

## **GUIDELINES**

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

for qualitative detection of *West Nile virus* RNA 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® WNV-FRT** PCR kit for qualitative detection of *West Nile virus (WNV)* RNA in the biological material 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 (Stratagene, USA);
- CFX96 (Bio-Rad, USA).

Table 1

### Correspondence of targets and detection channels

PCR-mix-1	Detection in channel	
	FAM/Green	JOE/Yellow/HEX
RT-PCR-mix-1-FRT <i>WNV</i>	IC	<i>WNV</i>

### Correspondence of fluorophores and detection channels

Channel for the fluorophore	Detection channel name for different instrument models <sup>1</sup>
FAM	FAM/Green
JOE	JOE/HEX/R6G/Yellow/Cy3

<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 instrument.

**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 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 **36-Well Rotor** and tick the **No Domed 0,2ml Tubes / Locking Ring Attached** option. Click the **Next** button.
4. In the opened window, set the operator and select the reaction mixture volume: **Reaction volume – 25 µl**. 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 for rotor-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
Hold 1	50	30 min	–	1
Hold 2	95	15 min	–	1
Cycling 1	95	5 s	–	5
	56	25 s	–	
	72	15 s	–	
Cycling 24	95	5 s	–	40
	56	25 s	FAM/Green, JOE/Yellow	
	72	15 s	–	

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 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**. 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-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 analyzed.

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<sub>t</sub>* (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 **Analysis** button in the menu, select the mode of the analysis **Quantitation**, activate **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 **More settings/Outlier Removal** parameter 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	Dynamic tube	Slope Correct	More Settings/Outlier Removal	Eliminate cycles before:
FAM/Green	0.03	on	on	5%	5
JOE/Yellow	0.03	on	on	5%	5

**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.

## **AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ and iCycler iQ5 (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, 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 iQ/iQ5 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.

### **Creating the template for the run**

1. Set the plate setup (set the order of the tubes in the reaction chamber and the detection of fluorescent signal).
  - In case of the **iCycler iQ5** instrument, to set the plate setup click the **Create New** or **Edit** button in the **Selected Plate Setup** window of the **Workshop** module. It is possible to edit plate setup in the **Whole Plate loading** mode. Set the reaction volume **Sample Volume – 25 µl**, type of the caps **Seal Type – Domed Cap**, and the type of tubes **Vessel Type – Tubes**. Save the plate setup by pressing the **Save&Exit Plate Editing** button.
  - For the **iCycler iQ** instrument, edit the plate setup in **Edit Plate Setup** window of the **Workshop** module. To do this, in option **Samples: Whole Plate Loading** set the order of the tubes in the reaction chamber and name each sample in the **Sample Identifier** window. In the **Select and load Fluorophores** option, set fluorescence measurement for all tubes in **FAM-490** and **JOE-530** channels.
  - Save the plate setup: enter the file name (with .pts extension) in the **Plate Setup Filename** window and click the **Save this plate setup** button (at the top of the display). The previously used **Plate Setup** can be edited: in the **Library** window,

open **View Plate Setup**, select the required **Plate Setup** (file with .pts extension), and click the **Edit** button in the right side. The edited file should be saved before use. Click the **Run with selected protocol** button to set the use of the selected plate setup.

2. Set the amplification program.

#### Amplification program for plate-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	50	30 min	–	1
2	95	15 min	–	1
3	95	5 s	–	5
	56	30 s	–	
	72	15 s	–	
4	95	5 s	–	40
	56	30 s	FAM/FAM-490, JOE/HEX/JOE-530	
	72	15 s	–	

- For the **iCycler iQ5** instrument, in order to create a protocol, click the **Create New** or **Edit** button in the **Selected Protocol** window of the **Workshop** module. Set the amplification parameters and save the protocol by clicking the **Save&Exit Protocol Editing** button. For further experiments, the file with this program can be selected in the **Protocol** block (protocol files are saved in the **Users** folder by default).
  - For the **iCycler iQ** instrument, create the amplification program: select the **Edit Protocol** option of **Workshop** module. Set the amplification parameters in the bottom window (cycle repeats, time, and temperature), in the right window specify the scanning step for the fluorescent signal: **Cycle 4 – Step 2**. Save the protocol, name the file in the **Protocol Filename** window (with .tmo extension), and click the **Save this protocol** button (in the upper part of the display). For further experiments, the file with this program can be selected in the **View Protocol** tab in the **Library** module. Click the **Run with selected plate setup** button after selecting or editing the required program to start it.
3. Insert the prepared tubes into the reaction chamber according to the selected plate setup.
- For the **iCycler iQ5** instrument, before a run it is obligatory to check if the selected protocol (**Selected Protocol**) and the plate scheme (**Selected Plate Setup**) are correct. To begin a run click the **Run** button. For the well factors measurement the **Use Persistent Well Factors** type is selected by default. Click the **Begin Run** button, save the experiment (the results of this experiment will be automatically saved in this file) and click **OK**. Select the type of the caps **Seal Type – Domed**

**Cap**, and the type of the tubes **Vessel Type – Tubes**.

- For the **iCycler iQ** instrument, before a run it is obligatory to check if the name of selected protocol and the plate scheme are correct in the **Run Prep** window. For the well factor measurement, select the **Persistent Plate** type in the **Select well factor source** menu. Set the reaction mix volume in the window **Sample Volume – 25 µl**. Click the **Begin Run** button to start the program, name the experiment (the results of this experiment will be automatically saved in this file), and click the **OK** button.
- 4. At the end of the run it is necessary to close the software and turn off the instrument (the thermocycler and the optical block).

### **Data analysis**

The obtained results are analyzed by the iCycler iQ5/iQ 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.

### **Data processing**

- For the **iCycler iQ5** instrument, select the required file with data of analysis (in the **Data File** window of the **Workshop** module) and click the **Analyze** button. Select data for the corresponding channel in the module window. Data analysis mode **PCR Base Line Subtracted Curve Fit** should be selected (by default).
- For the **iCycler iQ** instrument, activate the **View Post-Run Data** window in the **Library** module. In the **Data Files** window, select the required file with data of analysis and click the **Analyze Data** button. In the **PCR Quantification** option (**Select a Reporter** menu), select the icon of the corresponding channel. The **PCR Base Line Subtracted Curve Fit** data analysis mode should be selected (by default). In the **Threshold Cycle Calculation** menu, select manual setting the threshold line and automatic baseline calculation. Select the **Auto Calculated** in the **Baseline Cycles** submenu, select **User Defined** in the **Threshold Position** submenu. To set the threshold line level, click on it and drag it by cursor holding the left mouse button. Click the **Recalculate Threshold Cycles** button. *Ct* values will appear in the results grid.

### **Amplification data analysis for IC cDNA:**

1. Click the **FAM** button in the **Data Analysis** menu.
2. Select the **Baseline Threshold** option by clicking the right mouse button on the plot of fluorescence accumulation curves.

3. Select **User Defined, Select all, Edit Range** and set **Start Cycle = 2, Ending Cycle = 10** in the **Base Line Cycles** menu. Select **User Defined** and set **Threshold position = 50** in the **Crossing Threshold** menu. Click the **OK** button.
4. In the results grid (**Results** window), *Ct* values will appear.

#### **Amplification data analysis for WNV cDNA:**

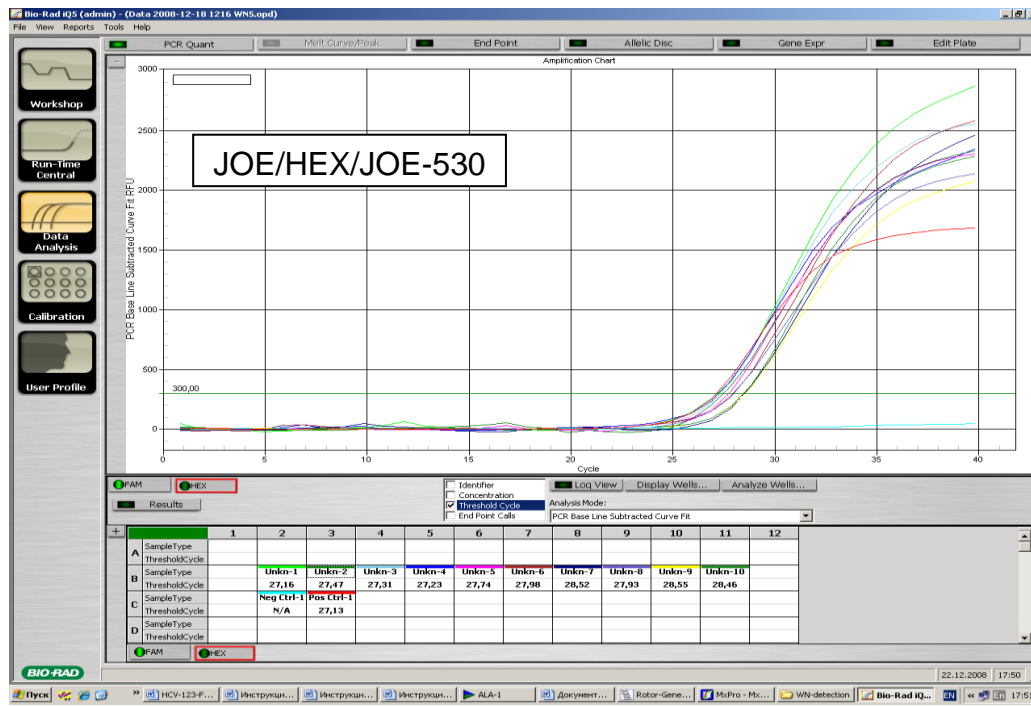
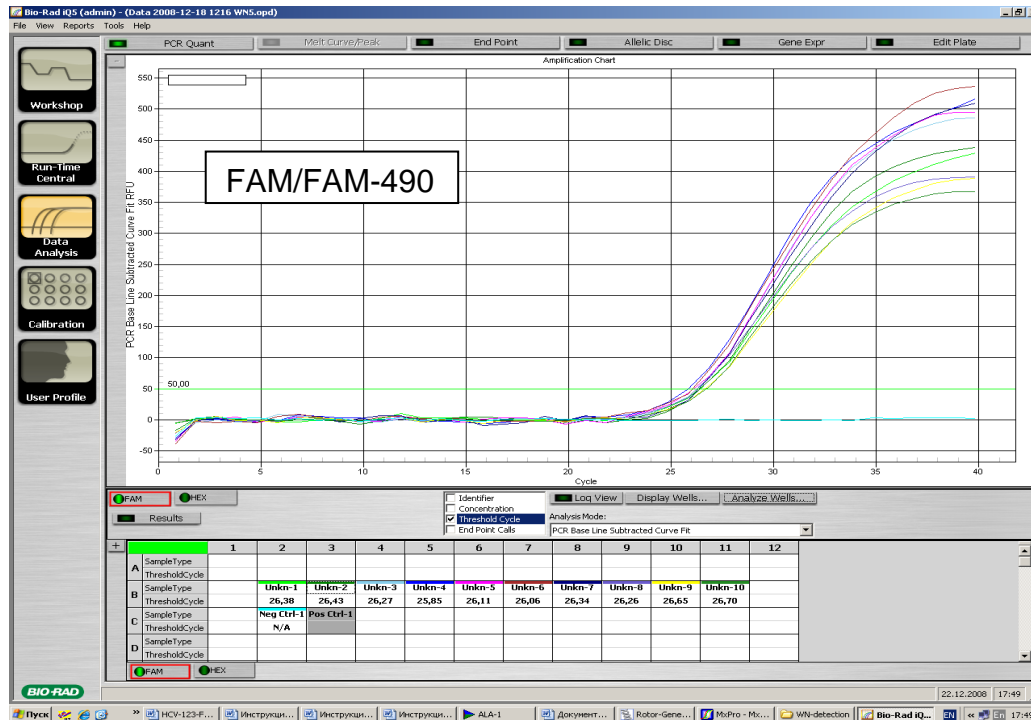
1. Click the **JOE** button in the **Data Analysis** menu.
2. Select the **Baseline Threshold** option by clicking the right mouse button on the plot of fluorescence accumulation curves.
3. Select **User Defined, Select all, Edit Range** and set **Start Cycle = 2, Ending Cycle = 10** in the **Base Line Cycles** menu. Select **User Defined** and set **Threshold position = 200** in the **Crossing Threshold** menu. Click the **OK** button.
4. In the results grid (**Results** window), *Ct* values will appear.

#### **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.

## Example of amplification with the use of iCycler iQ5 instrument



## AMPLIFICATION AND DATA ANALYSIS USING Mx3000P (Stratagene, USA) INSTRUMENT

Carry out the sample pretreatment and reaction mixture preparation stages according to the PCR kit *Instruction Manual*.

Program the thermocycler according to manufacturer's manual.

1. Switch on the instrument. Run the software Stratagene Mx3000P.
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, lock the fixing arm and the door of the instrument.
4. Select **Optics Configuration** in the **Options** menu and in the **Dye Assignment** tab set FAM parameter next to the **FAM filter set** item and JOE parameter next to the **HEX/JOE filter set**.
5. Set the fluorescence detection parameters in the **Plate Setup** menu. To do this, select all the cells with the test tubes and mark them as **Unknown** in the **Well type** field. Select **FAM** and **JOE** fluorophores in the **Collect fluorescence data** option.
6. Name each sample in the **Well Information** window.
7. Set the parameters for the fluorescence detection of the tubes in the **Plate Setup** tab. Select all the wells with the test tubes, click the **Unknown** and the **Collect fluorescence data** buttons in **Well type** menu. Select fluorophores **FAM** and **JOE**.
8. Set the amplification program in **Thermal Profile Setup** tab.

**Amplification program for plate-type instruments**

Step	Temperature, °C	Time	Fluorescence detection	Cycle repeats
1	50	30 min	–	1
2	95	15 min	–	1
3	95	5 s	–	5
	56	30 s	–	
	72	15 s	–	
4	95	5 s	–	40
	56	30 s	FAM, JOE/HEX	
	72	15 s	–	

9. Select the **Run** button, ensure that the specified amplification program is correct. Then click the **Start** button. Put a checkmark in the **Turn lamp off at end of run** window. Save the experiment.

### Data analysis

1. Open the saved file and proceed to the **Analysis** mode.
2. Activate the **Results** window in the menu.

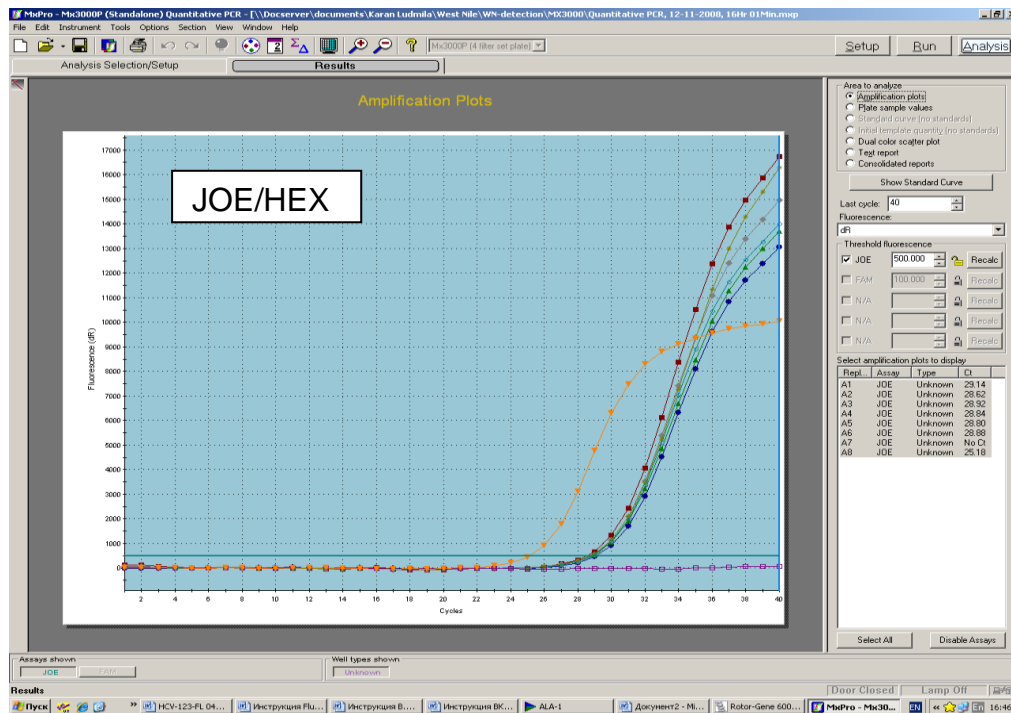
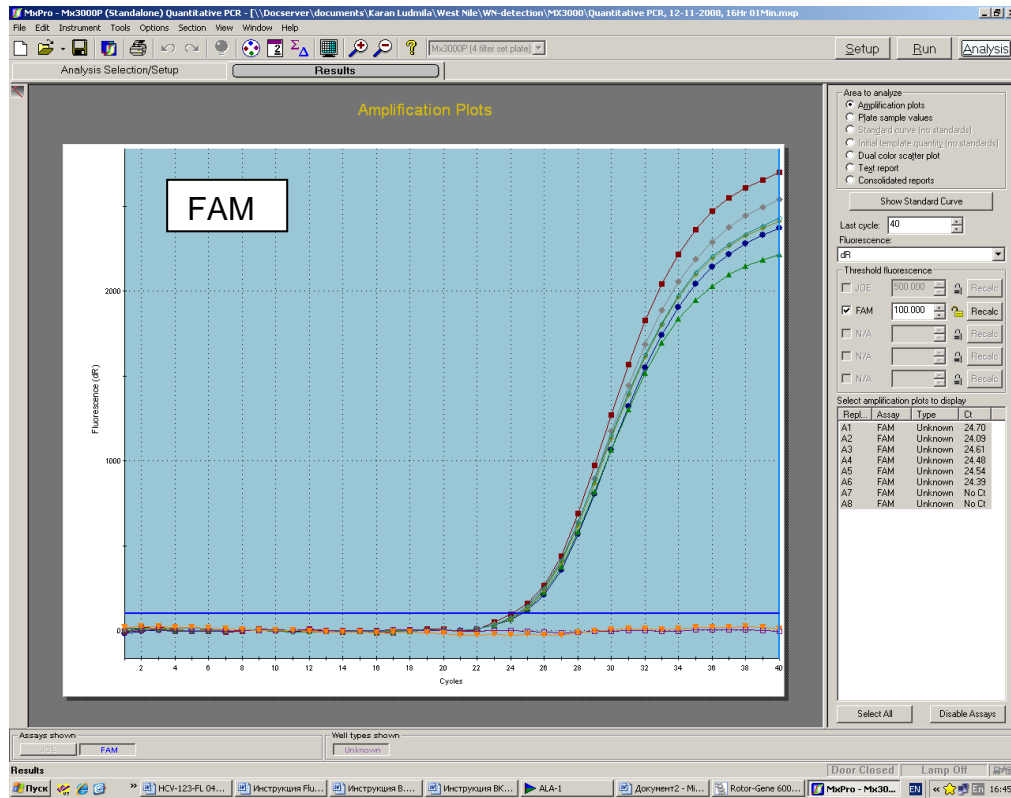
3. Select the ***Amplification plots*** line in the ***Area to analyze*** block.
4. In ***Threshold fluorescence*** menu, set the threshold line at a level where the fluorescence curves are linear. It is recommended to select the threshold line level equal to **150** for **FAM** channel and **500** for **JOE/HEX** 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.
5. Select the ***Text report*** line in the ***Area to analyze*** block.

### **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.

## Example of amplification with the use of Mx3000P instrument



## 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
1	50	30 min	–	1
2	95	15 min	–	1
3	95	5 s	–	5
	56	30 s	–	
	72	15 s	–	
4	95	5 s	–	40
	56	30 s	FAM, Hex	
	72	15 s	–	

**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 30:00
	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 0:05
	Slow Ramp Rate to 2,5 C per second	
4	56,0	C for 0:30
	Slow Ramp Rate to 2,5 C per second	
5	72,0	C for 0:15
	Slow Ramp Rate to 2,5 C per second	
6	GOTO 3 , 4 more times	
7	95,0	C for 0:05
	Slow Ramp Rate to 2,5 C per second	
8	56,0	C for 0:30
	+ Plate Read	
	Slow Ramp Rate to 2,5 C per second	
9	72,0	C for 0:15
10	GOTO 7 , 39 more times	
	END	

- Save the protocol: in the **Protocol Editor New** window select **File**, then **Save As**, name the file and click **Save**.
- 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.
- Save the plate setup: select **File** and then **Save as** in the **Plate Editor New** window. Enter the file name, click **Save**.
- 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.

- 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 3. 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 3

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 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.

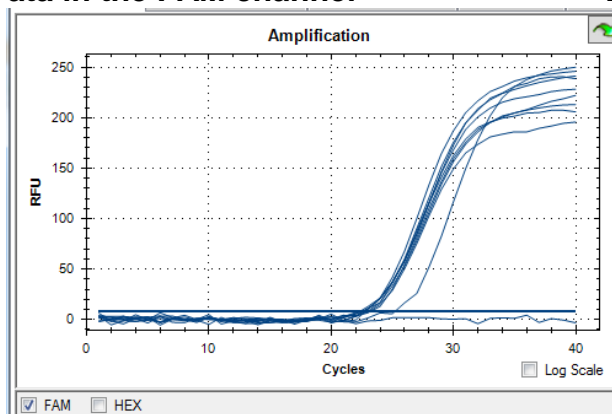
The interpretation of the test samples is to be carried out in accordance with the *Instruction Manual* and the *Important Product Information Bulletin* enclosed to the PCR kit.

**Possible problems and special aspects of results analysis with the use of Bio-Rad CFX software.**

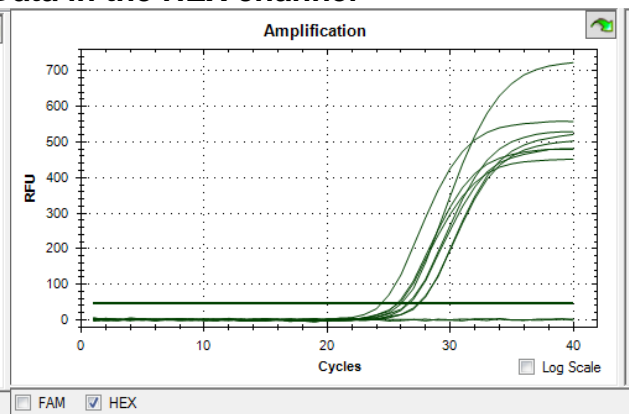
Possible problems	Characters	Ways to eliminate
The threshold level is set incorrectly	The threshold line passes with the negative samples or above some or all positive curves (curves are S-shaped)	Set the threshold line so that it crosses only the sigmoid curves of the fluorescence accumulation or at 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

**EXAMPLES OF OBTAINED RESULTS**

**Data in the FAM channel**



**Data in the HEX channel**



### List of Changes Made in the Guidelines

<b>VER</b>	<b>Location of changes</b>	<b>Essence of changes</b>
24.07.25 PS	Through the text	Misprints were corrected
	Amplification and data analysis using Rotor-Gene 3000/6000 instruments	Checkbox ticking was removed for <b>15 µl oil layer volume</b> position