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For Professional Use Only

GUIDELINES
to AmpliSens[®] *Yellow fever virus-FRT* PCR kit
using the PCR instruments with real-time
hybridization-fluorescence detection

AmpliSens[®]



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INTENDED USE

The guidelines describe the procedure of using **AmpliSens® Yellow fever virus-FRT** PCR kit using the following PCR instruments with real-time hybridization-fluorescence detection:

- Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia);
- Rotor-Gene Q (QIAGEN, Germany);
- CFX96 (Bio-Rad, USA).

Nucleic acid extraction kits recommended for DNA extraction

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

- **RIBO-prep** **REF** K2-9-Et-100-CE for DNA/RNA extraction from blood plasma, mosquitoes, urine, csaliva, tissue (autopsy, biopsy) material 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/DNA extraction from blood plasma, urine

Correspondence of targets and detection channels

Fluorophore	FAM	JOE
Detection channel name for different instrument models ¹	FAM/Green	JOE/HEX/R6G/ Yellow/Cy3
cDNA-target	Internal Control-FL (IC) cDNA	<i>Yellow fever virus</i> cDNA
Target gene	Artificially synthesized sequence	5'-UTR C-protein

¹ 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/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 (or 30 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:

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
	60	20 s	FAM/Green, JOE/Yellow	

NOTE: Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program.

Note – ROX/Orange, Cy5/Red, Cy5.5/Crimson channels are enabled when required if the “multiprime” format tests are performed.

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 optimization 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 1st Acquisition/ Perform Optimisation Before 1st 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**. 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** 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-optimization 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, 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, 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 *C_t* (threshold cycle) value of the DNA sample 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 **Cycling A. FAM/Cycling A. Green, Show** buttons.
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 **CT 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.

Channel	Threshold	More Settings/Outlier Removal	Slope Correct	Eliminate Cycles before
FAM/Green	0,03	5%	on	5
JOE/Yellow	0,03	5%	on	5

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

Possible problems	Reason	Characters	Ways to eliminate
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 Ignore First 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 gain parameter (10)	Clean the horizontal and vertical lenses of the instrument by a dry disposable cotton disc at least once a month

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 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
	60	20 s	FAM, Hex/R6G	

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
2	95.0	C for 15:00
3	95.0	C for 0:10
Slow Ramp Rate to 2.5 C per second		
4	60.0	C for 0:20
+ Plate Read		
Slow Ramp Rate to 2.5 C per second		
5	GOTO 3	.44 more times
END		

NOTE: Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program.

Note – The ROX, Cy5, Quasar 705 channels is enabled when required if the “multiprime” format tests are performed.

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, ROX, Cy5** 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.
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.

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** item (logarithmic scale selection) in the bottom of the window. 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 (the fluorescence level is equal to the proximate point marked with number).

Table 1

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

3. Click the **View/Edit Plate...** button on the toolbar and set the samples names and calibrators concentrations 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 r

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 (herewith, curves are S-shaped in a linear scale)	Set the threshold line so that it crosses only the sigmoid curves of the fluorescence accumulation or at the level of $\frac{1}{4}$ 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 Guidelines

VER	Location of changes	Essence of changes
30.06.11 LA	Cover page	The name of Institute was changed to Federal Budget Institute of Science “Central Research Institute for Epidemiology”
20.08.11 LA	Page footer	Reference number, R-V53(RG,iQ,Mx)-CE-B, was added
09.07.13 ME	Page footer	REF R-V53(RG,iQ,Mx)-CE-B was deleted
03.04.14 SA	Cover page	Address of European representative was added
09.06.16 ME	Text	Corrections according to the template
12.11.19 PM	Through the text	Corrections according to the template
	Amplification and data analysis using CFX96 (Bio-Rad, USA)	The chapter was added
25.12.20 EM	Through the text	The symbol  was changed to NOTE:
	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