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For Professional Use Only

GUIDELINES

to AmpliSens® *Toxoplasma gondii*-FRT PCR kit for qualitative detection *of Toxoplasma gondii* DNA in the biological material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

AmpliSens®

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INTENDED USE

The guidelines describe the procedure of the use of **AmpliSens® *Toxoplasma gondii*-FRT** PCR kit for qualitative detection of *Toxoplasma gondii* DNA in the biological material (white blood cells of whole peripheral blood, autopsy material, cerebrospinal fluid, amniotic fluid) by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection of amplification products using the following instruments:

- Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia);
- RotorGene Q (QIAGEN, Germany);
- iCycler iQ, iCycler iQ5 (Bio-Rad, USA);
- Mx3000P, Mx3005P (Stratagene, USA).

Correspondence of the fluorophores and detection channels

Channel for the fluorophore	Detection channel name for different instrument models[1]
FAM	FAM/Green
JOE	JOE/HEX/R6G/Yellow/Cy3

[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. If the tubes with reagents from different PCR kits or with different PCR-mixes are inserted into the rotor then the tubes' numbers for calibration in each detection channel should be indicated in the Rotor-Gene software. Recommendations about the calibration are described in the information list "Calibration priorities for the AmpliSens kits for Rotor-Gene 3000/6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany) thermocyclers".

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/Hydrolysis Probes*** template in the tab for edition and click The ***New*** button.
3. In the opened window, select ***36-Well Rotor*** (or ***72-Well Rotor***) and tick the ***No Domed 0.2 ml Tubes/Locking Ring attached*** option. Click ***Next*** button.
4. In the opened window, enter the operator name and select the reaction mixture volume: ***Reaction volume*** – **25 µl**. Click ***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 and fluorescent signal detection program

Step	*Temperature, °C*	*Time*	*Fluorescent signal detection*	*Cycle repeats*
1	95	15 min	–	1
2	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
3	95	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow	
	72	15 s	–	

NOTE: Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program (for example, together with the tests for detecting DNA of STI pathogens).

Note – The ROX/Orange, Cy5/Red и 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. In the ***New Run Wizard*** window, click the ***Calibrate/Gain Optimisation*** button. In the opened window:
   a) for signal measurement optimisation for the selected channels set calibration from **5Fl** to **10Fl** for all FAM/Green, JOE/Yellow channels.

      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.

   NOTE: The additional requirements for setting the channels’ calibration ranges are specified in the information list “Calibration priorities for the AmpliSens kits for Rotor-Gene 3000/6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany) thermocyclers”

   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-1**. 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 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 Positive Control of Amplification, the type ***Negative Control*** – for Negative Control of Amplification. Set the type ***None*** for the cells matching with the corresponding empty tubes. Click the ***Finish/OK*** button.

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

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

Data analysis

The obtained results are analyzed by the Rotor-Gene software. The results are interpreted according to the crossing (or not-crossing) of the S-shaped (sigmoid) fluorescence curve with the threshold line set at the specific level, that corresponds to the presence (or absence) of the *Ct* (threshold cycle) value 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 ***Quantitation*** analysis mode and 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 main window menu (***Quantitation analysis***).
5. In the ***CT Calculation*** menu (in the right part of the window), indicate the threshold line level ***Threshold* = 0.03**.
6. Choose the parameter ***More settings/Outlier Removal*** and set **10 %** for the value of negative samples threshold (***NTC/Threshold***).
7. Set **1** in the ***Eliminate cycles before:*** menu (in the right part of the window).
8. In the results grid (***Quantitation Results*** window), *Ct* values will appear.

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	10%	1
JOE/Yellow	0,03	on	on	10%	1

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 20%.

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 (Table 1).

The interpretation of the test samples is to be carried out in accordance with the Instruction Manual and the Table 2 of these Guidelines.

Table 1

Results for controls

Control	Stage for control	*Ct* value in the channel for fluorophore	
		FAM	JOE
C–	DNA extraction	≤ 30	absent
NCA	PCR	absent	absent
C+	PCR	≤ 30	≤ 30

Table 2

Results analysis interpretation for test samples

***Ct* value in the channel for fluorophore**		**Result**
FAM	**JOE**	
≤ 30	≤ 40	*Toxoplasma gondii* DNA **is detected**
≤ 30	absent	*Toxoplasma gondii* DNA **is NOT detected**
absent or > 30	detected or absent	Invalid
≤ 30	> 40	Equivocal

AMPLIFICATION AND DATA ANALYSIS USING 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 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).
   - click the ***Create New*** button in the ***Selected Plate Setup*** window of the ***Workshop*** module;
   - in the opened window click the ***Whole Plate loading*** button and set the plate setup using the buttons of the upper toolbar. Enter the samples' names in the ***Identifier/Condition*** column in the bar appeared in the screen bottom. Select the fluorescent signal detection in the FAM, JOE/HEX channels. Click the ***Select/Add Fluorophores*** button, select the fluorophore and tick it in the ***Selected*** column. Click ***OK***. The fluorophore name will appear in the ***Fluorophore*** window. For addition of fluorescence signal measuring for each sample it is necessary to click the fluorophore (activate it) and select the samples on the plate using the ***Fluorophore loading in whole Plate mode***  button under the scheme;
   - 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***;
   - Save the set plate setup by clicking the ***Save&Exit Plate Editing*** button. Enter the

file name and click ***Save***.

2. Set all the biological samples as ***Unknown***, positive controls as «**+**», and negative controls as «**–**».
3. Set the amplification program. To do this, in the ***Selected Protocol*** window of the ***Workshop*** module click the ***Create New*** button. Set the amplification parameters and save the protocol by activating the ***Save&Exit Protocol Editing*** button. Enter the name of the file and then click ***Save***.

AmpliSens-1 amplification program

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Number of cycles*
1	95	15 min	–	1
2	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
3	95	5 s	–	40
	60	20 s	FAM, JOE/HEX	
	72	15 s	–	

NOTE: Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program (for example, together with the tests for detecting DNA of STI pathogens).

Note – The ROX, Cy5 и Cy5.5 channels are enabled when required if the “multiprime” format tests are performed.

4. 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***.
5. At the end of the run it is necessary to close the software and turn off the instrument (the thermocycler and the optical block).

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:

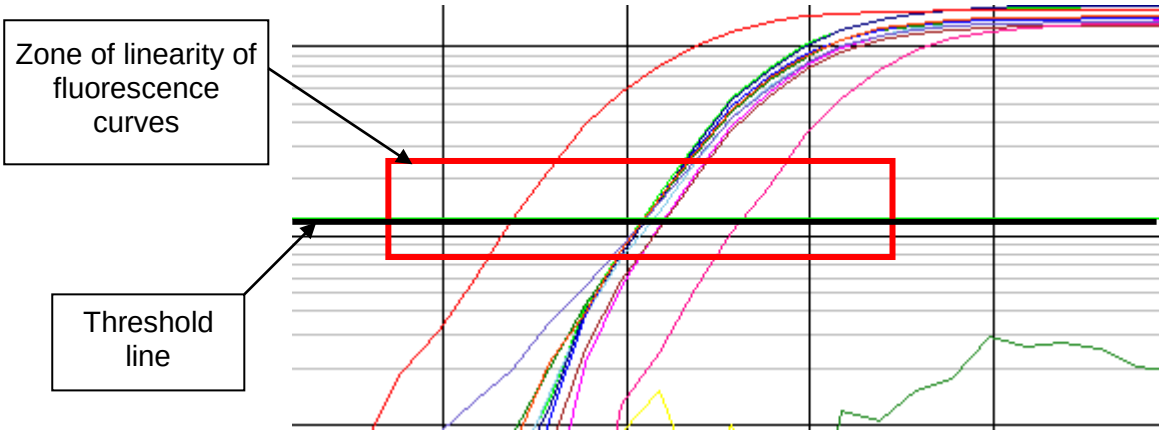
- select the needed file with the run in the upper left window of the ***Workshop*** module;
- click the ***Edit*** button in the ***Selected Plate Setup*** area of the ***Workshop*** module and edit the plate setup (the files of protocols are saved in the ***SampleFiles*** folder by default);
- click the ***Edit*** button in the ***Selected Protocol*** area of the ***Workshop*** module and

check the correctness of the selected protocol (the files of protocols are saved in the ***Users*** folder by default).

Data analysis

The obtained results are analyzed by the iCycler iQ5 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.

1. Start the software and open the needed file with data of the analysis in the ***Data File*** window of the ***Workshop*** module. Click the ***Analyze*** button.
2. Select the ***Analysis Mode: PCR Base Line Subtracted Curve Fit*** (is set by default).
3. Check the correctness of threshold line automatic choice for each channel. The threshold line is to cross only with S-shaped (sigmoid) 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 set the threshold line level for each channel manually. To do this, click the ***Log View*** (logarithmic scale selection) and set (with the left mouse button) the threshold line at the level where the fluorescence curves have a linear character and do not cross with the curves of the negative samples (see the figure below). As a rule, the threshold line is set at the level of 10-20 % of maximum fluorescence obtained for the Positive control in the last amplification cycle. Make sure that the fluorescence curve of the Positive control has the typical exponential growth of fluorescence.



4. In order to analyze the results click the ***Results*** button which is situated under the buttons with the fluorophores' names (for **iCycler iQ5**).

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 (Table 3).

The interpretation of the test samples is to be carried out in accordance with the Instruction Manual and the Table 4 of these Guidelines.

Table 3

Results for controls

Control	**Stage for control**	***Ct* value in the channel for fluorophore**	
		FAM	**JOE**
C–	DNA extraction	≤ 30	absent
NCA	PCR	absent	absent
C+	PCR	≤ 30	≤ 30

Table 4

Results analysis interpretation for test samples

***Ct* value in the channel for fluorophore**		**Result**
FAM	**JOE**	
≤ 30	≤ 40	DNA *Toxoplasma gondii* **is detected**
≤ 30	absent	DNA *Toxoplasma gondii* **is NOT detected**
absent or > 30	detected or absent	Invalid
≤ 30	> 40	Equivocal

AMPLIFICATION AND DATA ANALYSIS USING Mx3000P/Mx3005P (Stratagene, 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).

1. Switch on the instrument and start the Stratagene Mx3000P software.
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 and close the lid.
4. Select ***Optics Configuration*** in the ***Options*** menu and in the ***Dye Assignment*** tab set JOE/HEX parameter next to the ***HEX/JOE filter set*** item, parameter FAM next to ***FAM filter set***.

NOTE: Don’t turn the strips/plate upside down while inserting them into the instrument.

5. Lock the fixing arm and the door of the instrument
6. In the ***New Experiment Options*** window, select the ***Quantitative PCR (Multiple Standards)*** item and tick ***Turn lamp on for warm-up***.
7. Set fluorescence detection parameters in the ***Plate Setup*** menu. To do this:
   - Select all wells with the test tubes or strips (hold the Ctrl button down and select the necessary region with the mouse);
   - Mark all selected wells as ***Unknown*** in the ***Well type*** window. Tick ***FAM*** and ***JOE*** for ***Collect fluorescence data*** option. Name each sample in the ***Well Information*** window by double clicking with the mouse. *It is possible to name the samples during the amplification or to return to* ***Plate Setup*** *menu after amplification ends*.
8. Set the parameters for the fluorescence detection of the tubes in the ***Plate Setup*** tab. Select all the wells with test tubes (hold the Ctrl button and select necessary region by mouse). Click the ***Unknown*** button in the ***Well type*** menu, and tick ***FAM*** and ***JOE*** in the ***Collect fluorescence data*** tab. Name tubes in the ***Well Information*** window by double clicking with the mouse on each well: define Positive Control as ***«+»*** and Negative Control as ***«–»***.
9. Set the amplification program in the ***Thermal Profile Setup*** tab. To do this:

Using the template file for setting the amplification program (is recommended).

Click the ***Import…*** button which is to the right of the depicted thermocycling profile. Proceed to the folder containing previous experimental file and open it. The needed thermocycling profile will appear in the ***Thermal Profile*** window.

Individual programming

1. After setting all necessary values and parameters, select all wells with the test tubes once again. Proceed to the ***Thermal Profile Setup*** menu and set the amplification program specified in table below .

AmpliSens-1 amplification program

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Number of cycles*
1	95	15 min	–	1
2	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
3	95	5 s	–	40
	60	30 s	FAM, JOE/HEX	
	72	15 s	–	

NOTE: Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program (for example, together with the tests for detecting DNA of STI pathogens).

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

2. To set detection parameter of fluorescent signal at desired temperature, select the ***All points*** option for ***Data collection by marker dragging*** parameter and move it by mouse from right side to the shelf with desired temperature.
3. Select ***Run*** and ***Start*** and name the experiment file.

Data analysis

The obtained results (fluorescence accumulation curves for two channels) are analyzed by the instrument software.

The results are interpreted according to the crossing (or not-crossing) of the 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.

1. Proceed to the ***Analysis*** mode (select the corresponding button on the toolbar).
2. Make sure that all samples in the ***Analysis Selection/Setup*** tab are active (wells corresponding to samples should have another color). Otherwise, select all tested samples holding the ***Ctrl*** button down and marking the necessary region with the mouse.

3. Proceed to the ***Results*** tab.
4. Make sure that both HEX and FAM channels are active (***HEX*** and ***FAM*** buttons are pressed in the ***Dyes Shown*** field at the foot of the window).
5. Select the ***Threshold fluorescence*** field and make sure that tick marks are put against two fluorescence channels: JOE/HEX and FAM. Check the correctness of the automatically chosen threshold line. Normally, is to cross only with sigmoid[2] 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, the threshold level should be raised.

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 (Table 5).

The interpretation of the test samples is to be carried out in accordance with the Instruction Manual and the Table 6 of these Guidelines.

Table 5

Results for controls

Control	Stage for control	*Ct* value in the channel for fluorophore	
		FAM	JOE
C–	DNA extraction	≤ 30	absent
NCA	PCR	absent	absent
C+	PCR	≤ 30	≤ 30

Table 6

Results analysis interpretation for test samples

Ct value in the channel for fluorophore		Result
FAM	JOE	
≤ 30	≤ 40	DNA *Toxoplasma gondii* **is detected**
≤ 30	absent	DNA *Toxoplasma gondii* **is NOT detected**
absent or > 30	detected or absent	Invalid
≤ 30	> 40	Equivocal

[2] Curves of signal accumulation are displayed linear by default. To change curve from linear to logarithmic, click with the left mouse button on one of axis area (X or Y) and indicate ***Scale*** next to the ***Log*** item in ***Graph properties*** window (Y axis).

TROUBLESHOOTING

1. The *Ct* value determined for the Positive Control of amplification (C+) in the channels for the FAM and/or JOE fluorophores is greater than the boundary *Ct* value or absent. The amplification should be repeated for all the samples.
2. If the *Ct* value is determined for the Negative Control of Extraction (C–) in the channel for the FAM fluorophore is greater than the boundary *Ct* value or absent. The PCR analysis (beginning with the DNA extraction stage) should be repeated for all negative and invalid samples.
3. If the *Ct* value is determined for the Negative Control of Extraction (C–) in the channel for the JOE fluorophore. The contamination of laboratory with amplification fragments or contamination of reagents, test samples is probable at any stage of PCR analysis. Measures for detecting and elimination of contamination source must be taken. The PCR analysis (beginning with the DNA extraction stage) should be repeated for all positive and equivocal samples.
4. If the *Ct* value is determined for the Negative Control of amplification (NCA) in the channels for the FAM and/or JOE fluorophores. The contamination of laboratory with amplification fragments or contamination of reagents, test samples is probable at any stage of PCR analysis. Measures for detecting and elimination of contamination source must be taken. The PCR analysis should be repeated for all samples.
5. The *Ct* value is determined for the test sample, whereas the area of typical exponential growth of fluorescence is absent (the graphic looks like approximate straight line). It is necessary to check the correctness of selected threshold line level or parameters of base line calculation. If the result has been obtained with the correct level of threshold line (base line), the amplification and detection should be repeated for this sample.
6. The *Ct* value determined for the test sample in the channel for the FAM fluorophore is greater than the boundary *Ct* value or absent. The PCR analysis (beginning with the DNA extraction stage) should be repeated for the appropriate test sample.
7. The *Ct* value determined for the test sample in the channel for the JOE fluorophore is greater than the boundary *Ct* value, and the *Ct* value determined in the channels for the FAM fluorophore does not exceed the boundary *Ct* value. The PCR analysis (beginning with the amplification stage) should be repeated in two repeats for the appropriate test sample. If the same result has been obtained the sample is considered positive.

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
23.06.11 LA	Cover page	The name of Institute was changed to Federal Budget Institute of Science “Central Research Institute for Epidemiology”
31.08.15 ME	Text	Corrections according to the template
	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) instruments; Amplification and data analysis using iCycler iQ and iCycler iQ5 (Bio-Rad, USA) instruments; Amplification and data analysis using Mx3000P, Mx3005P (Stratagene, USA) instruments	The amplification data analysis was supplemented with the description of negative and invalid results
26.07.16 ME	Intended use	The clinical material was specified
31.05.21 VA	Front page	The phrase “For research use only. Not for diagnostic procedures” was added
	Through the text	The symbol  was changed to NOTE:
22.06.23 EM	Footer	**REF** R-P1(RG,iQ,Mx)-CE was added
01.08.25 PM	Through the text	Corrections according to the template
	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) AND Rotor-Gene Q (QIAGEN, Germany) instruments	The optimization of signal measurement using the FAM/Green, JOE/Yellow channels has been changed. The ability to increase the value of the threshold of negative samples (***NTC/Threshold***) to 20% and the value for ***Eliminate cycles before*** parameter have been added
	Troubleshooting	The section has been added