

GUIDELINES

to AmpliSens[®] *Coxiella burnetii*-FRT PCR kit

for qualitative detection of the DNA of *Coxiella burnetii* in the ticks, biological human material (blood, sputum, bronchial washing fluid, liquor, autopsy material) and animal material (blood, autopsy material, placenta and abortive material) using real-time hybridization-fluorescence detection

AmpliSens[®]



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INTENDED USE

The guidelines describe the procedure of using **AmpliSens® *Coxiella burnetii*-FRT** PCR kit for qualitative detection of *Coxiella burnetii* DNA in the ticks and 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, iQ5 (Bio-Rad, USA);
- Mx3000P (Stratagene, USA);

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 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.

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

Programming the thermocycler

1. Switch the instrument on.
2. Insert the tubes into the rotor of the thermocycler beginning with the Well 1 (the rotor wells are numbered, the numbers are used for the further programming of the samples' position in the thermocycler). Put the rotor into the instrument, close the lid.

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

3. Click the **New** button in the main menu of the program.
4. Select the **Advanced** template of the experiment's start and mark **Dual Labeled Probe/Hydrolysis probes**. Click the **New** button.
5. In the open window select **36-Well Rotor**, and mark that you do not use tubes with round caps (Rotor-Gene 3000) / the locking ring is fixed (Rotor-Gene 6000). Click the **New** button.
6. In the open window set the operator and the volume of the reaction mix: **Reaction volume – 25 µl**. For the Rotor-Gene 6000 instrument mark the **15 µl oil layer volume**

function with a tick. Click the **New** button.

- In the open window it is necessary to set the temperature profile of the experiment. To do this click the **Edit profile** button and set the following parameters (see Table 1).

Table 1

The amplification program for rotor-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
Hold	95	15 МИН	–	1
Cycling 1	95	5 с	–	5
	60	20 с	–	
	72	15 с	–	
Cycling 2	95	5 с	–	40
	56	20 с	FAM/Green, JOE/Yellow	
	72	15 с	–	

- Click the **OK** button.
- Click the **Calibrate/Gain Optimisation...** button in the **New Run Wizard** window:
 - perform the calibration through the FAM/Green, JOE/Yellow channels (click the **Calibrate Acquiring/Optimise Acquiring** button);
 - calibrate before the first acquisition (**Perform Calibration Before 1st Acquisition/Perform Optimisation Before 1st Acquisition**);
 - channel calibration setting for all the dyes from 5FI to 10FI (the **Edit...** button, the **Auto gain calibration channel settings** window). Click the **Close** button.
- Click the **Next** button, start the amplification by clicking the **Start run** button.
- Name the experiment and save it on the disc (the results of the experiment will be saved automatically in this file).
- Enter the data into the samples grid (*it opens automatically after/before the starting of the amplification*). In the **Name** column indicate the names/numbers of the test clinical and control samples. Set the **None** type for empty wells.

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

Data analysis:

Analysis of results is performed by the software of the real-time PCR instrument. Results are interpreted by the crossing (or not-crossing) the fluorescence curve with the threshold line set at the specific level that corresponds to the presence (or absence) of a *Ct* value of the DNA sample in the corresponding column of the results grid.

Amplification results analysis through the FAM/Green channel (Internal Control STI-87).

- Click the **Analysis** button, select the **Quantitation** analysis mode, activate the **Cycling A. FAM/Cycling A.Green**, click **Show**.

2. Cancel the automatic choice of the threshold line level **Threshold**.
3. The **Dynamic tube** and **Slope Correct** buttons are to be activated in the menu of the main window (**Quantitation analysis**).
4. In the **CT Calculation/Вычисление Ct** (in the right part of the window) menu set the threshold line level: **Threshold = 0.03, Eliminate Cycles before – 5**.
5. In the menu of the main window (**More settings/Outlier Removal**) set for the value of negative samples threshold (**NTC threshold**) – **5%**.
6. Ct values will appear in the results grid (the **Quant. Results** window).

The results analysis through the JOE/Yellow channel is to be carried out in the same way as through the FAM/Green channel.

NOTE: If the fluorescence curves in any channel does not correspond the exponential growth increase the value of the threshold of the negative samples for this channel (**NTC threshold**) by **10 %**.

Results analysis:

The result of the analysis is considered reliable only if the results obtained for Positive and Negative Controls of amplification as well as for the Negative Control of extraction are correct (see the “Results for control” table in the *Instruction Manual* and the Ct values given in the *Important Product Information Bulletin* enclosed to the PCR kit).

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

TROUBLESHOOTING

1. If the Ct value determined for the samples (except Negative Control of Amplification (NCA)) for the all channels is absent, the amplification and detection should be repeated. If the same result is obtained in the second run, the PCR analysis should be repeated starting from the DNA extraction stage. For NCA negative result for all channels is the norm.
2. If the Ct value for the Positive Control of Amplification (C+) through JOE/Yellow channel is absent or higher than the boundary value the amplification should be repeated for all samples in which the specific DNA was not detected.
3. If the Ct value is determined for the Negative Control of Extraction (C–) for the JOE/Yellow channel and/or Negative Control of Amplification (NCA) for the any channels, the PCR analysis should be repeated for all samples in which DNA was detected.

AMPLIFICATION AND DATA ANALYSIS WITH THE USE OF iCycler iQ and 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 optically transparent flat caps, or tubes (0.1 ml) with transparent caps from the eight-pieces-strips (e.g. Axygen, USA) (detection through the cap of the tube).

1. Switch on the instrument and the power supply unit of the optical part of the instrument.

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

2. Start the program **iCycler iQ/iQ5**.
3. Set the plate setup (set the order of the tubes in the reaction chamber and the detection of fluorescent signal).
 - For **iCycler iQ5** click the **Create New** or **Edit** buttons in the **Selected Plate Setup** window of the **Workshop** module. One can edit the plate setup in the **Whole Plate loading** mode. Set the reaction volume (**Sample Volume**) as **25 µl**, the caps type (**Seal Type**) as **Domed Cap**, and the tubes type (**Vessel Type**) as **Tubes**. Select the fluorescent signal detection through the FAM and JOE/HEX channels. Save the set plate setup by clicking the **Save&Exit Plate Editing** button.
 - For **iCycler iQ** select the setup of the samples' position in the reaction module by choosing the **Samples: Whole Plate Loading** option in the **Edit Plate Setup** window of the **Workshop** module. Name each sample in the **Sample Identifier** window. Set the fluorescence signal detection in all the tubes through **FAM-490** and **JOE-530** channels. Save the plate setup by naming the file in the **Plate Setup Filename** window (with .pts filename suffix) and clicking the **Save this plate setup** button (in the upper part of the screen). One can edit the plate setup which was used before. To do this, choose **View Plate Setup** in the **Library** window, select the needed setup in **Plate Setup** (the file with .pts filename suffix) and click the **Edit** button to the right. It is necessary to save the edited file before using. Set the using of the given plate setup by clicking the **Run with selected protocol** button.
4. Set the amplification program (see Table 2).

Table 2

The amplification program for plate-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
Hold	95	15 мин	–	1
Cycling 1	95	5 с	–	5
	60	25 с	–	
	72	15 с	–	

Cycling 2	95	5 c	–	40
	56	25 c	FAM/FAM-490, JOE/HEX/JOE-530	
	72	15 c	–	

- For **iCycler iQ5** click the **Create New** or **Edit** button in the **Selected Protocol** window of the **Workshop** module in order to create a protocol. Set amplification parameters and save the protocol by clicking the **Save&Exit Protocol Editing** button. One can select a file with this program for further runs in the **Protocol** column (files are saved as default in the **Users** folder).
 - For **iCycler iQ** select the **Edit Protocol** option of the **Workshop** module in order to create an amplification program. To do this set amplification parameters (number of cycles, time and temperature of cycling), and mark **Cycle 3 – Step 2** in the window on the right. Save the protocol by naming the file in the **Protocol Filename** window and clicking the **Save this protocol** button (in the upper part of the screen). One can select a file with this program for further runs in the **View Protocol** tab of the **Library** module. After choosing and editing of the necessary program click the **Run with selected plate setup** button.
5. Insert the tubes into the reaction module of the amplifier (thermocycler) and program 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 strips upside down while inserting them into the instrument.

- For **iCycler iQ5** before starting the program check whether the selected protocol (**Selected Protocol**) and plate setups (**Selected Plate Setup**) are correct. Click the **Run** button to start (results of the given experiment will be saved in this file automatically). Click **OK**. Select the type of caps (**Seal Type - Domed cap**), the type of tubes (**Vessel Type - Tubes**).
- For **iCycler iQ** before starting the program in the **Run Prep** window check whether the selected name of the protocol and plate setup are correct. Select the **Experimental Plate** variant in the **Select well factor source** menu for measuring well factors. Set the reaction mix volume in the **Sample Volume** window as 25 µl. Click the **Begin Run** button to start, name the experiment (results of the given experiment will be saved in this file automatically), and click the **OK** button.

After the program has been finished proceed to the results analysis.

Data analysis:

The analysis of the results is based on the presence (or absence) of the *C_t* (threshold cycle) value in the results grid. The sample curve of the fluorescence must be expressed

S-shaped on the fluorescence characteristic exponential rise and once crossing threshold line.

- For **iCycler iQ** t in **Library** module activate **View Post-Run Data** window. In window **Data Files** select the necessary file with the data of analysis and press **Analyse Data** button. In the **PCR Quantification** option in the **Select a Reporter** menu select the sign of one of the channels. The **PCR Base Line Subtracted Curve Fit** mode must be selected (it is selected as default). In the **Threshold Cycle Calculation** menu select the mode of manual setting of the threshold line and the automatic calculation of the threshold line. To do this select **Auto Calculated** in the **Baseline Cycles** submenu, and **User Defined** in the **Threshold Position** submenu. In order to set the threshold line level it is necessary to drag it with the mouse by pressing the left mouse button. Click the **Recalculate Threshold Cycles** button. *Ct* values will appear in the results grid.
- For **iCycler iQ5** select the necessary file with the data of analysis (in the **Data File** window of the **Workshop** module) and click the **Analyze** button. In the window of the module select the data through the corresponding channel. The **PCR Base Line Subtracted Curve Fit** mode must be selected (it is selected as default).

Amplification results analysis of IC DNA:

1. In **Data Analysis** menu click **FAM** button.
2. In the graph of fluorescence curves accumulation, right-click select **Baseline Threshold**.
3. Set the following parameters: the menu **Base Line Cycles** select **User Defined, Select all, Edit Range** and set **Start Cycle = 2, Ending Cycle = 20**; in the **Crossing Threshold** select **User Defined**, set **Threshold Position = 200**. Click **OK**.
4. In the results grid (**Results** window) *Ct* value will appear.

Amplification results analysis of *Coxiella burnetii* DNA:

1. In **Data Analysis** menu click **JOE** button.
2. In the graph of fluorescence curves accumulation, right-click select **Baseline Threshold**.
3. Set the following parameters: the menu **Base Line Cycles** select **User Defined, Select all, Edit Range** and set **Start Cycle = 2, Ending Cycle = 10**; in the **Crossing Threshold** select **User Defined**, set **Threshold Position = 250**. Click **OK**.
4. In the results grid (**Results** window) *Ct* value will appear.

Results analysis:

The result of the analysis is considered reliable only if the results obtained for Positive and Negative Controls of amplification as well as for the Negative Control of extraction are correct (see the “Results for control” table in the *Instruction Manual* and the *Ct* values given in the *Important Product Information Bulletin* enclosed to the PCR kit).

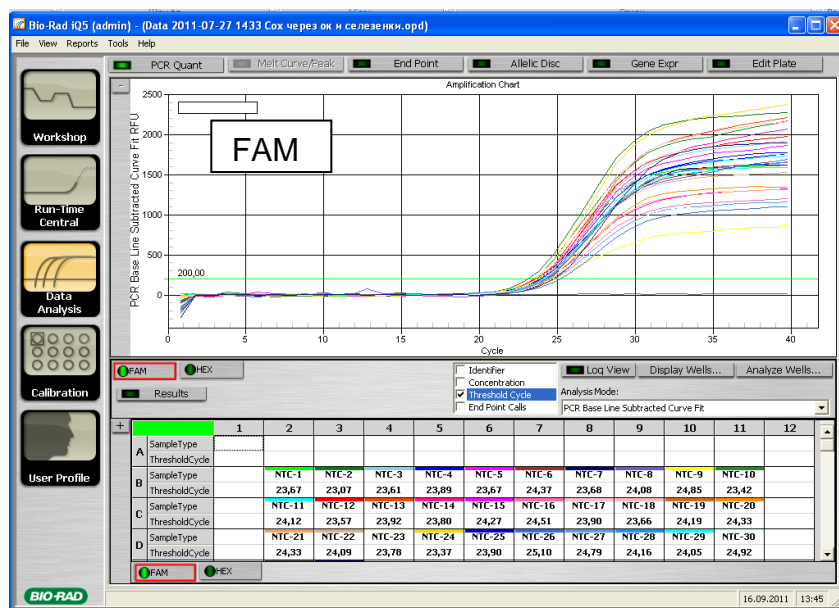
The interpretation of test samples should be performed in accordance with the *Instruction Manual* and the *Important Product Information Bulletin* enclosed to the PCR kit.

TROUBLESHOOTING

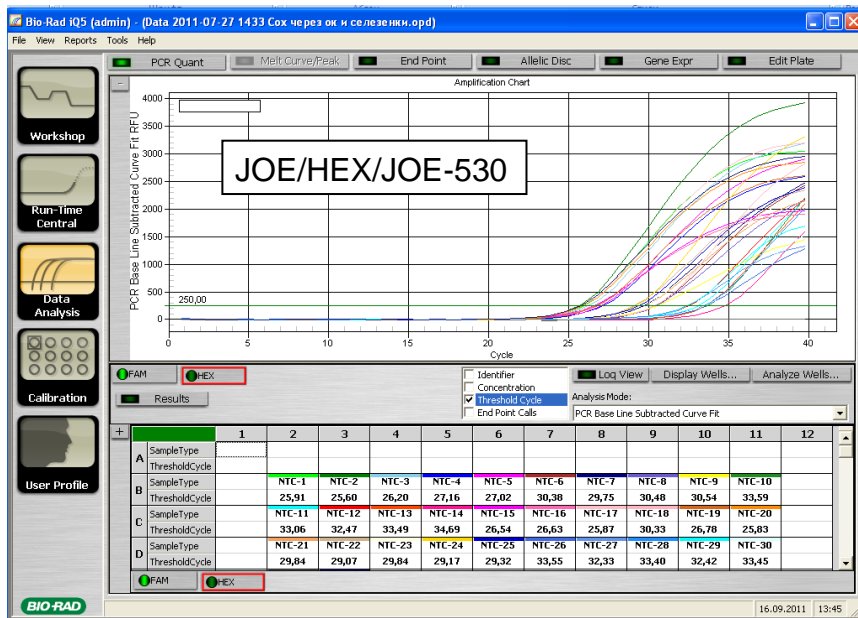
- If the *Ct* value determined for the samples (except Negative Control of Amplification (NCA)) for the all channels is absent, the amplification and detection should be repeated. If the same result is obtained in the second run, the PCR analysis should be repeated starting from the DNA extraction stage. For NCA negative result for all channels is the norm.
- If the *Ct* value for the Positive Control of Amplification (C+) through JOE/HEX/JOE-530 channel is absent or higher than the boundary value the amplification should be repeated for all samples in which the specific DNA was not detected.
- If the *Ct* value is determined for the Negative Control of Extraction (C-) for the JOE/HEX/JOE-530 channel and/or Negative Control of Amplification (NCA) for the any channels, the PCR analysis should be repeated for all samples in which DNA was detected.

EXAMPLE OF RESULTS

Data for FAM/FAM-490 channel



Data for JOE/HEX/JOE-530 channel



AMPLIFICATION AND DATA ANALYSIS USING Mx3000P (Stratagene, 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 optically transparent flat caps. (detection through the cap of the tube).

Program the thermocycler only according to the *Instruction Manual* given by the manufacturer of the instrument:

1. Switch the instrument on, start the program **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**.

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 strips/plate upside down while inserting them into the instrument.

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. In the **Well Information** window to name each sample.
7. Select the **Thermal Profile Setup** menu and set the amplification program (see Table 3).

Table 3

Amplification program for plate-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
Hold	95	15 МИН	–	1
Cycling	95	5 с	–	5
	60	25 с	–	
	72	15 с	–	
Cycling 2	95	5 с	–	40
	56	25 с	FAM, JOE/HEX	
	72	15 с	–	

8. Start the amplification by clicking the **Run** and **Start** buttons, then name the experiment. Tick the box **Turn lamp off at end of run**. Save experiment.

Data analysis:

1. Open the saved data file and switch to **Analysis**.
2. Activate in the menu the **Results** window.
3. In Block **Area to analyze** select a row **Amplification plots**.
4. In block **Threshold fluorescence** for each channel set threshold level at a level where the fluorescence curves are linear: for FAM channel is recommended to select the threshold line at 500, for JOE / HEX - 1000. Normally, the threshold line is to intersect only with sigma-shaped 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 raise the threshold level.
5. In Block **Area to analyze** select a row **Text report**.

Results analysis:

The result of the analysis is considered reliable only if the results obtained for Positive and Negative Controls of amplification as well as for the Negative Control of extraction are correct (see the “Results for control” table in the *Instruction Manual* and the *Ct* values given in the *Important Product Information Bulletin* enclosed to the PCR kit).

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

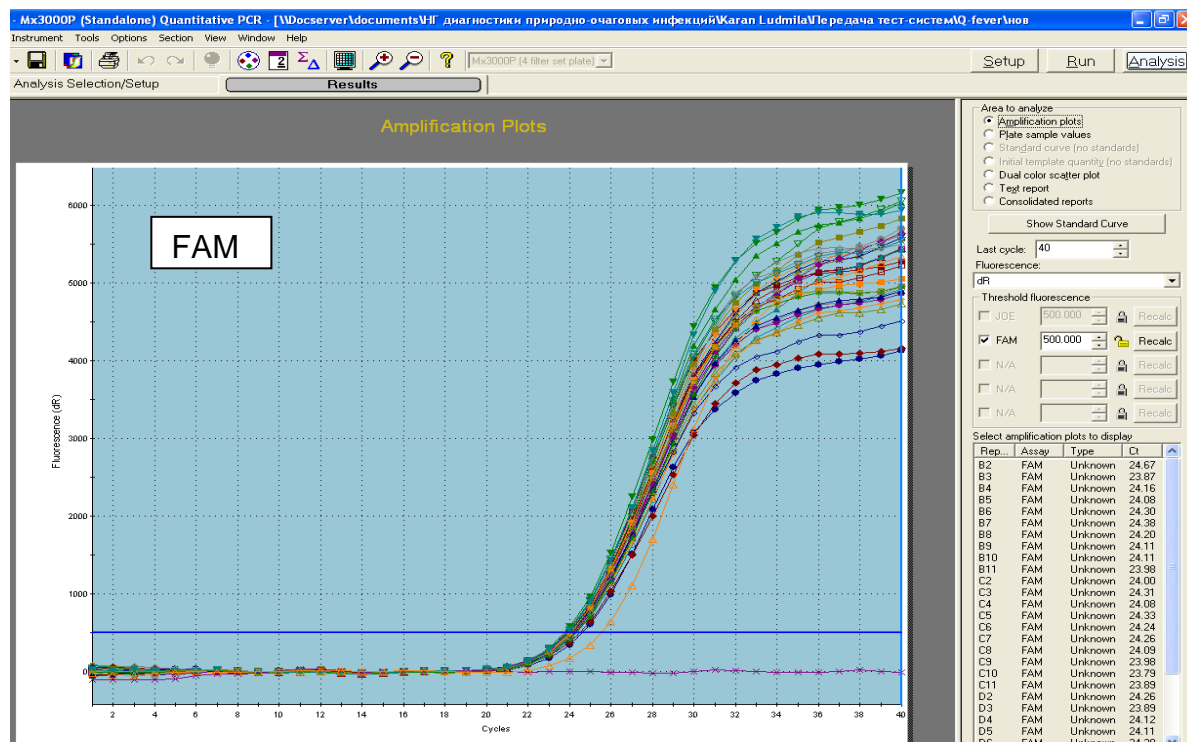
TROUBLESHOOTING

1. If the *Ct* value determined for the samples (except Negative Control of Amplification (NCA)) for the all channels is absent, the amplification and detection should be repeated. If the same result is obtained in the second run, the PCR analysis should be repeated starting from the DNA extraction stage. For NCA negative result for all channels is the norm.

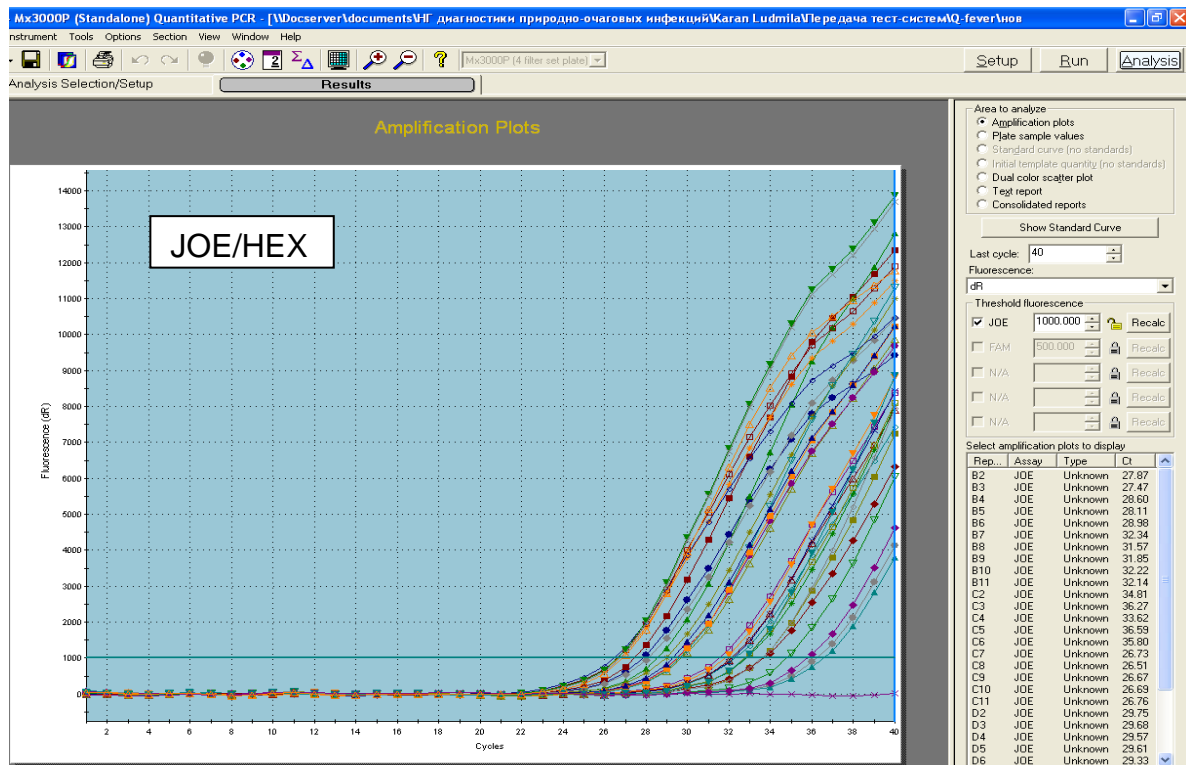
- If the C_t value for the Positive Control of Amplification (C+) through JOE/HEX channel is absent or higher than the boundary value the amplification should be repeated for all samples in which the specific DNA was not detected.
- If the C_t value is determined for the Negative Control of Extraction (C-) for the JOE/HEX channel and/or Negative Control of Amplification (NCA) for the any channels, the PCR analysis should be repeated for all samples in which DNA was detected.

EXAMPLE OF RESULTS


Data for FAM channel



Data for JOE/HEX channel



List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
29.12.20 KK	Through the text	The symbol  was changed to NOTE:
	Cover page	The phrase “Not for use in the Russian Federation” was added
18.03.21 VA	Front page	The name, address and contact information for Authorized representative in the European Community was changed