

IVD

For Professional Use Only

GUIDELINES

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

for qualitative detection of *Pneumocystis jirovecii* (*carinii*) DNA in the clinical 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 using **AmpliSens® *Pneumocystis jirovecii* (*carinii*)-FRT** PCR kit for qualitative detection of *Pneumocystis jirovecii* (*carinii*) DNA in the clinical 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, USA)
- Mx3000P, Mx3005P (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 6.1 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.
2. Insert the tubes 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).

NOTE: Well 1 must be filled with any test tube.

3. Set the amplification program for the rotor-type instrument (see Table 1).

AmpliSens-1 RG 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 RG universal amplification program (for example, together with tests for detecting DNA of STI pathogens). Analytical characteristics of this kit do not change with usage of universal amplification program.

4. Set calibration parameters (activate **Calibrate/Gain Optimisation...** in a new experiment module):
 - perform fluorescence measurement in FAM/Green and JOE/Yellow channels (activate **Calibrate Acquiring/Optimise Acquiring**);
 - perform calibration before the first measurement in FAM/Green and JOE/Yellow channels (activate **Perform Calibration Before 1st Acquisition/Perform Optimisation Before 1st Acquisition**);
 - set channel calibrations: for FAM/Green and JOE/Yellow channels by the first tube from **5FI** to **10FI** (activate the **Edit...** button, **Auto gain calibration channel settings** window);
5. Select the **Start run** button for amplification run and name the experiment.
6. During or after amplification, it is necessary to program the placement of test samples, negative control of extraction, positive and negative controls of DNA amplification. To do this, enter the data in the table for samples (opens automatically after the thermocycling process starts). Indicate the names/numbers of test samples in the **Name** column. Indicate Negative Control of Extraction as **C–**, Positive Control of Amplification as **C+**, and Negative Control of Amplification as **NCA**. Set the **Unknown** type next to all test samples, the **Positive Control** type next to the Positive Controls, the **Negative Control** type next to the Negative Control of Extraction, and the **NTC** type next to the Negative Control of Amplification. For empty wells, indicate **None**.

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

Data analysis

1. Activate the **Analysis** button in the menu, then select the **Quantitation** analysis mode and activate the **Cycling A. JOE/Cycling A. Yellow, Show** and **Cycling A. FAM/Cycling A. Green, Show** buttons.
2. Cancel the automatic choice of the threshold line level **Threshold** for each opened main window (FAM/Green and JOE/Yellow).
3. Activate the **Dynamic tube** and **Slope Correct** buttons in the main window menu (**Quantitation analysis**).
4. In the **CT Calculation** menu (in the right part of the window), indicate the threshold line level **Threshold = 0.03** and **More settings** as **10 %**.
5. In the results grid (**Quant. Results** window), *Ct* values will appear.
6. *Ct* values for the test samples are taken into account only if the results obtained for control samples are correct:
 - *Ct* value should be absent in the Negative Control of Extraction (C–) – Negative control (C–).
 - *Ct* value should be absent in the Negative Control of Amplification (NCA) –TE-buffer.
 - *Ct* value for the Positive Control of Amplification (C+) – Positive Control DNA *P.jirovecii* and human DNA (C+*P.jirovecii* and human DNA) – should not exceed the boundary *Ct* value specified in the *Important Product Information Bulletin*.
7. Samples are considered positive if *Ct* values for them do not exceed **35 cycles** in the JOE/Yellow channel. The result is **equivocal** if the *Ct* value in the JOE/Yellow channel exceeds this boundary *Ct* value. The PCR analysis of this sample should be repeated in duplicate. If a reproducible positive *Ct* value is obtained, the sample is considered positive. If the result is not reproduced in duplicate, the sample is considered equivocal.

Results of analysis are not taken into account in the following cases:

1. If any *Ct* value appears for the Negative Control of Extraction (C–) and for the Negative Control of Amplification (NCA) in the JOE/Yellow (*Pneumocystis jirovecii* (*carinii*)) and/or FAM/Green (IC Glob) channel in the results grid, it indicates the contamination of reagents or samples. In such cases, the results of analysis must be considered as invalid. Test analysis must be repeated and measures to detect and eliminate the source of contamination must be taken.
2. If the *Ct* value is absent for the Positive Control of Amplification (C+) in JOE/Yellow and FAM/Green channels, the results are invalid for all samples. PCR should be repeated.

- If *Ct* values for the IC Glob in the FAM/Green channel are absent for clinical samples in the results grid, this indicates the failure of DNA extraction. For these samples, PCR should be repeated starting from the DNA extraction stage.
- If the *Ct* value for the IC Glob in the tested sample exceeds **30** and the *Ct* value for *Pneumocystis jirovecii* (*carinii*) DNA exceeds **35**, analysis should be repeated starting from the DNA extraction stage. High *Ct* values may be due to the loss of DNA during extraction or may be caused by the presence of inhibitors.

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 0.2-ml disposable PCR tubes with domed caps (e.g. example, Axygen, USA).

Programming the thermocycler

- Turn on the instrument and optical block at least 20–30 min before measurement.
- Set the amplification program (**Protocol**) for plate-type instruments (see Table 2).

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). The analytical characteristics of this kit do not change when the universal amplification program is used.

- Set the order of samples in the plate (**Plate**), select fluorophores (**Select/add fluorophores**), activate fluorophores for the samples in the created protocol by the **Fluorophore loading in Whole Plate mode** button. Set **Sample Volume – 25 µl**, **Seal Type – Domed cap**, **Vessel Type – Tubes**. Save the created protocol clicking the **Save/Exit Plate Editing** button.
- Add reagents for amplification and DNA samples into the tubes and place them into the

instrument.

5. Click the **Run** button, select **Run Persistent Plate** and save the experiment.

Data analysis

1. Data are analyzed in JOE/HEX and FAM channels.
2. Activate the **Data Analysis** button in the menu window.
3. Select a range of 20–25 for **Base Line** in the **Crossing Threshold User Defined** menu. Normally, the threshold line should cross only the sigmoid curves of signal accumulation of positive samples and controls and should not cross curves of other shape. Otherwise, the threshold level should be raised.
4. *Ct* values for test samples are taken into account only if the results obtained for control samples are correct:
 - *Ct* value should be absent in the Negative Control of Extraction (C–) – Negative control (C–).
 - *Ct* value should be absent in the Negative Control of Amplification (NCA) –TE-buffer.
 - *Ct* value for the Positive Control of Amplification (C+) – Positive Control DNA *P.jirovecii* and human DNA (C+*P.jirovecii* and human DNA) – should not exceed the boundary *Ct* value specified in the *Important Product Information Bulletin*.
5. The results of testing the clinical samples are analyzed according to the *Important Product Information Bulletin* for the PCR kit. Samples are considered positive if *Ct* values for them do not exceed the boundary value specified in the *Important Product Information Bulletin*. The result is **equivocal** if the *Ct* value exceeds this boundary *Ct* value. The PCR analysis of this sample should be repeated in duplicate. If a reproducible positive *Ct* value is obtained, the sample is considered positive. If the result is not reproduced in duplicate, the sample is considered equivocal.

Results of analysis are not taken into account in the following cases:

1. If any *Ct* value appears for the Negative Control of Extraction (C–) and for the Negative Control of Amplification (NCA) in the JOE/HEX (*Pneumocystis jirovecii* (*carinii*)) and/or FAM (IC Glob) channel in the results grid, it indicates the contamination of reagents or samples. In such cases, the results of analysis must be considered as **invalid**. Test analysis must be repeated and measures to detect and eliminate the source of contamination must be taken.
2. If the *Ct* value is absent for the Positive Control of Amplification (C+) in JOE/HEX and FAM channels, the results are invalid for all samples. PCR should be repeated.

3. If *Ct* values for the IC Glob in the FAM channel are absent for clinical samples in the results grid, this indicates the failure of DNA extraction. For these samples, PCR should be repeated starting from the DNA extraction stage.
4. If the *Ct* value for IC Glob in the tested sample exceeds **30** and the *Ct* value for the *Pneumocystis jirovecii* (*carinii*) DNA exceeds **35**, analysis should be repeated starting from the DNA extraction stage. High *Ct* values may be due to the loss of DNA during extraction or may be caused by the presence of inhibitors.

AMPLIFICATION AND DATA ANALYSIS USING Mx3000P, Mx3005P (Stratagene, USA) INSTRUMENT

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).

The analytical characteristics of this kit do not change when the universal amplification program is used.

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

Data (fluorescence accumulation curves in two channels) are analyzed automatically by the real-time PCR instrument software. Accumulation of the amplified product of **β-globin gene DNA** region (IC Glob) is detected in the FAM channel, and accumulation of the amplified product of the *Pneumocystis jirovecii (carinii)* DNA is detected in the JOE/HEX channel.

The results are interpreted according to the crossing (or not-crossing) of the fluorescence curve with the threshold line set at the specific level and shown as the presence (or absence) of the *Ct* (threshold cycle) value in the results grid.

Data processing

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 sigmoid¹ 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 for the Positive Control of Amplification (C+) – Positive Control DNA *P.jirovecii* and human DNA (C+*P.jirovecii* and human DNA) – will appear in both channels in the results grid.
7. *Ct* values for the **IC Glob** should be present in each test sample.
8. *Ct* values for the Negative Control of Extraction (C–) – Negative control (C–) – should be absent.
9. *Ct* values for the Negative Control of Amplification (NCA) – TE-buffer – should be absent.
10. Clinical samples are considered positive if *Ct* values for them do not exceed **35 cycles** in the JOE/HEX channel. The result is **equivocal** if the *Ct* value exceeds this boundary *Ct* value. The PCR analysis of this sample should be repeated in duplicate. If a reproducible positive *Ct* value is obtained, the sample is considered positive. If the result is not reproduced in duplicate, the sample is considered equivocal.


Results of analysis are not taken into account in the following cases:

1. If any *Ct* value appears for the Negative Control of Extraction (C–) and/or for Negative Control of Amplification (NCA) in the FAM and JOE/HEX channels, it indicates the contamination of reagents or samples. In such cases, the results of analysis must be considered **invalid**. Test analysis must be repeated and measures to detect and eliminate the source of contamination must be taken.
2. If the *Ct* value is absent for the Positive Control of Amplification (C+) in the FAM and JOE/HEX channels, the results of analysis must be considered as **invalid** for all samples. PCR should be repeated for all samples.

¹ 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).

3. If *Ct* values for IC Glob in the FAM channel are absent in the results grid for the test samples, this indicates the failure of DNA extraction. For these samples, analysis should be repeated starting from the DNA extraction stage.
4. If the *Ct* value for IC Glob for a test sample exceeds **30 cycles** and the *Ct* value for the *Pneumocystis jirovecii* (*carinii*) DNA exceeds **35**, analysis should be repeated starting from the DNA extraction stage. High *Ct* values may be due to the loss of DNA during extraction or may be caused by the presence of inhibitors.

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
28.09.12 LA	Page footer	Reference number R-F2(RG,iQ,Mx)-CE-B was added
08.07.13 ME	Page footer	REF R-F2(RG,iQ,Mx)-CE-B was deleted
02.04.14 SA	Cover page	Address of European representative was added
13.10.15 ME	Text	Corrections according to the template
25.12.20 EM	Through the text	The symbol  was changed to NOTE:
	Cover page	The phrase "Not for use in the Russian Federation" was added
11.03.21 MM	Front page	The name, address and contact information for Authorized representative in the European Community was changed