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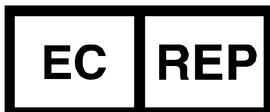
For Professional Use Only

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 and CFX384 (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

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 (e.g. Axygen, USA), or PCR tubes (0.1 ml) with caps from the four-pieces-strips (e.g. Corbett Research, Australia; QIAGEN, Germany) (detection through the bottom of the tube).

Creating the template for the run

1. For programming and creating new template choose the mode ***Advanced*** in ***New Run*** window. Choose any template (for example, ***Hydrolysis probes / Dual Labeled Probe***) for editing and click the ***New*** button.
2. In the opened window select the ***36-Well Rotor*** rotor-type. Tick the ***No Domed Tubes / Locking Ring Attached*** option.

[1] The detection channels names in each section of the guidelines are specified in accordance with the described instrument.

3. In the opened window select ***Reaction Volume (µL)*** as ***25 µl***, after that tick ***15 µL oil layer volume*** to activate this option.
4. In the opened window it is necessary to set the temperature profile of the experiment. To do this click the ***Edit profile*** button and set the parameters of programs for amplification and detection “AmpliSens-1", and click ***OK*** button.

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 clinical 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.

5. 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**.** For the ***FAM/Green*** channel enter the value **5** in the ***Min Reading*** column and the value **10** in the ***Max Reading*** column. For ***JOE/Yellow, ROX/Orange, Cy5/Red*** and ***Cy5.5/Crimson*** channels enter the value **4** in the ***Min Reading*** column and the value **8** in ***Max Reading*** column. 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.

6. Proceed to the next window. 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.
4. In the opened window select the ***36-Well Rotor*** or ***72-Well Rotor*** and tick the ***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. Proceed to the next window.
6. In the next window the correctness of the amplification and detection program and signal level auto-optimization parameters can be checked.
7. In the last window 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 file where the results will be saved and click the ***Save*** button.
8. To set the order of samples in the rotor enter the name and type (***Unknown)*** of each sample in the samples grid. Click ***OK***.

NOTE: 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.
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

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

NOTE: 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.
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 (in the ***Calculate automatic threshold*** window delete the tick from the ***Show automatically when opening a new channel*** string and click ***Cancel*** button).
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***.
5. 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***	***Outlier Removal***	***Slope Correct***
FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red	0.1	5-15 %	on

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

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 *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 clinical 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 AND CFX384 (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. Select ***Create a new Run*** in the start window (or select ***New*** and then ***Run…*** in the ***File*** menu). Select the universal amplification and detection program **AmpliSens-1.** For this purpose, select or create the program in the ***Experiment Setup*** module in the ***Protocol Editor*** 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.

2. Set the plate setup in the ***Plate*** tab: put the position of samples in reaction block and select fluorescent detection through five channels **FAM, HEX, ROX, Cy5, Quasar705** for all samples. To do this, click the ***Select Fluorophores…*** button and click the required fluorophores. In the ***Sample type*** menu select ***Unknown*** for all the samples. Define sample names in the ***Sample name*** window. Save the plate setup, click the ***OK*** button.
3. 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.

4. Click the ***Start Run*** button to start the program with the selected plate setup.
5. 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).

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.

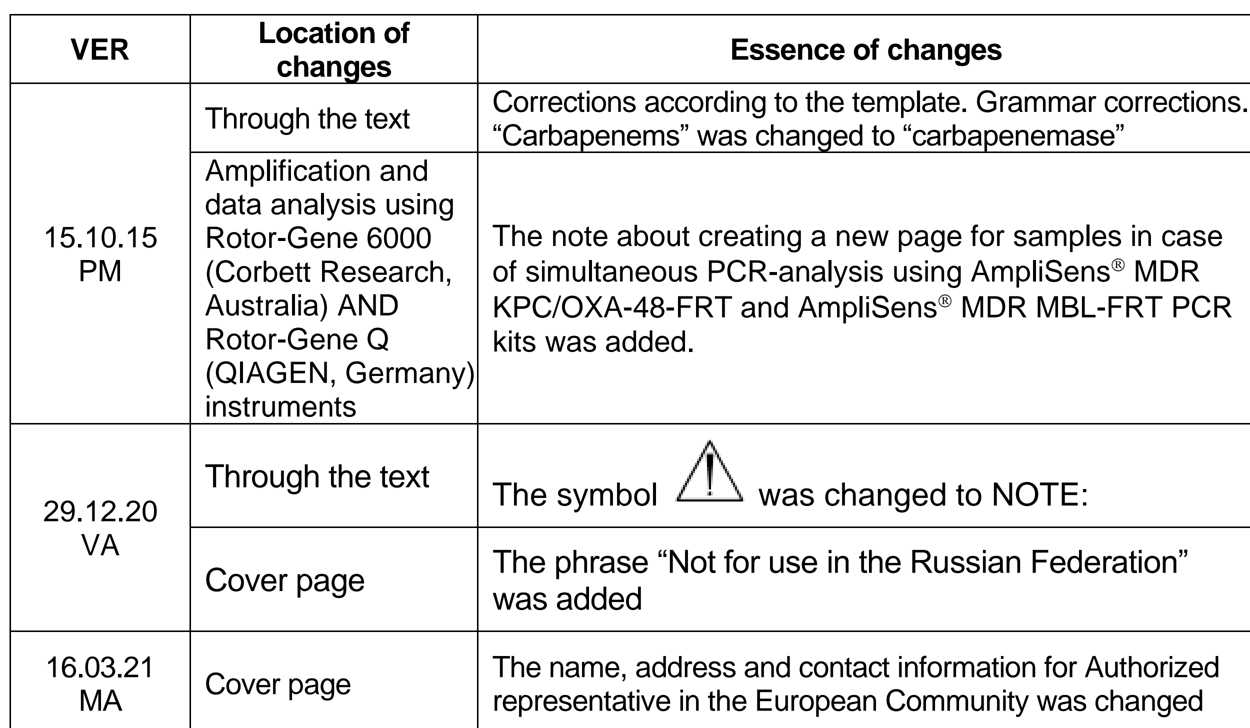
1. Start the software and open the saved file with data of the analysis. To do this, select ***File*** in the menu, then ***Open*** and ***Data file*** and select the needed file.
2. Analyze data separately for each channel turning off other channels (tick off the channel box under the main window with 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.

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 *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 clinical 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
15.10.15 PM	Through the text	Corrections according to the template. Grammar corrections. “Carbapenems” was changed to “carbapenemase”
	Amplification and data analysis using Rotor-Gene 6000 (Corbett Research, Australia) AND Rotor-Gene Q (QIAGEN, Germany) instruments	The note about creating a new page for samples in case of simultaneous PCR-analysis using AmpliSens® MDR KPC/OXA-48-FRT and AmpliSens® MDR MBL-FRT PCR kits was added.
29.12.20 VA	Through the text	The symbol ⚠ was changed to NOTE:
	Cover page	The phrase “Not for use in the Russian Federation” was added
16.03.21 MA	Cover page	The name, address and contact information for Authorized representative in the European Community was changed