginae</i> in the clinical material can cause the change of therapy tactics.		

2. The *G.vaginalis* and/or *A.vaginae* are absent or its amount is substantially less than the *Lactobacillus* spp. amount – ***Relative concentrations of microbial DNA do not correspond to bacterial vaginosis.***

Parameters	Results	Reference value
Bacteria DNA – total amount of bacteria (GE/ml)	1*10⁷	≥ 10 ⁶
<i>Lactobacillus</i> spp. DNA (GE/ml)	1*10⁷	no less than Bacteria DNA
<i>Gardnerella vaginalis</i> DNA (GE/ml)	not detected	no greater than <i>Lactobacillus</i> DNA concentration
<i>Atopobium vaginae</i> DNA (GE/ml)	not detected	no greater than <i>Lactobacillus</i> DNA concentration
Result	<i>Relative concentrations of microbial DNA do not correspond to bacterial vaginosis</i>	

3. The amount of *G.vaginalis* and/or *A.vaginae* is similar to the amount of *Lactobacillus* spp. but doesn't exceed the limit value – ***Relative concentrations of microbial DNA correspond to intermediate flora.***

Parameters	Results	Reference value
Bacteria DNA – total amount of bacteria (GE/ml)	8*10⁶	≥ 10 ⁶
<i>Lactobacillus</i> spp. DNA (GE/ml)	8*10⁶	no less than Bacteria DNA
<i>Gardnerella vaginalis</i> DNA (GE/ml)	2*10⁶	no greater than <i>Lactobacillus</i> DNA concentration
<i>Atopobium vaginae</i> DNA (GE/ml)	2*10⁴	no greater than <i>Lactobacillus</i> DNA concentration
Result	<i>Relative concentrations of microbial DNA correspond to intermediate flora</i>	

4. The amount of *Lactobacillus* spp. is reduced relative to the total amount of bacteria, whereas *G.vaginalis* and/or *A.vaginae* are absent or its amount is substantially less than total amount of bacteria – ***Relative concentrations of microbial DNA correspond to flora alteration, unspecified.***

Parameters	Results	Reference value
Bacteria DNA – total amount of bacteria (GE/ml)	1*10⁷	≥ 10 ⁶
<i>Lactobacillus</i> spp. DNA (GE/ml)	4*10³	no less than Bacteria DNA

<i>Gardnerella vaginalis</i> DNA (GE/ml)	not detected	no greater than <i>Lactobacillus</i> DNA concentration
<i>Atopobium vaginae</i> DNA (GE/ml)	not detected	no greater than <i>Lactobacillus</i> DNA concentration
Result	Relative concentrations of microbial DNA correspond to flora alteration, unspecified	

5. *G.vaginalis* and/or *A.vaginae* are absent or its amount is substantially less than *Lactobacillus* spp. amount, total amount of bacteria DNA is less than 10⁶ GE/ml and more than 10⁵ GE/ml – **Bacterial load decreased.**

Parameters	Results	Reference value
Bacteria DNA – total amount of bacteria (GE/ml)	9*10⁵	≥ 10 ⁶
<i>Lactobacillus</i> spp. DNA (GE/ml)	8*10⁵	no less than Bacteria DNA
<i>Gardnerella vaginalis</i> DNA (GE/ml)	not detected	no greater than <i>Lactobacillus</i> DNA concentration
<i>Atopobium vaginae</i> DNA (GE/ml)	not detected	no greater than <i>Lactobacillus</i> DNA concentration
Result	Bacterial load decreased	

6. Total amount of bacteria DNA is less than 10⁵ GE/ml – **Bacterial load insufficient for analysis.**

Parameters	Results	Reference value
Bacteria DNA – total amount of bacteria (GE/ml)	7*10⁴	≥ 10 ⁶
<i>Lactobacillus</i> spp. DNA (GE/ml)	1*10³	no less than Bacteria DNA
<i>Gardnerella vaginalis</i> DNA (GE/ml)	not detected	no more than concentration of <i>Lactobacillus</i> DNA
<i>Atopobium vaginae</i> DNA (GE/ml)	not detected	no more than concentration of <i>Lactobacillus</i> DNA
Result	Bacterial load insufficient for analysis	


TROUBLESHOOTING

Results of analysis are not taken into account in the following cases:

1. If **Calc Conc** value greater than 5 copies/reaction (that corresponds to 200 GE/ml) appears in the results grid for the Negative control of extraction (C-) and/or Negative control of amplification (NCA) in the FAM/Green and/or JOE/HEX/Yellow channel, it indicates contamination of reagents or samples. In such cases, the results of analysis must be considered as invalid. The analysis should be repeated from the extraction stage for all samples in which *Gardnerella vaginalis* and/or *Atopobium vaginae* DNA was detected. The measures for detection and elimination of contamination source should be assumed.
2. If the values (copies/reaction) of FC1 and FC2 calibrators differ from the specified ones by more than 30 %, check the tubes order in the instrument. For rotor-type instruments well 1 must be filled with any tube containing reaction mix.

3. If the value of the correlation coefficient, R^2 , is less than 0.9, calibration failure has occurred. Make sure that calibrators are set correctly and correct if necessary. If it does not help, repeat PCR for all samples and calibrators.
4. If the C_t value of the Positive control of extraction (BV-) is absent in the ROX/Orange or Cy5/Red channels, the results of analysis are considered invalid for all samples. Repeat PCR for all the samples.
5. If the C_t value of the Positive control of extraction (BV+) is absent in one or more channels (FAM/Green, JOE/HEX/Yellow, ROX/Orange, or Cy5/Red), the results of analysis are considered invalid for all samples. Repeat PCR for all the samples.
6. If a signal of a test sample is absent in the Cy5/Red channel or if value in the Cy5/Red channel is less than 10^5 GE/ml, the result is considered unreliable and PCR should be repeated for this sample. If the same result is reproduced, re-sampling is recommended.
7. If the signal of a sample is absent (C_t value is absent) in the Cy5/Red channel or if the quantity of *Lactobacillus* spp. is greater than the total quantity of bacteria by more than 0.5 Lg, the result for this sample is considered **invalid**. The analysis of the sample should be repeated starting from the extraction stage. If the same result is reproduced, re-sampling is recommended.

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
20.04.15 ME	Text	Corrections according to the template and Russian Guidelines. Grammar corrections
	Specimen type, DNA extraction	The sections was added
	Calculation of DNA concentration	All information from the section "Interpretation of results" was transferred to the subsection "Calculation algorithm" of section "Calculation of DNA concentration"
19.05.15 ME	10. Troubleshooting	For Negative Control of extraction (C-) and/or Negative Control of PCR (NCA) the translation into copies/ml was added
17.06.15 ME	Intended use	LineGene 9660 (BIOER TECHNOLOGY CO., LTD, China) instrument was added
	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) and Rotor-Gene Q (Qiagen, Germany)	The phrase "AmpliSens-1 program is a universal amplification program for carrying out tests with the help of AmpliSens kits to identify the DNA of STI pathogens and other reproductive organs infections. Any tests combinations can be carried out in one instrument simultaneously" was added after the amplification program. The subchapter "Results interpretation" was added. The subchapter "Data analysis" was complemented according to the template. The note about inclusion of the Cy5.5/Crimson / Quasar705 channel in case of need
	Amplification and data analysis using iCycler iQ and iQ5 (Bio-Rad, USA)	
	Amplification and data analysis using CFX96 and CFX384 (Bio-Rad, USA)	
Amplification and data analysis using LineGene 9660 (BIOER TECHNOLOGY CO., LTD, China) instrument	The chapter was added	
12.01.18 ME	Intended use	LineGene 9660 (BIOER TECHNOLOGY CO., LTD, China) instrument was deleted
	Amplification and data analysis using LineGene 9660 (BIOER TECHNOLOGY CO., LTD, China) instrument	The chapter was deleted
	Text	"The software in Microsoft Excel format" was changed to "the template of results calculation (the software) in Microsoft Excel format"
20.07.18 EM	Text	All the chapters were corrected according to the Russian Guidelines
13.02.19 EM	Calculation of DNA concentration	The information about concentrations which are calculated in GE/ml and the formula for conversion from GE/ml to copies/ml were added
08.05.20 EM	Specimen type	The information about Transport Medium TM-EDEM REF 1533-CE was deleted
	Text	"Log" was changed on "lg"
29.12.20 KK	Through the text	The symbol  was changed to NOTE:
	Cover page	The phrase "Not for use in the Russian Federation" was added
16.03.21 EM	Front page	The name, address and contact information for Authorized representative in the European Community

VER	Location of changes	Essence of changes
		was changed
06.04.21 MM	Through the text	"copies/ml" was changed on "GE/ml"
	Troubleshooting	The value was changed from 500 copies/ml to 200 GE/ml for Calc Conc value in point 1