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For Professional Use Only

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).

Correspondence of targets and detection channels

Fluorophore	**FAM**	**JOE**
Detection channel name for different instrument models[1]	**FAM/Green**	**JOE/HEX/R6G/Yellow/Cy3**
DNA target	Internal Control STI-87 DNA	*Coxiella burnetii* DNA

[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 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. 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.

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*** 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. 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-1 amplification program for rotor-type instruments

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

NOTE: **AmpliSens-1** program is a universal amplification program for carrying out tests with the help of AmpliSens kits to identify the DNA of STI pathogens. All the tests or any tests combinations can be carried out in one instrument simultaneously including the tests for identification and genotyping *human papilloma virus* (*HPV* HCR).

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:
   - for signal measurement optimization for the selected channels set calibration from **5Fl** to **10Fl** for FAM/Green channel, **2Fl** to **5Fl** for JOE/Yellow channel.

     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.
   - 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*** 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, 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 ***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.

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 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 ***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*	*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 by the FAM/Green and JOE/Yellow channels do not correspond to exponential growth (they do not have an S-shape), it is allowed to increase the value of the threshold of negative samples (***NTC/Threshold***) 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.

Principle of interpretation is the following:

– *Coxiella burnetii* DNA is **detected** if the *Ct* value determined in the results grid in the JOE/Yellow channel is less than the boundary *Ct* value specified in the *Important Product Information Bulletin*. Moreover, the fluorescence curve of the sample should cross the threshold line in the area of typical exponential growth of fluorescence.

– *Coxiella burnetii* DNA is **not detected** in a sample if the *Ct* value in the JOE/Yellow channel is not determined (absent) or greater than the specified boundary *Ct* value, whereas the *Ct* value determined in the FAM/Green channel is less than the boundary *Ct* value specified in the *Important Product Information Bulletin*.

– The result is **invalid** if the *Ct* value is not determined (absent) in the JOE/Yellow channel, whereas the *Ct* value in the FAM/Green channel is not determined (absent) or greater than the specified boundary *Ct* value. In such cases, the PCR analysis should be repeated starting from the DNA extraction stage.

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).

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 program iCycler iQ5.
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).
   - 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.

2. Set the amplification program:

AmpliSens-1 amplification program for plate-type instruments

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Number of cycles***
Hold	95	15 min	–	1
Cycling 1	95	5 s	–	5
	60	25 s	–	
	72	15 s	–	
Cycling 2	95	5 s	–	40
	56	25 s	FAM/FAM-490, JOE/HEX/JOE-530	
	72	15 s	–	

– 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.

3. 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 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.

- For **iCycler iQ** 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 sigh 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 interpretation

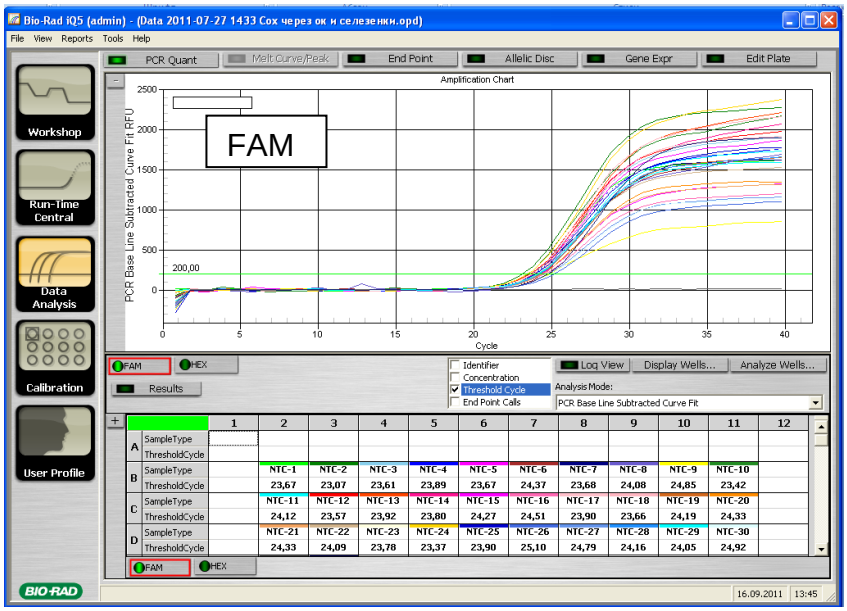
The result of the PCR analysis is considered reliable only if the results for the controls of the amplification and the extraction are correct in accordance with the table of assessment of results for controls (see the *Instruction manual*) and boundary values specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

Principle of interpretation is the following:

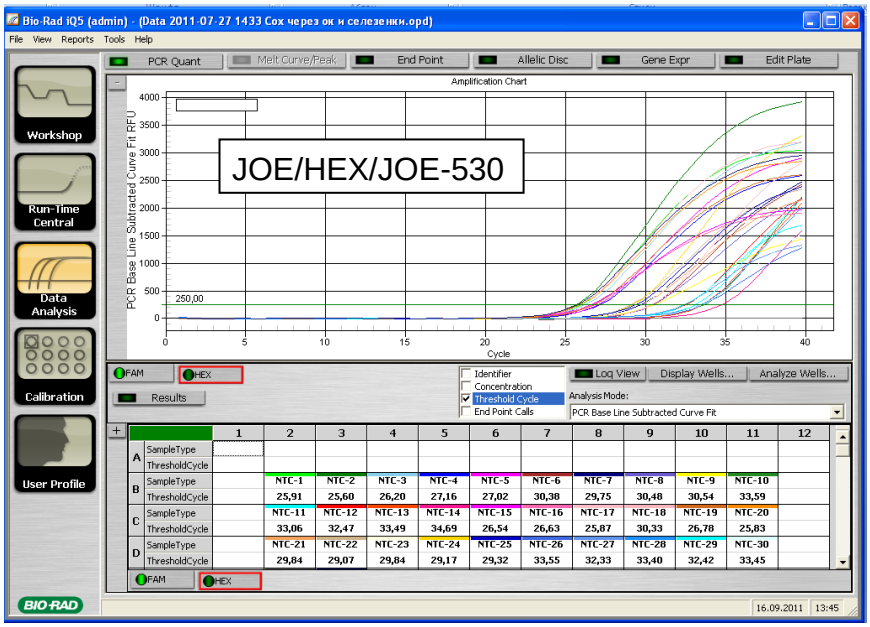
1. *Coxiella burnetii* DNA is **detected** if the *Ct* value determined in the results grid in the JOE/HEX/JOE-530 channel is less than the boundary *Ct* value specified in the *Important Product Information Bulletin*. Moreover, the fluorescence curve of the sample should cross the threshold line in the area of typical exponential growth of fluorescence.
2. *Coxiella burnetii* DNA is **not detected** in a sample if the *Ct* value in the JOE/HEX/JOE-530 channel is not determined (absent) or greater than the specified boundary *Ct* value, whereas the *Ct* value determined in the FAM/FAM-490 channel is less than the boundary *Ct* value specified in the *Important Product Information Bulletin*.
3. The result is **invalid** if the *Ct* value is not determined (absent) in the JOE/HEX/JOE-530 channel, whereas the *Ct* value in the FAM/FAM-490 channel is not determined (absent) or greater than the specified boundary *Ct* value. In such cases, the PCR analysis should be repeated starting from the DNA extraction stage.

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:

AmpliSens amplification program for plate-type instruments

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

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:

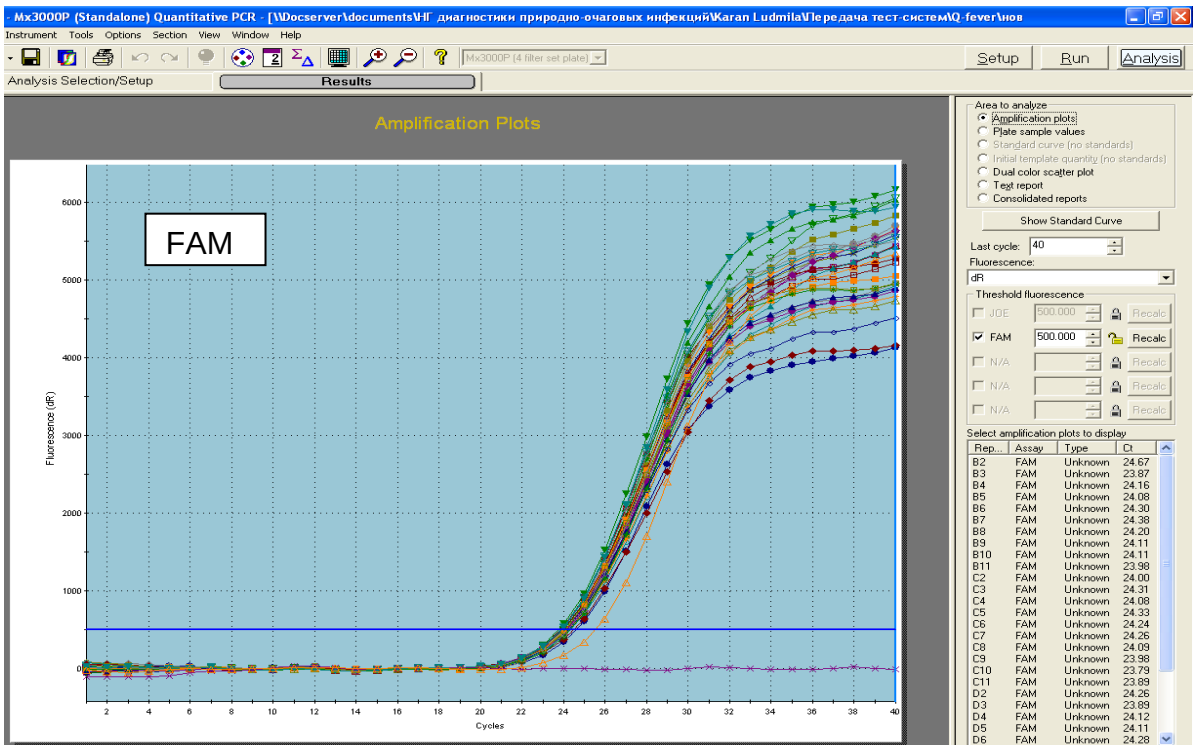
The result of the PCR analysis is considered reliable only if the results for the controls of the amplification and the extraction are correct in accordance with the table of assessment of results for controls (see the *Instruction manual*) and boundary values specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

Principle of interpretation is the following:

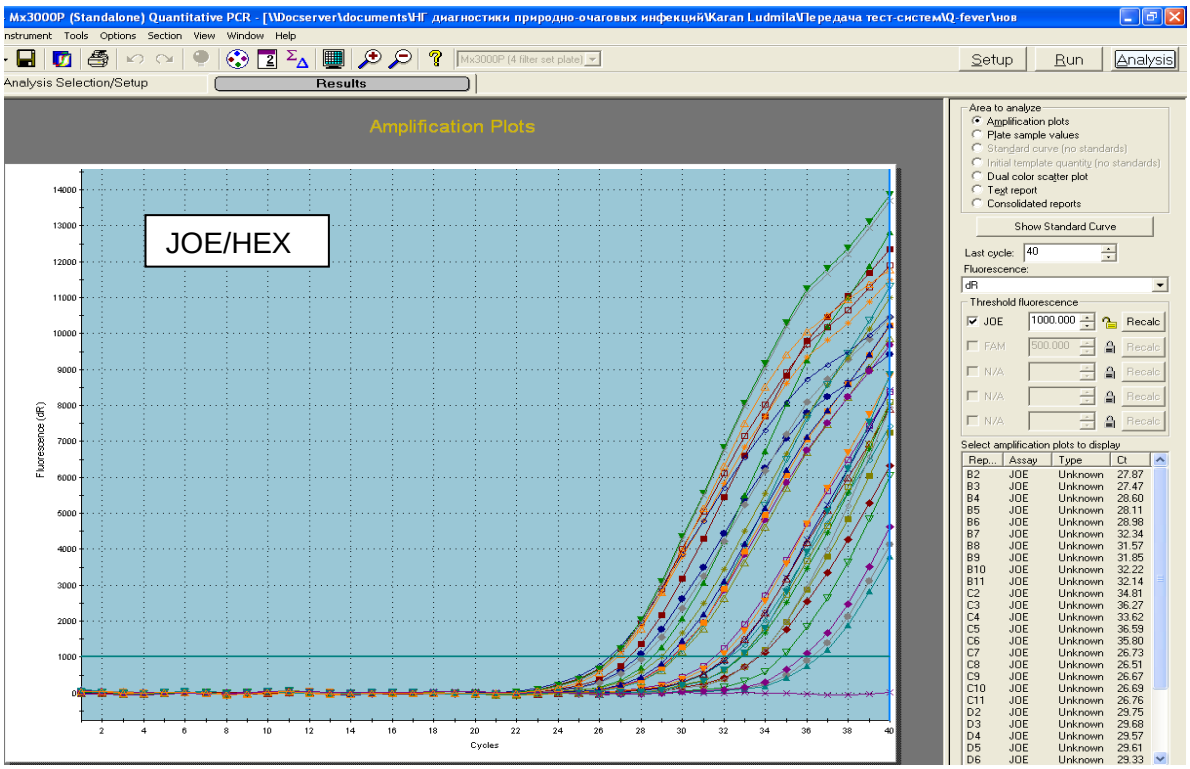
1. *Coxiella burnetii* DNA is **detected** if the *Ct* value determined in the results grid in the JOE/HEX channel is less than the boundary *Ct* value specified in the *Important Product Information Bulletin*. Moreover, the fluorescence curve of the sample should cross the threshold line in the area of typical exponential growth of fluorescence.
2. *Coxiella burnetii* DNA is **not detected** in a sample if the *Ct* value in the JOE/HEX channel is not determined (absent) or greater than the specified boundary *Ct* value, whereas the *Ct* value determined in the FAM channel is less than the boundary *Ct* value specified in the *Important Product Information Bulletin*.
3. The result is **invalid** if the *Ct* value is not determined (absent) in the JOE/HEX channel, whereas the *Ct* value in the FAM channel is not determined (absent) or greater than the specified boundary *Ct* value. In such cases, the PCR analysis should be repeated starting from the DNA extraction stage.

EXAMPLE OF RESULTS

Data for FAM channel



Data for JOE/HEX channel



TROUBLESHOOTING

1. If the *Ct* value determined for the Positive Control of Amplification (C+) in the channel for the JOE fluorophore is greater than the boundary *Ct* value or absent, the amplification and detection should be repeated for all samples in which specific DNA was not detected.
2. If the *Ct* value is determined for the Negative Control of Extraction (C–) in the channel for the JOE fluorophore, the PCR analysis should be repeated for all samples in which *Coxiella burnetii* DNA was detected.
3. If the *Ct* value is determined for the Negative Control of Amplification (NCA) in the channels for the FAM or/and JOE fluorophores, the PCR analysis should be repeated for all samples in which *Coxiella burnetii* DNA was detected, with carrying out the NCA at least three times.

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
14.08.25 PM	Through the text	Corrections according to the template. Added information about the principle of interpretation of the results
	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) AND Rotor-Gene Q (QIAGEN, Germany) instruments	The optimization of signal measurement for JOE/Yellow channel has been changed from «5Fl to 10Fl» to « 2Fl to 5Fl». The value for ***Eliminate cycles before*** parameter have been added
	Troubleshooting	The section has been added