

## GUIDELINES

### to AmpliSens<sup>®</sup> *Zika virus*-FRT PCR kit

for qualitative detection of RNA of *Zika virus* 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 using **AmpliSens® Zika virus-FRT** PCR kit for qualitative detection of RNA of *Zika virus* in the biological material (blood plasma, saliva, urine, spermatic fluid, histological material (autopsy/biopsy material, placenta), amniotic fluid, mosquitos, oropharyngeal swabs) 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);
- CFX96 (Bio-Rad, USA).

## Nucleic acid extraction kits recommended for RNA extraction

The Federal Budget Institute of Science “Central Research Institute for Epidemiology” recommends the following nucleic acid extraction kits for RNA extraction for further PCR analysis with the use AmpliSens® *Zika virus*-FRT PCR kit:

- RIBO-prep, **REF** K2-9-Et-50-CE for RNA extraction from blood plasma, saliva, urine, spermatic fluid, histological material (autopsy/biopsy material, placenta), amniotic fluid, mosquitos, oropharyngeal swabs in accordance with the Instruction manual for the nucleic acid extraction kit;
- MAGNO-sorb, **REF** K2-16-200-CE, **REF** K2-16-1000-CE for RNA extraction from blood plasma and urine in accordance with the Instruction manual for the nucleic acid extraction kit.

## Correspondence of targets and detection channels

Fluorophore	FAM	JOE
Detection channel name for different instrument models <sup>1</sup>	FAM/Green	JOE/HEX/R6G/Yellow/Cy3
DNA target	IC cDNA	<i>Zika virus</i> cDNA
Target gene	Artificially synthesized sequence	NS3

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

## **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 (e.g. Axygen, USA), or 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 thermocycler**

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 (the rotor wells are numbered, the numbers are used for the further programming of the samples' order in the thermocycler). Insert the rotor into the instrument, close the lid.

**NOTE:** Well 1 must be filled with any test tube except for an empty one.

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 software main menu. To create the template select the **Advanced** tab in the opened window **New run**.
2. Select the **TwoStep/Hidrolisis 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:

### Amplification program

<b>Step</b>	<b>Temperature, °C</b>	<b>Time</b>	<b>Fluorescence detection</b>	<b>Number of cycles</b>
Hold	50	15 min	–	1
Hold	95	15 min	–	1
Cycling	95	10 s	–	45
	55	20 s	FAM/Green, JOE/Yellow	

6. After setting up the temperature profile click the **OK** button.
7. Click the **Calibrate/Gain Optimisation...** button in the **New Run Wizard** window. In the opened window:
  - a) for signal measurement optimisation for the selected channels set calibration from **5FI to 10FI** for all the channels (FAM/Green, JOE/Yellow).  
To do this, click the **Calibrate Acquiring/Optimise Acquiring** button. In the opened window for first channel (**Auto Gain Optimisation Channel Settings/Auto Gain Calibration Channel Settings**) indicate the values of minimum and maximum signal in the **Target Sample Range** line. Click the **OK** button. The window for the next channel will open automatically. The selected values for all the channels can be checked in the **Min Reading, Max Reading** boxes.
  - b) perform the calibration in the selected channels before the first detection (tick the **Perform Calibration Before 1<sup>st</sup> Acquisition/ Perform Optimisation Before 1<sup>st</sup> Acquisition** option). Click the **Close** button.
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® Zika virus-FRT**. 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. Click the **New** button in the software main menu. In the opened **New Run** window select the **Advanced** tab. Then select the **AmpliSens® Zika virus-FRT** template (which is programmed as described in the “Creating the template for the run” section) in the template list.
2. 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.
3. 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.
4. 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).

5. 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, the Positive control of amplification as C+. Set the type **Unknown** opposite all the test samples, the type **Positive control** – for the Positive control of amplification, the type **Negative control** – for the Negative control of amplification. 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 analysed.

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

#### **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 in the corresponding column of the results table.

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

1. Activate the button **Analysis** in the menu, select the mode of the analysis **Quantitation**, activate the buttons **Cycling A. FAM/Cycling A. Green, Show**.
2. Cancel the automatic choice of the threshold line level **Threshold**.
3. Select the **Linear scale**.
4. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of main window (**Quantitation analysis**).
5. In the **Calculation** menu (in the right part of the window) indicate the threshold line level **0.03** in the **Threshold** box.
6. Choose the parameter **More settings/Outlier Removal** and set **5 %** for the value of negative samples threshold (**NTC/Threshold**).
7. Set **5** in the **Eliminate cycles before:** menu (in the right part of the window).
8. In the results grid (the **Quantitation Results** window) one will be able to see the *Ct*

values.

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

<b>Channel</b>	<b>Threshold</b>	<b>More Settings/Outlier Removal</b>	<b>Slope Correct</b>
FAM/Green	0.03	5 %	on
JOE/Yellow	0.03	5 %	on

**NOTE:** If the fluorescence curves in the FAM/Green, JOE/Yellow channels do not correspond to the exponential growth, then **NTC threshold** value can be increased up to 10 %.

### **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.

**Possible problems and special aspects of results analysis with the use of Rotor-Gene 3000/6000/Q software**

<b>Possible problems</b>	<b>Reason</b>	<b>Characters</b>	<b>Ways to eliminate</b>
Negative samples are analyzed as positive by Rotor-Gene software	Incorrect mathematical processing of negative samples in the presence of the fluorescence decrease section at the initial cycles	A typical positive sample has a characteristic S-shaped curve of fluorescence accumulation. Incorrectly processed negative samples are viewed as quite straight bottom-up lines	One must use the <b>Ignore First</b> by selecting the value 5 cycles. If it does not lead to a proper result, try to increase this value by 1 - 5
Sensitivity decrease due to impurity of the instrument lenses	Impurity of lenses leads to reduction of effectiveness of fluorescence excitation and detection. It primarily affects to samples with small quantity of specific RNA, which show low fluorescence growth	Low values of the background signal in all 4 detection channels (<1) at the maximum value of the <b>gain</b> parameter (10)	Clean the horizontal and vertical lenses of the instrument by a dry disposable cotton disc at least once a month
The absence of the fluorescence curves when the fluorescence values are less than 1 or more than 100	The autocalibration parameter from 5FI to 10FI is not set or it is a failure of the first tube in the rotor (tube is absent, RNA sample or reaction mixture is added incorrectly)	Most of fluorescence background signals are less than 1 or greater than 20	Repeat the PCR after setting the autocalibration parameters. Make sure that the well 1 is filled with any test tube except for an empty one

The information about all set parameters of the experiment and the autocalibration report can be found by browsing the experiment settings (the **Settings** button). Particularly, **Autocalibration Log Messages** point in the **Messages** tab is the autocalibration report.

## AMPLIFICATION AND DATA ANALYSIS USING CFX96 (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. 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...** Set the amplification parameters (time, temperature, cycles, and fluorescence acquiring cycle) in the opened **Protocol Editor – New** window. Set **Sample Volume – 25 µl**.

#### AmpliSens unified amplification program

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
Hold	50	15 min	–	1
Hold	95	15 min	–	1
Cycling	95	10 s	–	45
	55	20 s	FAM, HEX	

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

1	50,0	C for 15:00
		Slow Ramp Rate to 2,5 C per second
2	95,0	C for 15:00
		Slow Ramp Rate to 2,5 C per second
3	95,0	C for 0:10
		Slow Ramp Rate to 2,5 C per second
4	55,0	C for 0:20
		+ Plate Read
		Slow Ramp Rate to 2,5 C per second
5	GOTO 3	,44 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 and click the **Selected** checkbox next to the **FAM, HEX** fluorophores and click **OK**. In the **Sample type** menu select **Unknown** for all the samples. 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.
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**.

### Using the ready template for the run

The test parameters and the plate setup set earlier can be used for the further runs. To do this:

- click the **Select Existing...** button in the **Run Setup** window of the **Protocol** tab. Select the needed file with the amplification program in the **Select Protocol** window. Click **Open**.
- go to the **Plate** tab in the **Run Setup** window. Click the **Select Existing...** button. Select the needed file with the plate setup in the **Select Plate** window. Click **Open**. Click the **Edit selected** button to edit the plate setup.

### 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 *Ct* (threshold cycle) value in the corresponding column of the results table.

1. Start the software and open the saved file with data of the analysis. To do this, select **File** in the menu, then **Open** and **Data file** and select the needed file.
2. The fluorescence curves, the tube order in the plate and the table with the *Ct* values are represented in the **Data Analysis** window of the **Quantification** tab.

3. For each channel at a time set the threshold line at the specific level in accordance with the table 1. To do this tick the **Log Scale** box in the lower part of the window (switching to logarithmic view). The threshold line is set as % of maximum fluorescence obtained in the corresponding channel for the Positive control of amplification (C+) in the last amplification cycle. For threshold line setting at the specific level drag it with a cursor while pressing the left mouse button.

Table 1

Channel	Threshold line level, % of maximum fluorescence obtained for the C+ sample
FAM	10
HEX	5

4. Click the **View/Edit Plate...** button on the toolbar and set the samples names in the opened window.

### **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.

### **Possible problems and special aspects of results analysis with the use of CFX96 for research software**

Possible problems	Characters	Ways to eliminate
The threshold level is set incorrectly	The threshold line passes with the negative samples or above some or all positive curves (curves are S-shaped)	Set the threshold line so that it crosses only the sigmoid curves of the fluorescence accumulation or at the level of ¼ of height between the final fluorescence values of positive and negative samples
The drops are not sedimented from the walls of the tubes before the run	The appearance of negative or positive “steps” in the curves of the fluorescence accumulation	Repeat the amplification for the sample

### List of Changes Made in the Instruction Manual

<b>VER</b>	<b>Location of changes</b>	<b>Essence of changes</b>
28.12.20 MA	Through the text	The symbol  was changed to NOTE:
29.12.20 MA	Cover page	The phrase "Not for use in the Russian Federation" was added
12.03.21 MM	Front page	The name, address and contact information for Authorized representative in the European Community was changed