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GUIDELINES

to AmpliSens[®] MDR MBL-FRT and

AmpliSens[®] MDR KPC/OXA-48-FRT PCR kits

for detection of carbapenemase genes using the polymerase chain reaction (PCR) with real-time fluorescence-hybridization detection

AmpliSens[®]



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INTENDED USE

The guidelines describe the procedure of using **AmpliSens® MDR MBL-FRT** and **AmpliSens® MDR KPC/OXA-48-FRT** PCR kits for detection of carbapenemase genes by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using the following instruments:

- Rotor-Gene 6000 (Corbett Research, Australia);
- Rotor-Gene Q (QIAGEN, Germany);
- CFX96 (Bio-Rad, 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
ROX	ROX/Orange/TxR
Cy5	Cy5/Red
Cy5.5	Cy5.5/Crimson/Quasar705

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 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 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, or PCR tubes (0.1 ml) with caps from the four-pieces-strips (detection through the bottom of the tube).

Creating the template for the run

1. Click the **New** button in the software main menu. To create the template select the **Advanced** tab in the opened window **New run**.
2. Select the **TwoStep/Hidrolysis Probes** template in the tab for edition and click The **New** button.
3. In the opened window select the **36-Well Rotor** rotor-type. Tick the **No Domed 0,2ml Tubes / Locking Ring Attached** option. Click the **Next** button.
4. In the opened window enter the operator name, select the reaction volume – 25 µl. Tick the **15 µl oil layer volume** option. Click the **Next** button.
5. In the **New Run Wizard** window set the temperature profile of the experiment. To do this click the **Edit profile** button and set the amplification program:

Table 1

AmpliSens-1 amplification program for rotor-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Cycles
Hold	95	15 min	–	1
1 Cycling	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
2 Cycling	95	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red and Cy5.5/Crimson	
	72	15 s	–	

NOTE: The Cy5.5/Crimson channel is enabled when required if the “multiprime” format tests are performed

AmpliSens-1 amplification program is used for PCR analysis of DNA samples obtained from the original biological material or DNA samples obtained by the extraction from the hemoculture, pure culture or mixture of bacterial cultures using DNA-sorb-AM nucleic acid extraction kit. **AmpliSens-1** is universal program for conducting tests for detection carbapenemase genes and the DNA of the major Gram-negative pathogens of suppurative-septic infections using AmpliSens® PCR kits.

6. After setting up the temperature profile click the **OK** button.
7. Set automatic calibration in order to select **gain** parameter. To do this, click **Calibrate/Gain Optimisation** button in the **Channel Setup** window. In the newly opened **Auto Gain Calibration Setup** window click the **Calibrate Acquiring/Optimize Acquiring** button. In the **Target Sample Range** line set for the **FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red u Cy5.5/Crimson** channels enter the value **5** in the **Min Reading** column and the value **10** in the **Max Reading** column (it is acceptable to set **Calibrate/Gain Optimisation...** value from 4 FI to 8 FI for the **JOE/Yellow, ROX/Orange, Cy5/Red** and **Cy5.5/Crimson** channels). In the **Tube position** column specify the number of the tube as **1** and the **gain** parameter will be selected automatically according to the signal. Tick the **Perform Calibration Before 1-st Acquisition/Perform Optimisation Before 1-st Acquisition** box. Close the **Auto Gain Calibration Setup** window.
8. Click the **Next** button. For saving the programmed template it is necessary to click the **Save Template** button to save the test template. Enter the template name according to the amplification program. Save the file to the **Templates** folder (in the **Quick Start Templates** subfolder) and close the **New Run Wizard** window. After that the created template will appear in the list of templates in the **New Run** window.

Using the ready template for the run

1. Turn on the instrument.
2. Insert the tubes into the rotor so that the first well will be loaded with a tube filled with the reaction mixture for the run. Put the locking ring, attach the rotor by uniting of the fixing arm hole with the fixing arm and close the lid.

NOTE: The first tube in the rotor is used for automatic optimization of the signal level, that's why the tube with the reaction mixture should be inserted at the first position of the rotor. If different tests are carried out simultaneously, the tube with the reaction mixture of the kit with a greater number of used channels should be inserted at the first well (for example, in case of conducting tests using **AmpliSens® MDR MBL-FRT** and **AmpliSens® MDR KPC/OXA-48-FRT** reagents kits, in the first position of the rotor should be inserted the tube with reaction mixture from the **AmpliSens® MDR MBL-FRT** kit).

NOTE: It is forbidden to use the tubes with PCR mixture passed through amplification earlier for rotor filling. It is allowed to leave the empty rotor wells.

3. In the **New Run** menu select the **Advanced** tab at the top of **New Run Wizard** window to start run using the ready template. Select the template with the **AmpliSens-1** amplification program from the list of templates. 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.
4. 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. Proceed to the next window.
5. In the opened window check that the reaction volume is correct and the **15 µL oil layer volume** option is activated. Click the **Next** button.
6. In the next window the correctness of the amplification and detection program and signal level auto-optimization parameters can be checked. Go to the next window clicking the **Next** button. Start the amplification by the **Start run** button. Herewith, the rotor with the samples should be already fixed and the lid should be closed. Name the experiment and save it to the disc (the results of the experiment will be automatically saved in this file).
7. 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.

In case of simultaneous PCR analysis in one instrument using **AmpliSens® MDR KPC/OXA-48-FRT** and **AmpliSens® MDR MBL-FRT** PCR kits or other **AmpliSens®** kits, create a new page in the table of samples and name it, for example, **KPC/OXA-48**. Set the **Unknown** type for samples, which results of analysis is performed using **AmpliSens® MDR KPC/OXA-48-FRT** PCR kit, for all another samples set the **None** type. The new page in the table of sample is created using the **New** button. It is recommended to set (a checkmark will appear) the **Synchronize pages** option (under the table) for convenience.

NOTE: This page is used at data analysis for **AmpliSens® MDR KPC/OXA-48-FRT** PCR kit.

NOTE: To edit the samples table 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

8. Analyze results after the amplification program is completed.

NOTE: The tubes should be removed from the rotor and disposed after the amplification program is completed.

Data analysis:

Analysis of results is performed separately (sequentially) for each channel according to the Instruction Manual and description given in this section.

The results of amplification of carbapenemase genes fragments for each type are registered on the appropriate channel (see Table 2).

Table 2

Analysis of results for groups of genes

Channel	FAM/Green	JOE/Yellow	ROX/Orange	Cy5/Red
DNA target for AmpliSens® MDR MBL-FRT PCR kit	VIM-type MBL genes	IMP-type MBL genes	Internal Control	NDM-type MBL genes
DNA target for AmpliSens® MDR KPC/OXA-48-FRT PCR kit	KPC-type carbapenemase genes	OXA-48-type carbapenemase genes	Internal Control	-

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 target in the corresponding column of the results table.

Sequentially analyze the data obtained for each used channel as follows:

1. Activate the **Analysis** button in the menu, select the mode of the analysis **Quantitation**. Select the required channel and click the **Show** button.

In case of simultaneous PCR analysis in one instrument using **AmpliSens® MDR KPC/OXA-48-FRT** and **AmpliSens® MDR MBL-FRT** PCR kits or other **AmpliSens®** kits, create a new page in the table of samples and name it, for example, **KPC/OXA-48**. Set the **Unknown** type for samples, which results of analysis is performed using **AmpliSens® MDR KPC/OXA-48-FRT** PCR kit, for all another samples set the **None** type. The new page in the table of sample is created using the **New** button. It is recommended to set (a checkmark will appear) the **Synchronize pages** option (under the table) for convenience.

NOTE: This page is used at data analysis for **AmpliSens® MDR KPC/OXA-48-FRT** PCR kit.

2. Cancel the automatic choice of the threshold line level **Threshold**.
3. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of main window (**Quantitation analysis**).
4. In the **CT Calculation** menu (in the right part of the window), indicate the threshold line

level **0.1** in the **Threshold box**.

- Choose the parameter **Outlier Removal** and set the values from 5% to 15% for all channels (see Table 3).

Table 3

Parameters for results analysis

Channel	Threshold	Slope Correct	Outlier Removal
FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red	0.1	on	5-15 %

- In the results grid (the **Quantitation Results** window) one will be able to see the *Ct* values.

Results interpretation

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 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.

NOTE: For the samples of pure bacterial culture, positive blood culture, mixture of bacterial cultures, obtained by primary inoculation of biological material, the result of carbapenemase genes detection is **valid** if the *Ct* value determined in the corresponding channel is less than the boundary *Ct* value specified in the *Important Product Information Bulletin*.

NOTE: The result is **equivocal** if the *Ct* value determined in the corresponding channel is greater than the boundary *Ct* value specified in the *Important Product Information Bulletin*. In such cases, the PCR analysis should be repeated starting from the DNA extraction stage.

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

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

Programming the thermocycler

1. Turn on the instrument and start the Bio-Rad CFX Manager software.
2. Program the instrument according to the *Instruction Manual* provided by the manufacturer.

Creating the template for the run

1. In the **Startup Wizard** window it is necessary to select **Create a new Run** in the start window (or select **New** and then **Run...** in the **File** menu). Click **OK**. Select the universal amplification and detection program **AmpliSens-1**.
2. In the **Run Setup** window, select **Protocol** tab and click the **Create new....** Set the amplification parameters (time, temperature, cycles, and fluorescence acquiring cycle) in the opened **Protocol Editor – New** window. Set **Sample Volume – 25 µl**.

Table 4

AmpliSens-1 amplification program for plate-type instruments

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, HEX, ROX, Cy5, Quasar705	
	72	15 s	–	

NOTE: Set **Ramp Rate 2,5 °C/s** by clicking the **Step Options** button for each step of cycling (see the figure below). Click **OK**.

```

1  95,0 C for 15:00
2  95,0 C for 0:05
   Slow Ramp Rate to 2,5 C per second
3  60,0 C for 0:20
   Slow Ramp Rate to 2,5 C per second
4  72,0 C for 0:15
   Slow Ramp Rate to 2,5 C per second
5  GOTO 2, 4 more times
6  95,0 C for 0:05
   Slow Ramp Rate to 2,5 C per second
7  60,0 C for 0:30
   + Plate Read
   Slow Ramp Rate to 2,5 C per second
8  72,0 C for 0:15
   Slow Ramp Rate to 2,5 C per second
9  GOTO 6, 39 more times
END

```

NOTE: The channel Quasar705 is enabled when required if the “multiprime” format tests are performed.

3. Save the protocol: in the **Protocol Editor New** window select **File**, then **Save As**, name the file and click **Save**.
4. Set the plate setup in the **Plate** tab click the **Create new...** button. Set the tube order in the opened **Plate Editor – New** window. Click the **Select Fluorophores...** button and click the **Selected** checkbox next to the **FAM, HEX, ROX, Cy5, Quasar705** fluorophores and click **OK**. In the **Sample type** menu select **Unknown** for all the samples. Define sample names in the **Sample name** window. Then in the **Load** column (in the right part of the window) tick the fluorescence signal acquiring for all the samples in the required channels. Define sample names in the **Sample name** window, moreover the **Load** function is to be ticked.
5. Save the plate setup: select **File** and then **Save as** in the **Plate Editor New** window. Enter the file name, click **Save**.
6. Select the **Start Run** tab. Open the lid of the instrument by the **Open Lid** button. Insert the reaction tubes in the wells of the instrument in accordance with the entered plate setup. Close the lid of the instrument by the **Close Lid** button.

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.

7. Click the **Start Run** button and start the program with the selected plate setup. Select the directory for the file saving, name the file, click **Save**.
8. Analyze results after the amplification program is completed.

Data analysis

Analysis of results is performed separately (sequentially) for each channel according to the Instruction Manual and description given in this section.

The results of amplification of carbapenemase genes fragments for each type are registered on the appropriate channel (see Table 5).

Analysis of results for groups of genes

Channel	FAM	HEX	ROX	Cy5
DNA target for AmpliSens® MDR MBL-FRT PCR kit	VIM-type MBL genes	IMP-type MBL genes	Internal Control	NDM-type MBL genes
DNA target for AmpliSens® MDR KPC/OXA-48-FRT PCR kit	KPC-type carbapenemase genes	OXA-48-type carbapenemase genes	Internal Control	-

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* (**Cq**) (threshold cycle) value in the corresponding column of the results table.

1. Start the software and open the saved file with data of the analysis. To do this, select **File** in the menu, then **Open** and **Data file** and select the needed file.
2. Analyze data separately for each channel turning off other channels (tick off the channel box under the main window with the **Amplification** curve).
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. Set the threshold line for each channel (except the ROX channel) at the level of 10-20 % of maximum fluorescence obtained for the Positive Control in the last amplification cycle. For the ROX channel set the threshold line at the level of 10-20 % of maximum fluorescence obtained for the Negative Control of Extraction (C-).
4. In the result grid **Cq** values for the analysed channel will appear.

Results interpretation

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 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.

NOTE: For the samples of pure bacterial culture, positive blood culture, mixture of bacterial cultures, obtained by primary inoculation of biological material, the result of carbapenemase genes detection is **valid** if the *Ct* value determined in the corresponding channel is less than the boundary *Ct* value specified in the *Important Product Information Bulletin*.

NOTE: The result is **equivocal** if the *Ct* value determined in the corresponding channel is greater than the boundary *Ct* value specified in the *Important Product Information Bulletin*. In such cases, the PCR analysis should be repeated starting from the DNA extraction stage.

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
14.07.23 EM	Footer	REF R-C1(RG,CFX)-CE, REF R-C2(RG,CFX)-CE were added
25.07.25 HM	Through text	Changings according to the template
	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany) instruments	The Calibrate /Gain Optimisation... value for JOE/Yellow, ROX/Orange, Cy5/Red and Cy5.5/Crimson channels was changed from "4 FI to 8 FI" to "5 FI to 10 FI" . The note "it is acceptable to set Calibrate/Gain Optimisation... value from 4 FI to 8 FI for the JOE/Yellow, ROX/Orange, Cy5/Red and Cy5.5/Crimson channels" was added

