

GUIDELINES

to AmpliSens[®] *Mycoplasma pneumoniae* / *Chlamydophila pneumoniae*-FRT PCR kit

for qualitative detection of *Mycoplasma pneumoniae* and
Chlamydophila pneumoniae DNA in the biological material by the
polymerase chain reaction (PCR) with real-time hybridization-
fluorescence detection

AmpliSens[®]



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INTENDED USE

Guidelines describe the procedure of the use of **AmpliSens® *Mycoplasma pneumoniae* / *Chlamydophila pneumoniae*-FRT** PCR kit for qualitative detection of *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* DNA in the biological material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using the following instruments:

- Rotor-Gene 3000 (three or more channels) (Corbett Research, Australia);
- Rotor-Gene 6000 (five or six channels) (Corbett Research, Australia);
- Rotor-Gene Q (QIAGEN, Germany);
- iCycler iQ, iCycler iQ5 (Bio-Rad, USA);
- CFX96 (Bio-Rad, USA).

and also in combination with the automatic station for the nucleic acids extraction NucliSENS easyMAG (bioMérieux, France).

WORK WITH AUTOMATED INSTRUMENT NucliSENS easyMAG NUCLEIC ACID EXTRACTION PLATFORM

Variant 1. DNA extraction with off-board sample lysis (off-board mode)

This method of extraction allows reducing the consumption of NucliSens lysis buffer. It is preferred for working with test samples which contain clots (sputum, aspirates).

1. Switch on the NucliSENS easyMAG instrument and prepare it for the DNA extraction according to the instruction manual.
2. In the window for input of test samples, enter the following parameters for each sample:
 - Sample name;
 - **Matrix** for DNA extraction (select **Other**);
 - **Volume** – 0.1 ml;
 - **Eluate** – 25 µl;
 - **Type** – Lysed;
 - **Priority** – Normal.
3. Create a new protocol of DNA extraction and save it. In protocol select **On-board Lysis Buffer Dispensing – No, On-board Lysis Incubation – No**.
4. Transfer the sample table into the created protocol.
5. Take the required number of special disposable tubes intended for DNA extraction in the NucliSENS easyMAG instrument (including negative control of DNA extraction). Add **550 µl** of **NucliSens lysis buffer**.

NOTE: When working with the material which contains clots, lysis should be carried out in 1.5-ml tubes. After finishing the incubation, tubes should be centrifuged at 10,000 rpm for 1 min. Then transfer the supernatant into special tubes intended for DNA extraction in the NucliSENS easyMAG instrument.

6. Add **100 µl** of the prepared samples into the tubes with **NucliSens lysis buffer** using disposable tips with aerosol barriers and carefully mix by pipetting. Avoid adding mucus clots and large particles into the tube.
7. Add **100 µl** of **Negative Control (C-)** into the tube with Negative Control of Extraction (C-).
8. Incubate the tubes for 10 min at room temperature.
9. Resuspend the tube with **magnetic silica NucliSens** by intensive vortexing. Add **25µl** of **magnetic silica** using disposable tips with aerosol barriers and carefully mix by pipetting. Magnetic silica should be distributed evenly over the whole tube volume.
10. Place the tubes with the samples into the instrument and start the DNA extraction program with lysis of samples by selecting **off board** mode.
11. After finishing DNA extraction, take the tubes out of the instrument.

For storage the purified DNA should be transferred into a sterile tube for 30 min after extraction. Purified DNA can be stored for one week at 2 to 8 °C, for 1 year at minus 24 to minus 16 °C, for a long time at the temperature no more than minus 68 ° C.

Variant 2. DNA extraction on-board sample lysis (on-board mode)

1. Switch on the NucliSENS easyMAG instrument and prepare it for the DNA extraction according to the instruction manual.
2. In the window for input of test samples enter the following parameters:
 - Sample name;
 - **Matrix** for DNA isolation (select **Other**);
 - **Volume** – 0.1 ml;
 - **Eluate** – 25 µl;
 - **Type** – Primary;
 - **Priority** – Normal.
3. Create a new protocol of DNA extraction and save it. Select **On-board Lysis Buffer Dispensing – Yes, On-board Lysis Incubation – Yes**.
4. Transfer the sample table into the created protocol.
5. Take the required number of special disposable tubes intended for DNA extraction in the NucliSENS easyMAG instrument (including negative control of DNA extraction).
6. Add **100 µl** of prepared samples into the tubes intended for DNA extraction in

NucliSENS easyMAG instrument by using of disposable tips with aerosol barriers.

7. Add **100 µl** of **Negative Control (C–)** into the tube with Negative Control of Extraction (C–).
8. Place tubes with samples into the instrument and start up the DNA extraction program with lysis of samples by selecting the **on board** mode.
9. Wait until the NucliSENS easyMAG instrument stops working at the **Instrument State – Idle** position.
10. Resuspend the tube with **magnetic silica NucliSens** by intensive vortexing. Open the lid of the instrument and add into each tube **25µl** of **magnetic silica** using disposable tips with aerosol barriers and carefully mix by pipetting. Magnetic silica should be distributed evenly over the whole tube volume.
11. Close the lid of the instrument. Continue the DNA extraction program.
12. After the extraction is completed, take the tubes out of the instrument.

For storage the purified DNA should be transferred into a sterile tube for 30 min after extraction. Purified DNA can be stored for one week at 2 to 8 °C, for 1 year at minus 24 to minus 16 °C, for a long time at the temperature no more than minus 68 ° C.

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 (detection through the bottom of the tube) or 0.1 ml tubes.

Programming the thermocycler

1. Switch on the instrument.
2. Insert the tubes into the rotor ensuring that the first tube is in well no. 1, place the rotor into the instrument, and close the lid (the wells are numbered, these numbers are used for subsequent tube order programming).

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

3. Click the **New** button in the main menu of the program.
4. In the opened window select **Advanced** menu and **Dual Labeled Probe/Hydrolysis probes**. Activate the **New** button.
5. Select **36-Well Rotor** and **No Domed 0.2 ml Tubes/Locking ring attached**. Click the **Next** button.
6. Set an operator and specify the **Reaction volume** as **25 µl**. For Rotor-Gene 6000 select **15 µl oil layer volume** with a checkmark. Click the **Next** button.
7. Select the **Edit profile** button and set the amplification program (see table 1).

Table 1

Amplification program for rotor type instruments

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	95	15 min	–	1
2	95	10 s	–	10
	60	20 s	–	
	72	10 s	–	
3	95	10 s	–	35
	60	20 s	FAM/Green, JOE/Yellow ROX/Orange	
	72	10 s	-	

8. Click **OK**.
9. In the **New Run Wizard** window select the **Calibrate/Gain Optimisation**.
 - For calibration in FAM/Green, JOE/Yellow, and ROX/Orange channels select **Calibrate Acquiring/Optimise Acquiring**.
 - Check the **Perform Calibration Before 1st Acquisition/Perform Optimisation Before 1st Acquisition**.
 - For all channels set calibration from **5FI** to **10FI** (**Edit...** button, **Auto gain calibration channel settings** window). Press the **Close** button.
10. Click the **Next** button. Select the **Start run** button.
11. Name the experiment and save it to the disk (results of the run will be automatically saved in this file).
12. Enter the data in the table of samples (it opens automatically after amplification run). Indicate the names/numbers of test samples in the **Name** column. Mark the Negative control of amplification as **NCA**, Positive control of amplification as **C+**. Set the **Unknown** type for all test samples, the **Positive control** type for Positive control of amplification and the **Negative control** type for Negative control of amplification. For empty wells set **None**.

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

Data analysis

Results are analyzed by the software of the used real time PCR instrument. Results are interpreted by the crossing (or not-crossing) of the fluorescence curve with the threshold line and shown as the presence (or absence) of the *Ct* (threshold cycle) value in the results grid.

Data analysis of the *Mycoplasma pneumoniae* DNA amplification (FAM/Green channel)

1. Click the **Analysis** button in the menu, select **Quantitation**, and then select **Cycling A.FAM/Cycling A.Green**. Click **Show**.
2. Cancel the **Threshold** automatic choice.
3. Activate the **Dynamic tube** button in the menu of the main window (**Quantitation analysis**).
4. In the **CT Calculation** menu, set **Threshold = 0.05**.
5. Select the **More Settings/Outlier Removal** parameter and set **NTC threshold** value **20 %**.
6. In the results grid (**Quant. Results** window) the *Ct* (cycle threshold) values will appear.

Data analysis of the human DNA amplification (JOE/Yellow channel)

1. Click the **Analysis** button in the menu, select **Quantitation**, and then select **Cycling A.JOE/Cycling A.Yellow**. Click **Show**.
2. Cancel the **Threshold** automatic choice.
3. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of the main window (**Quantitation analysis**).
4. In the **CT Calculation** menu, set **Threshold = 0.1**.
5. Select the **More Settings/Outlier Removal** parameter and set **NTC threshold** value **10 %**.
6. In the results grid (**Quant. Results** window) the *Ct* (cycle threshold) values will appear.

Data analysis of the *Chlamydomonas pneumoniae* DNA amplification (ROX/Orange channel)

1. Click the **Analysis** button in the menu, select **Quantitation**, and then select **Cycling A.ROX/Cycling A.Orange**. Click **Show**.
2. Cancel the **Threshold** automatic choice.
3. Activate the **Dynamic tube** button in the menu of the main window (**Quantitation analysis**).
4. In the **CT Calculation** menu, set **Threshold = 0.1**.
5. Select the **More Settings/Outlier Removal** parameter and set **NTC threshold** value **5 %**.
6. In the results grid (**Quant. Results** window) the *Ct* (cycle threshold) values will appear.

Interpretation of results for the control samples

The result of the analysis is considered reliable only if the results obtained for Positive and Negative controls of amplification as well as for the Negative control of extraction are correct (see the “Results for control” table in the Instruction manual and *Ct* boundary values in the *Important Product Information Bulletin*).

Interpretation of test samples should be carried out in accordance with the Instruction Manual and the *Important Product Information Bulletin* enclosed to the PCR kit.

Interpretation of results for the test samples

Mycoplasma pneumonia is **detected** in a sample if the *Ct* value determined in the results grid in the FAM/Green channel does not exceed 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.

Chlamydomphila pneumoniae is **detected** in a sample if the *Ct* value determined in the results grid in the ROX/Orange channel does not exceed 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.

Mycoplasma pneumonia and *Chlamydomphila pneumoniae* DNA is **not detected** in a sample if the *Ct* value is not determined (absent) in one of the channels (FAM/Green and ROX/Orange), whereas the *Ct* value determined in the JOE/Yellow channel (human DNA) does not exceed the boundary *Ct* value specified in the *Important Product Information Bulletin*.

The result is **invalid** if the *Ct* value is not determined (absent) or greater than specified boundary *Ct* value (see the bulletin) in the FAM/Green or ROX/Orange channel, whereas the *Ct* value in the JOE/Yellow channel (IC) is absent or greater than the specified boundary *Ct* value. In such cases, the PCR analysis should be repeated starting from the DNA extraction stage. If the same result is obtained in the second run, re-sampling of material is recommended.

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

1. For samples (except for C– and NCA) where a negative result is obtained in all channels, PCR analysis should be repeated starting from the DNA extraction stage. If a negative result is obtained again, sampling for analysis should be repeated. For Negative control of amplification (NCA) and Negative Control of extraction (C–) the negative result is normal.

2. If *Ct* value determined for the Positive control of amplification (C+) in any channel is absent or greater than the boundary value, amplification should be repeated for Positive Control of Amplification (C+) and all negative test samples.
3. If *Ct* value determined for the Negative control of amplification (NCA) and/or Negative control of extraction (C-) in the target gene detection channels is less than the boundary value of the positive result, the analysis should be repeated from the extraction stage for all samples in which the given target was detected in order to exclude the consequence of possible contamination.

AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ, 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 optically transparent caps (detection through the cap of the tube).

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

Programming the thermocycler:

1. Switch on the instrument and power unit of the optical module.

NOTE: The lamp should be warmed up for at least 30 min before the experiment starts.

2. Start the iCycler iQ or iCycler iQ5 software in accordance with the instrument used.

NOTE: Make sure there are no drops left on the tube walls. Drop fall during amplification run can cause signal detection error. Do not rotate strips/plate when loading into the instrument.

3. Set the plate setup (set the order of the tubes in the reaction chamber and the detection of fluorescent signal).
 - **iCycler iQ5.** Click the **Create New** or **Edit** button in the **Selected Plate Setup** window of the **Workshop** module. Edit plate setup in the **Whole Plate loading** mode. Set the **Sample Volume** as **25 µl**, **Seal Type** as **Domed Cap**, and **Vessel Type** as **Tubes**. Click the **Save &Exit Plate Editing**.
 - **iCycler iQ.** Edit the plate setup in the **Edit Plate Setup** window of the **Workshop** module. To do this, set the order of the tubes in the reaction chamber in the **Samples: Whole Plate Loading** module and indicate the name of each sample in the **Sample Identifier** window. Set fluorescent signal detection in **FAM-490, JOE-530 and ROX-**

575 channels for all tubes in the **Select and Load Fluorophores** option. Save the plate setup. Name the file in the **Plate Setup Filename** window (use .pts extension) and activate the **Save this plate setup** button (at the top of the screen). It is possible to edit the earlier used plate and in order to do this, open the **View Plate Setup** in the **Library** window, select the appropriate **Plate Setup** (a file with .pts extension) and activate the **Edit** button. Prior to using, save the edited file. Select **Run with selected protocol** in order to use the plate setup.

4. Set the amplification program (see the table below).

Table 2

Amplification program of plate type instruments

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	95	15 min	–	1
2	95	10 s	–	10
	60	25 s	–	
	72	25 s	–	
3	95	10 s	–	35
	60	25 s	FAM, JOE/HEX, ROX	
	72	25 s	–	

- **iCycler iQ5**. To create the protocol, click the **Create New** or **Edit** button in the **Selected Protocol** window of the **Workshop** module. Enter amplification parameters and click the **Save&Exit Protocol Editing**. For further runs the file with this program can be selected from the **Protocol** tab (protocol files are saved in the **Users** folder by default).
 - **iCycler iQ**. To create the protocol, select the **Edit Protocol** option in the **Workshop** module. Enter the amplification parameters (cycles, time, temperature) at the bottom window and indicate **Cycle 3 – Step 2** in the window at the right. Name the file in the **Protocol Filename** window and click the **Save this protocol** button (at the top of the display). For further runs, the file with this program can be selected from the **View Protocol** tab of the **Library** module. Click **Run with selected plate setup**.
5. Insert the tubes into the module of the instrument in accordance with the defined order and start the selected protocol with the selected plate setup.
 - **iCycler iQ5**. Check the correctness of the **Selected Protocol** and **Selected Plate Setup** before starting the program. Click **Run** to start. For detection of the well factor select the **Collect Well Factors from Experimental Plate** option and click the **Begin Run** button. Name the experiment (the results of the experiment will be automatically saved in this file) and click **OK**.
 - **iCycler iQ**. Check the correctness of the selected protocol and plate setup in the

Run Prep window. For the detection of the well factor select **Experimental Plate** in the **Select well factor source** menu. Set the **Sample Volume** as **25 µl**. Press **Begin Run** to start. Name the experiment (the results of the experiment will be automatically saved to this file) and click **OK** button.

6. Proceed to the analysis of results at the end of the program.

Analysis of results

Results are interpreted by the crossing (or not-crossing) of the fluorescence curve (S-shaped) with the threshold line (set in the middle of the linear fragment of fluorescence growth of the positive control in the log scale) and shown as the presence (or absence) of the *Ct* (threshold cycle) value in the results grid.

For FAM, JOE/HEX, ROX channels set the threshold (drag it with the cursor while holding the left mouse button) at 10-20 % level of the maximal fluorescence of the positive control sample in the last amplification cycle. Moreover, the fluorescence curve of the positive control sample should cross the threshold line at the area of exponential fluorescence growth coming into the linear growth phase.

The result of the analysis is considered reliable only if the results obtained for Positive and Negative controls of amplification as well as for the Negative control of extraction are correct (see the “Results for control” table in the Instruction manual and the boundary values in the *Important Product Information Bulletin*).

Data analysis of the *Mycoplasma pneumoniae* DNA amplification

- **iCycler iQ5**. Select the required data file (**Data File** window of the **Workshop** module) and click the **Analyze** button. Select data in the **FAM** channel. The **PCR Base Line Subtracted Curve Fit** mode should be activated (set by default). The threshold line is set manually. Press the **Results** button to display the results grid.
- **iCycler iQ**. In the **PCR Quantification** option (**Select a Reporter** menu) select the **FAM-490** icon. The **PCR Base Line Subtracted Curve Fit** mode should be activated (set by default). In the **Threshold Cycle Calculation** menu select manual setting of the threshold line and automatic baseline calculation. To do this, select **Auto Calculated** in the **Baseline Cycles** submenu and select **User Defined** in the **Threshold Position** submenu. The threshold line is set manually. Press the **Recalculate Threshold Cycles** button. *Ct* values will appear in the results grid.

Data analysis of the human DNA amplification

- **iCycler iQ5**. Select **JOE** channel data from the module window. The **PCR Base Line Subtracted Curve Fit** mode should be activated (set by default). The threshold line is

set manually. Press the **Results** button to display the results grid.

- **iCycler iQ**. Activate the **View Post-Run Data** window in the **Library** module. In the **Data Files** window select the required file with results of analysis and press the **Analyse Data** button. In the **PCR Quantification** option (**Select a Reporter** menu), select the **JOE-530** icon. Make sure that the **PCR Base Line Subtracted Curve Fit** mode is activated (set by default). In the **Threshold Cycle Calculation** menu select manual setting of the threshold line and automatic baseline calculation. To do this, select the **Auto Calculated** in the **Baseline Cycles** submenu and select **User Defined** in the **Threshold Position** submenu. The threshold line is set manually. Press the **Recalculate Threshold Cycles** button. *Ct* values will appear in the results grid.

Data analysis of the *Chlamydomphila pneumoniae* DNA amplification

- **iCycler iQ5**. Select **ROX** channel data in the module window. Disable **FAM** and **JOE** buttons. The **PCR Base Line Subtracted Curve Fit** mode should be activated (set by default). The threshold line is set manually. Press the **Results** button to display the results grid.
- **iCycler iQ**. In the **PCR Quantification** option (**Select a Reporter** menu), select the **ROX-575** icon. Make sure that the **PCR Base Line Subtracted Curve Fit** mode is activated (set by default). In the **Threshold Cycle Calculation** menu select manual setting of the threshold line and automatic baseline calculation. To do this, select the **Auto Calculated** in the **Baseline Cycles** submenu and select **User Defined** in the **Threshold Position** submenu. The threshold line is set manually. Press the **Recalculate Threshold Cycles** button. *Ct* values will appear in the results grid.

Interpretation of results for the test samples

Mycoplasma pneumonia is **detected** in a sample if the *Ct* value determined in the results grid in the FAM channel does not exceed 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.

Chlamydomphila pneumoniae is **detected** in a sample if the *Ct* value determined in the results grid in the ROX channel does not exceed 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.

Mycoplasma pneumonia and *Chlamydomphila pneumoniae* DNA is **not detected** in a sample if the *Ct* value is not determined (absent) in one of the channels (FAM and ROX), whereas the *Ct* value determined in the JOE/HEX channel (human DNA) does not exceed

the boundary *Ct* value specified in the *Important Product Information Bulletin*.

The result is **invalid** if the *Ct* value is not determined (absent) or greater than specified boundary *Ct* value (see the bulletin) in the FAM or ROX channel, whereas the *Ct* value in the JOE/HEX channel (IC) is absent or greater than the specified boundary *Ct* value. In such cases, the PCR analysis should be repeated starting from the DNA extraction stage. If the same result is obtained in the second run, re-sampling of material is recommended.

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

1. For samples (except for C– and NCA) where a negative result is obtained in all channels, PCR analysis should be repeated starting from the DNA extraction stage. If a negative result is obtained again, sampling for analysis should be repeated. For Negative control of amplification (NCA) and Negative Control of extraction (C–) the negative result is normal.
2. If *Ct* value determined for the Positive control of amplification (C+) in any channel is absent or greater than the boundary value, amplification should be repeated for Positive Control of Amplification (C+) and all negative test samples.
3. If *Ct* value determined for the Negative control of amplification (NCA) and/or Negative control of extraction (C–) in the target gene detection channels is less than the boundary value of the positive result, the analysis should be repeated from the extraction stage for all samples in which the given target was detected in order to exclude the consequence of possible contamination.

AMPLIFICATION AND DATA ANALYSIS USING CFX96 (Bio-Rad, USA) INSTRUMENT

Carry out the pretreatment and reaction mixture preparation stages according to the PCR kit *Instruction Manual*. When carrying out the amplification it is recommended to use PCR tubes (0.2 ml) with optically transparent caps (detection through the cap of the tube).

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.

Programming the thermocycler:

Program the instrument in accordance with the Operation Manual provided by the manufacturer.

1. Turn on the instrument and start the *Bio-Rad CFX Manager* software.
2. Select **Create a new Run** (or select **New** and then **Run...** in the **File** menu).
3. In the **Run Setup** window, select **Protocol** and click the **Create new...** button. Set amplification parameters (time, temperature, cycles, and fluorescence acquiring cycle) in the opened **Protocol Editor – New** window (see table 3). Set **Sample Volume – 25 µl**.

Table 3

Amplification program of plate type instrument

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	95	15 min	–	1
2	95	10 s	–	10
	60	25 s	–	
	72	25 s	–	
3	95	10 s	–	35
	60	25 s	FAM, HEX, ROX	
	72	25 s	–	

NOTE! Set **Ramp Rate 2,5 °C/sec** by clicking the **Step Options** button for each step of cycling.

4. In the **Protocol Editor New** window select **File**, then **Save As**, and name the protocol. This protocol can be used for further runs by clicking the **Select Existing...** button in the **Protocol** tab. This file can be selected for further runs from the **Protocol** tab by clicking the **Select Existing...** button. When the required program is entered or edited, click **OK** at the bottom of the window.
5. In the **Plate** tab click the **Create new...** button. Set the tube order in the opened **Plate Editor – New** window. In the **Sample type** menu select **Unknown**; click the **Select Fluorophores...** button and tick the required fluorophores; click **OK**; then tick the

fluorescence signal acquiring for the selected wells in the required channels. Define sample names in the **Sample name** window and click **Load** after entering name of each sample.

6. In the **Plate Editor New** window select **File**, then **Save As**, and name the plate. When the required plate is entered or edited, click **OK** at the bottom of the window.
7. Place the reaction tubes in the wells of the instrument in accordance with the entered plate setup. In the **Start Run** tab click the **Start Run** button then save the file of the experiment.
8. Proceed to the analysis of results after the end of the run.

Analysis of results

Obtained data are interpreted by the real-time PCR instrument software by the crossing of a fluorescence curve with the threshold line set at the specific level (that corresponds to the presence of *Ct* value in the results grid).

Fluorescence curves, plate setup, and the results grid with *Ct* values are displayed in the **Quantification** tab.

For all the channels (FAM, HEX, ROX) set the threshold line (drag it with a cursor while pressing the left mouse button) at the level of 10-20 % of maximum fluorescence obtained for the positive control samples in the last amplification cycle. Make sure that fluorescence curve of the positive control samples crosses the threshold line at the zone of exponential growth of fluorescence passing onto linear growth.

The result of the analysis is considered reliable only if the results obtained for Positive and Negative controls of amplification as well as for the Negative control of extraction are correct (see the “Results for control” table in the Instruction manual and threshold values in the Important Product Information Bulletin for **CFX (Bio-Rad, USA)**).

Click **Tools** on the toolbar, then **Reports** and then save the generated report.

Interpretation of results for the test samples

Mycoplasma pneumonia is **detected** in a sample if the *Ct* value determined in the results grid in the FAM channel does not exceed 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.

Chlamydomphila pneumoniae is **detected** in a sample if the *Ct* value determined in the results grid in the ROX channel does not exceed 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.


Mycoplasma pneumonia and *Chlamydomphila pneumoniae* DNA **is not detected** in a sample if the *Ct* value is not determined (absent) in one of the channels (FAM and ROX), whereas the *Ct* value determined in the HEX channel (human DNA) does not exceed the boundary *Ct* value specified in the *Important Product Information Bulletin*.

The result is **invalid** if the *Ct* value is not determined (absent) or greater than specified boundary *Ct* value (see the bulletin) in the FAM or ROX channel, whereas the *Ct* value in the HEX channel (IC) is absent or greater than the specified boundary *Ct* value. In such cases, the PCR analysis should be repeated starting from the DNA extraction stage. If the same result is obtained in the second run, re-sampling of material is recommended.

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

1. For samples (except for C– and NCA) where a negative result is obtained in all channels, PCR analysis should be repeated starting from the DNA extraction stage. If a negative result is obtained again, sampling for analysis should be repeated. For Negative control of amplification (NCA) and Negative Control of extraction (C–) the negative result is normal.
2. If *Ct* value determined for the Positive control of amplification (C+) in any channel is absent or greater than the boundary value, amplification should be repeated for Positive Control of Amplification (C+) and all negative test samples.
3. If *Ct* value determined for the Negative control of amplification (NCA) and/or Negative control of extraction (C–) in the target gene detection channels is less than the boundary value of the positive result, the analysis should be repeated from the extraction stage for all samples in which the given target was detected in order to exclude the consequence of possible contamination.

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
28.07.16 ME	Cover page	Title was corrected
	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany); Instruments amplification and data analysis using iCycler iQ, iCycler iQ5 (Bio-Rad, USA) instruments; Amplification and data analysis USING CFX96 (Bio-Rad, USA) instrument	Interpretation of results for the test samples was added
27.02.20 EM	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany); Instruments Amplification and data analysis using iCycler iQ, iCycler iQ5 (Bio-Rad, USA) instruments; Amplification and data analysis using CFX96 (Bio-Rad, USA) instrument	Information about Slope correct button was added to data analysis of the human DNA amplification in JOE/Yellow channel
07.06.21 EM	Through the text	The symbol  was changed to NOTE:
	Cover page	The phrase "For research use only. Not for diagnostic procedures" was added
22.06.23 EM	Footer	REF R-B42-100-F-CE was added