

## GUIDELINES

to **AmpliSens<sup>®</sup> Florocenosis / *Mycoplasma*-FRT**

PCR kit for simultaneous qualitative detection and quantitation of STIs in the biological material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

# AmpliSens<sup>®</sup>



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## INTENDED USE

The guidelines describe the procedure of simultaneous qualitative detection and quantitation of *Ureaplasma parvum* DNA, *Ureaplasma urealyticum* DNA, and *Mycoplasma hominis* DNA in biological material (urogenital swabs from cervical canal, vagina, urethra and first pass of urine) by the polymerase chain reaction (PCR) with 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, iCycler iQ5 (Bio-Rad, USA),
- Mx3000P, Mx3005 (Stratagene, USA),
- CFX 96 (Bio-Rad, USA).

### Correspondence of targets and detection channels

Fluorophore	FAM	JOE	ROX	Cy5
Detection channel name for different instrument models <sup>1</sup>	FAM/ Green	JOE/ HEX/ R6G/ Yellow/ Cy3	ROX/ Orange/ TxR	Cy5/ Red
DNA target	<i>U.parvum</i> DNA	<i>U.urealyticum</i> DNA	<i>M.hominis</i> DNA	$\beta$ -globin gene DNA (IC Glob)

## AMPLIFICATION AND DATA ANALYSIS USING Rotor-Gene 3000/6000 (Corbett Research, Australia) AND Rotor-Gene Q (QIAGEN, Germany) INSTRUMENTS

When working with Rotor-Gene 3000 one should use the Rotor-Gene version 6.1 and higher software 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/Q.**

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, or PCR tubes (0.1 ml) with caps from the four-pieces-strips (detection through the bottom of the tube).

### Creating the template for the run

1. Click the **New** button in the software main menu. To create the template select the **Advanced** tab in the opened window **New run**.

<sup>1</sup> The detection channels names in each section of the guidelines are specified in accordance with the described instrument.

2. Select the **TwoStep/Hidrolysis Probes** template in the tab for edition and click the **New** button.
3. In the opened window select the **36-Well Rotor (or 72-Well Rotor)** and tick the **No Domed 0,2ml Tubes / Locking Ring Attached** option. Click the **Next** button.
4. In the opened window enter the operator name, select the **Reaction Volume – 25 µl**. Tick the **15 µL oil layer volume** option. Click the **Next** button.
5. In the **New Run Wizard** window set the temperature profile of the experiment. To do this click the **Edit profile** button and set the amplification program.

Table 1

**AmpliSens-1 amplification program**

<b>Step</b>	<b>Temperature, °C</b>	<b>Time</b>	<b>Fluorescence detection</b>	<b>Number of cycles</b>
1	95	15 min	–	1
2	95	5 s	–	5
	60	20 s		
	72	15 s		
3	95	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red	
	72	15 s	–	

**NOTE:** **AmpliSens-1** is a universal program for conducting tests for detection of STIs and other reproductive tract infections with AmpliSens PCR kits. Therefore, any combination of tests including tests for identifying *human papillomaviruses (HPV HCR)* can be carried out simultaneously in the same instrument.

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

6. After setting up the temperature profile click the **OK** button.
7. Adjust the fluorescence channel sensitivity. In the **Channel Setup** window, select the **Calibrate/Gain Optimisation** button. In the opened **Auto Gain Calibration Setup** window, click the **Calibrate Acquiring/Optimise Acquiring** button. Tick the **Perform Calibration Before 1<sup>st</sup> Acquisition/ Perform Optimisation Before 1<sup>st</sup> Acquisition** box. For the **FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red and Cy5.5/Crimson** channels, enter **5** in the **Min Reading** line and **10** in the **Max Reading** line (it is acceptable to set **Calibrate/Gain Optimisation...** value from 4 FI to 8 FI for **JOE/Yellow, ROX/Orange, Cy5/Red, and Cy5.5/Crimson** channels).  
In the **Tube position** column, specify the number of the tube by which the **Gain** parameter will be selected automatically. By default, this is the first tube in the rotor. The first rotor well should be loaded with a test tube with any reaction mixture (see Note 1). Close the **Auto Gain Calibration Setup** window.

**NOTE:** The additional requirements for setting the channels' calibration ranges are specified in the information list "Calibration priorities for the AmpliSens kits for Rotor-Gene 3000/6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany) thermocyclers"

8. Click the **Next** button. For saving the programmed template it is necessary to click the **Save Template** button and enter the template file name corresponding to the amplification program: **Amplisens-1**. Save the file into a proposed folder: **Templates/Quick Start Templates**; close the **New Run Wizard** window. After that the programmed template will appear in the template list in the **New Run** window.

### Using the ready template for the run

1. Insert the tubes into the rotor (the rotor wells are numbered, the numbers are used for the further programming of the samples' order in the thermocycler). Fix the locking ring, secure the rotor, and close the lid.

**NOTE:** The first well of the rotor should be filled with a test tube with the reaction mixture from the current experiment (see Notes 1 and 2).

2. Click the **New** button in the software main menu. In the opened **New Run** window select the **Advanced** tab. Then select the **AmpliSens-1** template (which is programmed as described in the "Creating the template for the run" section) in the template list.
3. In the opened window select the **36-Well Rotor** (or **72-Well Rotor**) and tick the **No Domed 0.2 ml Tubes/Locking ring attached** option. Click the **Next** button.
4. In the opened window check that the **Reaction Volume** is **25 µl** and the **15 µl oil layer volume** option is activated. Click the **Next** button.
5. In the next window the correctness of the amplification program and signal level auto-optimisation parameters can be checked. Go to the next window clicking the **Next** button. Start the amplification by the **Start run** button. Herewith, the rotor with the samples should be already fixed and the lid should be closed. Name the experiment and save it to the disc (the results of the experiment will be automatically saved in this file).
6. Enter the data into the grid of the samples (it opens automatically after the amplification has been started). Enter the names/numbers of the test samples in the **Name** column. Define the Negative control of amplification as NCA. Set the type **Unknown** opposite all the test samples, the type **Negative control** – for the Negative control of amplification. Set the type **Standard** for DNA-calibrators C1 and C2 and enter the values from the *Important product information bulletin* enclosed to the given lot of the PCR kit into the **Given conc.**

column. Set the type **None** for the cells matching with the corresponding empty tubes.  
Click the **Finish/OK** button.

**NOTE:** Samples indicated as **None** won't be analyzed.

To edit the table of samples before the start it is needed previously to select  
**NOTE:** the **Edit Samples Before Run Started** option in the **User preferences** submenu of the **File** menu. See also Note 3

**Note 1.** The first tube in the rotor is used for automatic optimization of the level of signal; therefore, the first tube should contain the reaction mixture. If several tests for detection of STIs with PCR kits manufactured by FBIS CRIE are conducted within the same run, any tube containing the reaction mixture can be placed in the first well of the rotor. If several MULTIPRIME tests are simultaneously carried out, the first well should be loaded with the tube analyzed in the maximum number of channels.

**Note 2.** Do not place tubes that already passed amplification run in the rotor iteratively. It is acceptable to leave some rotor wells unloaded.

**Note 3.** If tests for detection of *human papillomavirus* (HPV) DNA and different tests for detection of STI with PCR kits manufactured by FBIS CRIE are simultaneously conducted, it is necessary to create the second page in the table of samples. On this page, all samples tested for *HPV* should be defined (**Unknown** type) while the other samples should have the **None** type. This is important for data analysis.

### **Data analysis**

The obtained results are analyzed by the Rotor-Gene software. 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 of the DNA sample in the corresponding column of the results table.

The calibration curve plotting and calculation of the DNA concentration are performed automatically in accordance with the *Ct* values of the calibrators.

### **Amplification data analysis in the FAM/Green channel.**

1. Make sure that calibrators are defined and their concentrations are entered in the table of samples.
2. Activate the button **Analysis** in the menu, select the mode of the analysis **Quantitation**, activate the **Cycling A. FAM/Cycling A. Green, Show** buttons.
3. Cancel the automatic choice of the threshold line level **Threshold**.
4. Select the **Linear scale**.

5. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of main window (**Quantitation analysis**).
6. In **CT Calculation** menu (in the right part of the window), indicate the threshold line **Threshold = 0.1** (see Tables 3 and 4).
7. Choose the parameter **More Settings/Outlier Removal** and set **10-20 %** for the value of negative samples threshold (**NTC/Threshold**) (see Tables 3 and 4).
8. In the results grid (the **Quantitation Results** window) one will be able to see the *Ct* values.
9. For the Negative Control of Extraction (C-) - *Ct* values should be absent.
10. For the Negative Control of Amplification (NCA) - *Ct* values should be absent.
11. For DNA calibrators - *Ct* values should appear.
12. Check the **Standard Curve** window:  $R^2$  (correlation coefficient) should exceed 0.99.

The difference between cycles *Ct* (UG2) – *Ct* (UG1) should be in the range  $10 \pm 1$  cycle.

Results analysis in the JOE/Yellow, ROX/Orange, Cy5/Red is carried out similarly to results analysis in the FAM/Green channel in accordance with the settings in the table below.

Table 2

Channel	Threshold	More Settings / Outlier Removal	Slope Correct
FAM/Green	0,1	10-20 %	on
JOE/Yellow	0,1	10-20 %	on
ROX/Orange	0,1	10-20 %	on
Cy5/Red	0,1	10-30 %	on

### **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.

Final result of the DNA concentration of the analyzed microorganism may be in two variants – absolute and relative (normalized) values.

In both cases, the concentration of the internal control DNA (Glob) should be estimated: it should exceed  $10^3$  and  $10^2$  GE/reaction in urethral and cervical swabs for women and men, respectively.

**NOTE:** The number of GE per reaction can be less than  $10^3$  for women and less than  $10^2$  for men if the following biological material is used: swabs taken from rectum, throat, and eye conjunctiva; urine; and prostate secretion.

### **Absolute values of analyzed microorganism DNA concentration**

Absolute values of analyzed microorganism DNA concentrations correspond to total content of microorganisms in the biological material, placed into the transport media.

### **Relative (normalized) values of analyzed microorganism DNA concentration**

Normalized values of analyzed microorganism DNA concentrations correspond to analyzed microorganism cell number relative to human mucous cells. Obtained values of the number of GE normalize on 100,000 of human cells. In addition, the human DNA concentration values represent the quality of sampling.

Quantitation can be performed using software in Microsoft Excel format **AmpliSens® Florocenosis**. Copy the *Ct* values for all channels in the **AmpliSens® Florocenosis** table, name the controls and calibrators according to the software instruction, select **Calculate**.

### **Troubleshooting**

1. If the value determined in the results grid for Negative Control of extraction (C-) and/or Negative Control of amplification (NCA) in the channel assigned for IC detection (JOE/Yellow or ROX/Orange or Cy5/Red according to the used PCR kit) is more than 5 copies/reaction (500 copies/ml), it indicates the contamination of reagents or samples. In such cases the results of analysis are considered invalid for all samples. The PCR analysis (beginning with the DNA extraction stage) should be repeated for all samples in which specific DNA was detected. Measures for detecting and elimination of contamination source must be taken.
2. If the signal is detected for Negative Control of Extraction (C-) and/or Negative Control of amplification (NCA) in the channels for detection of microorganisms' DNA, PCR analysis should be repeated for all samples in which *Ct* values are determined in corresponding channels.
3. If *Ct* value is absent for DNA calibrators (UG1 and UG2) in the FAM/Green and/or JOE/Yellow channels (as well as in the ROX/Orange channel in case of using PCR kits for detection of DNA of two microorganisms, and ROX/Orange and Cy5/Red channel in case of using PCR kits for detection of three microorganisms) amplification should be repeated for all samples.
4. If the  $Ct(UG2) - Ct(UG1)$  difference is more than  $10 \pm 1$ , it indicates calibration failure. It is necessary to check calibrators' parameters and correct inaccuracy. In case of repeated insufficient results, analysis must be repeated for all probes and calibrators.

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

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 (detection through the cap of the tube) or tubes (0.2 ml) with transparent caps from the eight-pieces-strips (detection through the cap of the tube).

### Programming the thermocycler

1. Turn on the instrument and the lamp for 20–30 min before the experiment starts.
2. Set the **AmpliSens-1** (Table 3) universal amplification program.

Table 3

### AmpliSens-1 amplification program for iCycler iQ or iCycler iQ5 PCR instruments

Step	Temperature, °C	Time	Fluorescence detection	Number of 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	–	

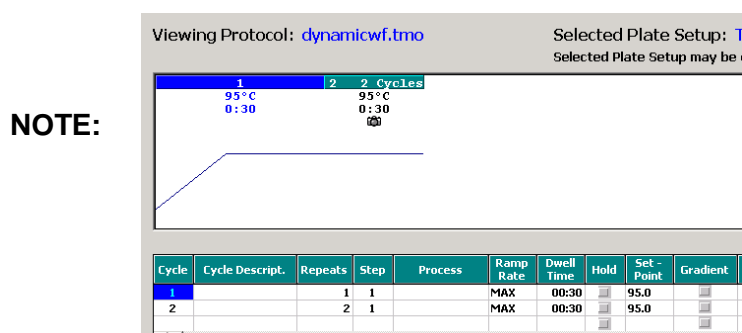
To do this, select or create this program in the **Protocol** module (**View Protocols** for iCycler iQ). For iCycler iQ, click the **Run with selected Plate Setup** button to start the program.

**NOTE:** **AmpliSens-1** is a universal program for conducting tests for detection of STIs and other reproductive tract infections with PCR kits manufactured by FBIS CRIE.

It is not recommended running the MULTIPRIME-format test and single pathogen detection tests (tests with different combinations of detection channels)

**NOTE:** simultaneously in the iCycler iQ instrument. If these tests are to be conducted within the same run, then the **External Well Factors Plate** option should be selected for the well factor determination and the tube kit with a special **External Well Factor Solution** (Bio-Rad) should be used for start up.

For iCycler iQ instrument, make sure that the **dynamicwf.tmo** protocol complies with a standard protocol, as shown below:



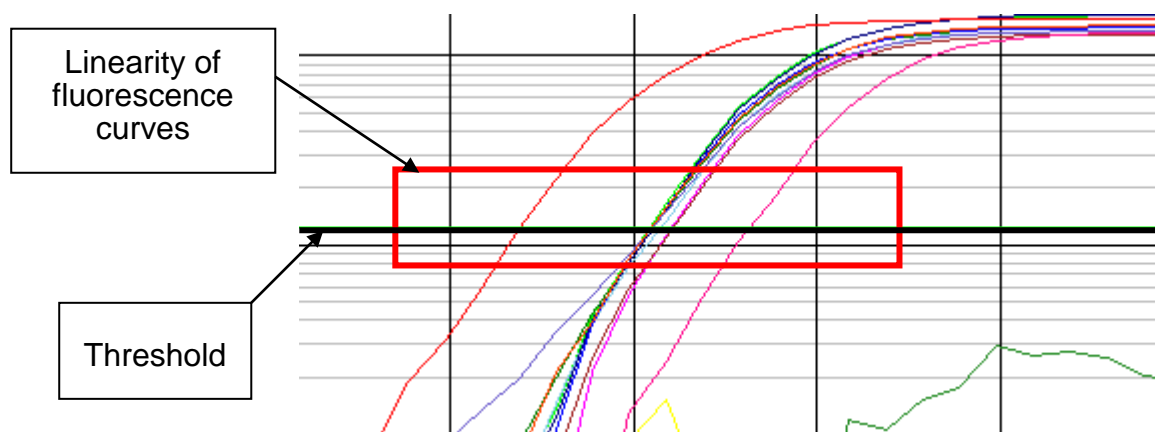
3. In the opened window, define all test samples as **Unknown** and negative controls as “–”. Define UG1 and UG2 calibrators as **Standards** in the **FAM, JOE/HEX, ROX, and Cy5** channels and enter concentration values specified in the *Important Product Information Bulletin*. Make sure that the **Whole Plate Loading** button is disabled when setting calibrator parameters. Enable fluorescence detection for all samples and calibrators in the channels: **FAM, JOE/HEX, ROX, and Cy5**.
4. Name the plate setup and save it.
5. To start the program, click **Run** (iCycler iQ5) or **Run with selected protocol** (for iCycler iQ). Set the reaction volume as **25 µl** in the opened window.  
For determination of the well factor select **Experimental Plate** (iCycler iQ) or **Use Persistent Well Factor** (iCycler iQ5). Click the **Begin Run** button and save the experiment.
6. Insert the tubes into the wells in accordance with the created plate setup. Close the lid.
7. Start the **AmpliSens-1** program with the created plate setup.
  - **iCycler iQ5 instrument.** Ensure that the **Selected Protocol** and **Selected Plate Setup** are set correctly before starting the program. To start the program, click the **Run** button. Select the **Use Persistent Well Factor** option (set by default) for detection of a well factor.
  - **iCycler iQ instrument.** Ensure that the selected protocol and plate setup are set correctly in the **Run Prep** window. To determine the well factor, select the **Experimental Plate** option (set by default) below the **Select well factor source** line (see item 2). Set the reaction mix volume as **25 µl**. Press **Run** to start.
8. Proceed to data analysis when the program is completed.
9. At the end of the work, close the program and shut down the instrument.

#### **Data analysis:**

The obtained results are analyzed by the iCycler iQ5 software. 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 DNA concentration are performed automatically in accordance with the *Ct* values of the calibrators.

1. Start the software and open the needed file with data of the analysis in the **Data File** window of the **Workshop** module. Click the **Analyze** button.
2. Select the **Analysis Mode: PCR Base Line Subtracted Curve Fit** (is set by default).

3. Check the correctness of threshold line automatic choice for each channel. The threshold line is to cross only the 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** button (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 DNA-calibrator **UG1** in the last amplification cycle. Make sure that the fluorescence curve of the DNA-calibrator **UG1** has the typical exponential growth of fluorescence.



The *Ct* values detected in the analyzed channel will appear in the results grid in the **Quant. Result** window.

4. For analysis of results, click the **PCR Quant** button (for iCycler iQ) or **Results** (for iCycler iQ5).

To continue work with data, click the right mouse button on the appeared table with results. In the drop-down menu select **Export to Excel**. Save the file to the needed folder.

### **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.

Final result of the DNA concentration of the analyzed microorganism may be in two variants – absolute and relative (normalized) values.

In both cases, the concentration of the internal control DNA (Glob) should be estimated: it should exceed  $10^3$  and  $10^2$  GE/reaction in urethral and cervical swabs for women and men, respectively.

**NOTE:** The number of GE per reaction can be less than  $10^3$  for women and less than  $10^2$  for men if the following biological material is used: swabs taken from rectum, throat, and eye conjunctiva; urine; and prostate secretion.

### **Absolute values of analyzed microorganism DNA concentration**

Absolute values of analyzed microorganism DNA concentrations correspond to total content of microorganisms in the biological material, placed into the transport media.

### **Relative (normalized) values of analyzed microorganism DNA concentration**

Normalized values of analyzed microorganism DNA concentrations correspond to analyzed microorganism cell number relative to human mucous cells. Obtained values of the number of GE normalize on 100 thousands of human cells. In addition, the human DNA concentration values represent the quality of sampling.

Quantitation can be performed using software in Microsoft Excel format **AmpliSens® Florocenosis**. Copy the *Ct* values for all channels in the **AmpliSens® Florocenosis** table, name the controls and calibrators according to the software instruction, select **Calculate**.

### **Troubleshooting**

1. If the value determined in the results grid for Negative Control of extraction (C-) and/or Negative Control of amplification (NCA) in the channel assigned for IC detection (JOE/HEX or ROX or Cy5 according to the used PCR kit) is more than 5 copies/reaction (500 copies/ml), it indicates the contamination of reagents or samples. In such cases the results of analysis are considered invalid for all samples. The PCR analysis (beginning with the DNA extraction stage) should be repeated for all samples in which specific DNA was detected. Measures for detecting and elimination of contamination source must be taken.
2. If the signal is detected for Negative Control of Extraction (C-) and/or Negative Control of amplification (NCA) in the channels for detection of microorganisms' DNA, PCR analysis should be repeated for all samples in which *Ct* values are determined in corresponding channels.
3. If *Ct* value is absent for DNA calibrators (UG1 and UG2) in the FAM and/or JOE/HEX (as well as in the ROX channel in case of using PCR kits for detection of DNA of two

microorganisms, and ROX/Orange and Cy5 channel in case of using PCR kits for detection of three microorganisms) amplification should be repeated for all samples.

4. If the  $Ct(UG2) - Ct(UG1)$  difference is more than  $10 \pm 1$ , it indicates calibration failure. It is necessary to check calibrators parameters and correct inaccuracy. In case of repeated insufficient results, analysis must be repeated for all probes and calibrators.

## AMPLIFICATION AND DATA ANALYSIS USING Mx3000P, Mx3005P (Stratagene) INSTRUMENTS

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 (detection through the cap of the tube).

### Programming the thermocycler

1. Switch on the instrument. Start the **Stratagene Mx3000P, Mx3005P** program.
2. Select **Quantitative PCR (Multiple Standards)** in the **New Experiment Options** window and check the **Turn lamp on for warm-up** box.

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

3. Insert the tubes into the instrument, close the lid.
4. Select **Optics Configuration** in the **Options** menu. In the **Dye Assignment** window, set **FAM** parameter next to the **FAM filter set** item, set **JOE** parameter next to the **HEX/JOE filter set** item, set **ROX** parameter next to the **ROX filter set** item, set **Cy5** parameter next to the **Cy5 filter set** item.
5. Lock the fixing arm and the door of the instrument
6. Set fluorescence detection parameters in the **Plate Setup** menu. To do this:
  - select all the cells with the test tubes or strips (hold down **Ctrl** and select the required area with the mouse),
  - define the cells as **Unknown** in the **Well type** field. Select **FAM** and **JOE** fluorophores (or **FAM, JOE** and **ROX** fluorophores if two microorganisms are detected, or **FAM, JOE, ROX**, and **Cy5** fluorophores if three microorganisms are detected) in the **Collect fluorescence data** option.
  - Enter the name of each test sample in the **Well Information** window (double click on the well before data inserting). Indicate **Standard** for DNA calibrators UG1 and UG2 in the **Well Type** window in FAM, JOE/HEX, ROX, and Cy5 channels and enter the concentration in the **Standard Quantity** window. These data can be entered at the end of the program as well.
7. Set the amplification program. This may be performed by one of two methods:

### Using of the template file for setting the amplification program (is recommended).

Proceed to the **Thermal Profile Setup** tab. Click the **Import...** button left to the thermal profile. Proceed to the folder with the previous saved file and open it. The required profile will appear in the **Thermal Profile** window.

## Individual programming

Select all wells with the test tubes after all parameters were set. Proceed to the **Thermal Profile Setup** tab and set the amplification program:

Table 4

### Amplisens-1 amplification program for plate-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Number of 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:** Any combination of the tests for detection of STIs, including tests for detection and genotyping of *human papillomaviruses (HPV HCR)*, can be performed in one instrument simultaneously with the use of the **AmpliSens-1** universal program and PCR kits manufactured by FBIS CRIE in which not more than 4 detection channels are used.

To enable fluorescence detection at the required temperature, select the **All points** option for **Data collection marker by dragging** parameter and move it from the left part of the display to the cell with the desired temperature using the mouse.

8. Start the amplification by clicking the **Run** and **Start** buttons, then name the experiment.

### Data analysis:

The results are interpreted with software of the PCR instrument used by the crossing (or not-crossing) of the fluorescence curve with the threshold line set at a specific level (it corresponds to the presence (or absence) of **Ct** (cycle threshold) in the results grid). The calibration curve plotting and calculation of the DNA concentration are performed automatically in accordance with the *Ct* values of the calibrators.

1. Select the **Analysis** by clicking the corresponding button of the tool bar of Mx3000P program.
2. The **Analysis Selection/Setup** tab will open. Make sure that all the test samples are active (the cells corresponding to the samples should be of a different colour). Otherwise select all the test samples by holding down the **Ctrl** button and selecting the needed range with the mouse.
3. Select the **Results** grid.
4. Make sure that the automatic setting of the threshold line is correct for each channel. Normally, the threshold line should cross only the sigmoid curves of signal

accumulation of positive samples and controls and should not cross the baseline; otherwise, the threshold level should be raised. As a rule, the threshold line is set at the level of 10-20 % of maximum fluorescence obtained for the DNA-calibrator **UG1** in the last amplification cycle. Make sure that the fluorescence curve of the DNA-calibrator **UG1** has the typical exponential growth of fluorescence. To do this, activate the channel displayed in the **Dyes shown** panel (separately for each channel). Check the level of the threshold and change it, if necessary.

By default, all fluorescence curves are displayed as linear. To shift from the linear to the logarithmic mode, double click one axis (X or Y) with the left mouse button and tick the **Log** option for Y axis in the **Scale** field of the **Graph properties** window.

5. Activate displaying two, three or four channels depending on PCR kit in use (make sure that **FAM**, **JOE**, **ROX**, and **Cy5** buttons are activated in the **Dyes Shown** field at the bottom of the program window).
6. Select the **Text Report** option in the **Area to analyze** field. Make sure that all data are sorted by the dye (the **Dye** column). To do this, click the name of the **Dye** column.
7. Proceed to the **File** menu, **Export Text Report**, and **Export Text Report to Excel**. Microsoft Excel window will appear.

### Quantitation of results

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.

Final result of the DNA concentration of the analyzed microorganism may be in two variants – absolute and relative (normalized) values.

In both cases, the concentration of the internal control DNA (Glob) should be estimated: it should exceed  $10^3$  and  $10^2$  GE/reaction in urethral and cervical swabs for women and men, respectively.

**NOTE:** The number of GE per reaction can be less than  $10^3$  for women and less than  $10^2$  for men if the following biological material is used: swabs taken from rectum, throat, and eye conjunctiva; urine; and prostate secretion.

### **Absolute values of analyzed microorganism DNA concentration**

Absolute values of analyzed microorganism DNA concentrations correspond to total content of microorganisms in the biological material, placed into the transport media.

### **Relative (normalized) values of analyzed microorganism DNA concentration**

Normalized values of analyzed microorganism DNA concentrations correspond to analyzed microorganism cell number relative to human mucous cells. Obtained values of the number of GE normalize on 100 thousands of human cells. In addition, the human DNA concentration values represent the quality of sampling.

Quantitation can be performed using software in Microsoft Excel format **AmpliSens® Florocenosis**. Copy the *Ct* values for all channels in the **AmpliSens® Florocenosis** table, name the controls and calibrators according to the software instruction, select **Calculate**.

### **Troubleshooting**

1. If the value determined in the results grid for Negative Control of extraction (C-) and/or Negative Control of amplification (NCA) in the channel assigned for IC detection (JOE/HEX or ROX or Cy5 according to the used PCR kit) is more than 5 copies/reaction (500 copies/ml), it indicates the contamination of reagents or samples. In such cases the results of analysis are considered invalid for all samples. The PCR analysis (beginning with the DNA extraction stage) should be repeated for all samples in which specific DNA was detected. Measures for detecting and elimination of contamination source must be taken.
2. If the signal is detected for Negative Control of Extraction (C-) and/or Negative Control of amplification (NCA) in the channels for detection of microorganisms' DNA, PCR analysis should be repeated for all samples in which *Ct* values are determined in corresponding channels.
3. If *Ct* value is absent for DNA calibrators (UG1 and UG2) in the FAM and/or JOE/HEX (as well as in the ROX channel in case of using PCR kits for detection of DNA of two microorganisms, and ROX and Cy5 channel in case of using PCR kits for detection of three microorganisms) amplification should be repeated for all samples.
4. If the  $Ct(UG2) - Ct(UG1)$  difference is more than  $10 \pm 1$ , it indicates calibration failure. It is necessary to check calibrators parameters and correct inaccuracy. In case of repeated insufficient results, analysis must be repeated for all probes and calibrators.

## AMPLIFICATION AND DATA ANALYSIS USING CFX96 (Bio-Rad, USA) INSTRUMENT

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 (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. In the **Startup Wizard** window it is necessary to select the **Create a new Run/Experiment** (or select **New** in the **File** menu and then select **Run.../Experiment...**). Click **OK**.
2. In the **Run Setup** window select **Protocol** tab and click the **Create new...** button. Set the parameters in the opened **Protocol Editor – New** window. Set **Sample Volume – 25 µl**.

Table 5

**AmpliSens-1 amplification program for CFX96**

Step	Temperature, °C	Time	Fluorescence detection	Number of 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	<b>Fam, Hex, Rox, Cy5</b>	
	72	15 s	–	

**NOTE:** **AmpliSens-1** is a universal program for conducting tests for detection of STIs and other infections of reproductive system with AmpliSens PCR kits. Therefore, any combination of tests including tests for detection and genotyping of *human papillomaviruses (HPV HCR)* can be carried out simultaneously in the same instrument.

Note – The **Quasar 705** channel is enabled when required if the “multiprime” format tests are performed

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

3. Save the protocol: in the **Protocol Editor New** window select **File**, then **Save As**, name the file and click **Save**.
4. Set the plate setup: in the **Plate** tab click the **Create new...** button. Set the tube order in the opened **Plate Editor – New** window. Click the **Select Fluorophores...** button; click **OK**; and click the **Selected** checkbox next to the required fluorophores and click **OK**. In the **Sample type** menu select **Unknown** for all the samples, except for the DNA-calibrators. Then in the **Load** column (in the right part of the window) tick the fluorescence signal acquiring for all the samples in the required channels. Define sample names in the **Sample name** window, moreover the **Load** function is to be ticked.  
Set the calibrators C1 and C2 for all the channels as **Sample type – Standard**. Enter a concentration in the **Concentration** box according to the *Important Product Information Bulletin* enclosed to the PCR kit, moreover the **Load** function is to be ticked.
5. Save the plate setup: select **File** and then **Save as** in the **Plate Editor New** window. Enter the file name, click **Save**.
6. Select the **Start Run** tab. Open the lid of the instrument by the **Open Lid** button. Insert the reaction tubes in the wells of the instrument in accordance with the entered plate setup. Close the lid by the **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.

7. Click the **Start Run** button and start the program with the selected plate setup. Select the directory for the file saving, name the file, click **Save**.

### **Data analysis**

The obtained results are analyzed by the software of the 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 DNA concentration are performed automatically in accordance with the  $C_t$  values of the calibrators.

Set the threshold for **FAM, HEX, ROX, Cy5** channels (with the left mouse button) at a level where fluorescence curves are linear: select data for appropriate channels in the **Quantitation** window of the **Data Analysis** module. As a rule, the threshold line is set at the level of 10-20 % of maximum fluorescence obtained for the DNA-calibrator **UG1** in the last amplification cycle. Make sure that the fluorescence curve of the DNA-calibrator **UG1** has the typical exponential growth of fluorescence.

### **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.

Final result of the DNA concentration of the analyzed microorganism may be in two variants – absolute and relative (normalized) values.

In both cases, the concentration of the internal control DNA (Glob) should be estimated: it should exceed  $10^3$  and  $10^2$  GE/reaction in urethral and cervical swabs for women and men, respectively.

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### **Troubleshooting**

1. If the value determined in the results grid for Negative Control of extraction (C–) and/or Negative Control of amplification (NCA) in the channel assigned for IC detection (HEX or ROX or Cy5 according to the used PCR kit) is more than 5 copies/reaction (500 copies/ml), it indicates the contamination of reagents or samples. In such cases the results of analysis are considered invalid for all samples. The PCR analysis (beginning with the DNA extraction stage) should be repeated for all samples in which specific DNA was detected. Measures for detecting and elimination of contamination source must be taken.
2. If the signal is detected for Negative Control of Extraction (C–) and/or Negative Control of amplification (NCA) in the channels for detection of microorganisms' DNA, PCR analysis should be repeated for all samples in which *Ct* values are determined in corresponding channels.
3. If *Ct* value is absent for DNA calibrators (UG1 and UG2) in the FAM and/or HEX (as well as in the ROX channel in case of using PCR kits for detection of DNA of two microorganisms, and ROX and Cy5 channel in case of using PCR kits for detection of three microorganisms) amplification should be repeated for all samples.
4. If the  $Ct(UG2) - Ct(UG1)$  difference is more than  $10 \pm 1$ , it indicates calibration failure. It is necessary to check calibrators parameters and correct inaccuracy. In case of repeated insufficient results, analysis must be repeated for all probes and calibrators.

### List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
31.05.21 MM	Through the text	The symbol  was changed to NOTE:
	Cover page	The phrase "For research use only. Not for diagnostic procedures" was added
13.07.23 EM	Footer	<b>REF</b> R-B75-100-FT(RG,iQ,Mx)-CE was added
04.08.25 HM	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) AND Rotor-Gene Q (QIAGEN, Germany) instruments	The <b>Calibrate /Gain Optimisation.../</b> value for JOE/Yellow, ROX/Orange, <i>Cy5/Red</i> and <i>Cy5.5/Crimson</i> channels was changed from "4 FI to 8 FI" to "5 FI to 10 FI" . The note "it is acceptable to set <b>Calibrate/Gain Optimisation...</b> value from 4 FI to 8 FI for the <b>JOE/Yellow, ROX/Orange, Cy5/Red</b> and <b>Cy5.5/Crimson</b> channels" was added



