RUO

For Professional Use Only

GUIDELINES

to **AmpliSens® *Pneumocystis jirovecii (carinii)*-FRT PCR kit**

for qualitative detection of *Pneumocystis jirovecii (carinii)* DNA in the biological material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

AmpliSens®

Federal Budget Institute of Science “Central Research Institute for Epidemiology”
3A Novogireevskaya Street
Moscow 111123 Russia

For research use only. Not for diagnostic procedures

TABLE OF CONTENTS

INTENDED USE

The guidelines describe the procedure of using **AmpliSens® *Pneumocystis jirovecii (carinii)*-FRT** PCR kit for qualitative detection of *Pneumocystis jirovecii (carinii)* DNA 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 iQ5 (Bio-Rad Laboratories, Inc., 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 (OIAGEN, 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 transparent 0.2-ml PCR tubes or 0.1-ml tubes with flat caps (detection trough 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:

Table 1

AmpliSens-1 amplification program for rotor-type instruments

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*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/Green, JOE/Yellow	
	72	15 s	–	

NOTE: Any combination of the tests can be performed in one instrument simultaneously with the use of the AmpliSens-1 amplification program (for example, together with tests for detecting DNA of STI pathogens).

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

6. After setting up the temperature profile click the ***OK*** button.
7. Click the ***Calibrate/Gain Optimisation…*** button in the ***New Run Wizard*** window. In the opened window:
   a. for signal measurement optimisation for the selected channels set calibration from **5Fl** to **10Fl** for all the channels (FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red, Cy5.5/Crimson).

      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**. 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 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 button ***Analysis*** in the menu, select the mode of the analysis ***Quantitation***, activate the buttons ***Cycling A. FAM/Cycling A. Green***, ***Show***.
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 ***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 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 (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	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 (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 iQ5 (Bio-Rad, USA) INSTRUMENT

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***.

Table 2

AmpliSens-1 amplification program for plate-type instruments

Step	***Temperature, °C***	***Time***	***Fluorescence 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	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 AmpliSens-1 universal amplification program (for example, together with tests for detecting DNA of STI pathogens).

Note – The ROX and Cy5 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. 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 analyse the results click the ***Results*** button which is situated under the buttons with the fluorophores' names

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 Mx3000P, Mx3005P (Stratagene, USA) INSTRUMENT

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. Switch on the instrument and run the Stratagene Mx3000P/Mx3005P software.
2. Select ***Quantitative PCR (Multiple Standards)*** and ***Turn lamp on for warm-up*** in the ***New Experiment Options*** window.

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** parameter next to ***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 select ***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. Set **FAM** and **JOE** fluorophores for the ***Collect fluorescence data*** option. Name each sample in the ***Well Information*** window by double clicking with the mouse. Designate Positive Control as ***«+»*** and Negative Control as ***«–»***. It is possible to name the samples during the amplification or to return to ***Plate Setup*** menu after the amplification ends.
8. Set the amplification program in the ***Thermal Profile Setup*** tab. To do this:

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

1. Click the ***Import…*** button right to the thermocycling profile picture.
2. Proceed to the folder containing previous experimental file and open it.
3. In the ***Thermal Profile*** window, the necessary thermocycling profile appears.

Manual programming

1. After setting all necessary values and parameters, select all wells with the tested tubes

once again. Proceed to the ***Thermal Profile Setup*** menu and set the amplification program (see Table 3).

Table 3

AmpliSens-1 amplification program for plate-type instruments

Step	*Temperature, °C*	*Time*	*Fluorescence 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	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 AmpliSens-1 universal amplification program (for example, together with tests for detecting DNA of STI pathogens).

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

Data analysis

The obtained results are analyzed by the software of the Mx3000P/Mx3005P 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. 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 by holding the ***Ctrl*** button down and marking the necessary region with the mouse.
3. Proceed to the ***Results*** tab.
4. Make sure that both JOE and FAM channels are active (***JOE*** and ***FAM*** buttons are pressed in the ***Dyes Shown*** field at the foot of the window).
5. Make sure that ***JOE*** and ***FAM*** buttons are selected in the ***Threshold fluorescence*** field. Make sure that the automatic selection of the threshold level is correct. Normally, the

threshold line should cross only the sigmoid[2] curves of signal accumulation of positive samples and controls and should not cross the baseline. Otherwise, the threshold level should be raised.

6. *Ct* values in both channels will appear in the results grid.

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.

[2] Curves of signal accumulation are displayed linear by default. To change curve from linear to logarithmic, click twice with the left mouse button in the area of one of the axes (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 specified in the *Important Product Information Bulletin* or absent. The PCR analysis (beginning with the amplification) should be repeated for all samples.
2. If the *Ct* value is determined for the Negative Control of Extraction (C–) 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 (beginning with the DNA extraction stage) should be repeated for all samples in which specific DNA was detected.
3. If the *Ct* value is determined for the Negative Control of amplification (NCA) in any of 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 (beginning with the DNA extraction stage) should be repeated for all samples in which specific DNA was detected.
4. If 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.
5. The *Ct* value determined for the test sample in the channels for the FAM and/or JOE fluorophores is greater than the boundary *Ct* value specified in the *Important Product Information Bulletin* or absent. The PCR analysis (beginning with the DNA extraction stage) should be repeated for the appropriate test sample.
6. The *Ct* value determined for the test sample in the channel for the JOE fluorophore is greater than the boundary *Ct* value specified in the *Important Product Information Bulletin*, and the *Ct* value determined in the channel for the FAM fluorophore does not exceed the boundary *Ct* value specified in the *Important Product Information Bulletin*. The PCR analysis 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 Guidelines

VER	Location of changes	Essence of changes
13.07.23 EM	Footer	**REF** R-F2-Mod(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