

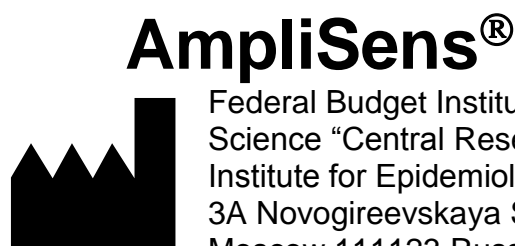
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GUIDELINES

to AmpliSens[®] *MRSA-screen-titre-FRT* PCR kit

for qualitative and quantitative detection of methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* DNA and methicillin-resistant coagulase-negative *Staphylococcus* spp. DNA in the biological material by polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection



For research use only. Not for diagnostic procedures

TABLE OF CONTENTS

INTENDED USE 3

WORK WITH THE NucliSENS easyMAG AUTOMATED NUCLEIC ACID EXTRACTION SYSTEM..... 3

AMPLIFICATION AND DATA ANALYSIS USING Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany) 5

AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ and iQ5 (Bio-Rad, USA)..... 9

INTENDED USE

The guidelines describe the procedure of using the **AmpliSens® MRSA-screen-titre-FRT** PCR kit for qualitative detection and quantitation of methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* DNA and methicillin-resistant coagulase-negative *Staphylococcus* spp. DNA in the biological material (oropharyngeal swabs, bronchoalveolar lavage (BAL), sputum, endotracheal aspirate, bronchial washing fluid, urine (first portion pellet), blood, blood plasma, cerebrospinal fluid (CSF), affected organs and tissues aspirates, washes from healthcare equipment and instruments) by the polymerase chain reaction (PCR) with 'real-time' fluorescence-hybridization detection using the following instruments:

- Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia);
- Rotor-Gene Q (QIAGEN, Germany);
- iCycler iQ, iCycler iQ5 (Bio-Rad, USA).

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

WORK WITH THE NucliSENS easyMAG AUTOMATED NUCLEIC ACID EXTRACTION SYSTEM

Variant 1

DNA extraction with lysis of sample outside of the instrument (off-board lysis mode)

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

1. Switch on the NucliSENS easyMAG instrument and prepare it to the RNA/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 extraction (select **Plasma**)
 - **Volume – 0.1 ml**
 - **Eluate – 55 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. Relocate sample table into the created protocol.
5. Take the required quantity of special disposable tubes intended for DNA extraction in

NucliSENS easyMAG instrument (including negative and positive control of extraction).
Add **10 µl** of **Internal Control STI-87 (IC)** into each tube, on the internal walls. Add **550 µl** of **NucliSens lysis buffer**.

NOTE: When working with material which contains clots, lysis is recommended to be carried out in 1.5-ml tubes. After incubation finish (item 8), tubes should be centrifuged at 10,000 rpm for 1 min. Then transfer the supernatant into special tubes intended for DNA extraction in NucliSENS easyMAG instrument.

6. Add **100 µl** of prepared samples into tubes with **NucliSens lysis buffer** and **Internal Control STI-87 (IC)** using disposable filter tips and carefully mix by pipetting. Avoid entering mucus clots and big particles into the tube.
7. Add **100 µl** of **Negative Control (C-)** into the tube with Negative Control of Extraction (C-). Add **90 µl** of **Negative Control (C-)** and **10 µl** of **Positive Control DNA MRSA** into the tube with Positive Control of Extraction (PCE).
8. Incubate tubes for 10 min at room temperature.
9. Resuspend the tube with **NucliSens magnetic silica** (bioMérieux) by intensive vortexing. Add **10 µl** of **magnetic silica** by using disposable filter tips and carefully mix by pipetting. Magnetic silica should be distributed evenly on whole tube volume.
10. Place tubes with samples into the instrument and start the DNA extraction program with lysis of samples by selecting **off board**.
11. After the extraction procedure is completed take the tubes out of the instrument. Transfer the tubes with DNA-samples to the amplification area.

Variant 2

DNA extraction with lysis of sample in the instrument (on-board lysis mode)

1. Switch on the NucliSENS easyMAG instrument and prepare it to 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 extraction (select **Plasma**)
 - **Volume – 0.1-1 ml**
 - **Eluate – 55 µl**
 - **Type – Primary**
 - **Priority – Normal.**
3. Create a new protocol of DNA extraction and save it. In protocol select **On-board Lysis Buffer Dispensing - yes, On-board Lysis Incubation - yes**.
4. Relocate programmed sample into created protocol.
5. Add **100 µl** of prepared samples into tubes intended for DNA extraction in NucliSENS

easyMAG instrument using of disposable filter tips.

6. Add **100 µl** of **Negative Control C–**) into tube with Negative Control of Extraction (C–) intended for DNA extraction in NucliSens easyMAG instrument. Add **90 µl** of **Negative Control C–**) and **10 µl** of **Positive Control DNA MRSA** into tube with Positive Control of Extraction (PCE).
7. Mix **NucliSens magnetic silica** and **Internal Control STI-87 (IC)** in individual sterile 2 ml volume tube by sterile disposable filter tips in following ratio:

Quantity of samples for DNA extraction	Quantity of NucliSens magnetic silica (µl)	Quantity of Internal Control STI-87 (IC) (µl)
1	10	10
24 (complete use of instrument)	250 (with reserve for 25 samples)	250 (from two tubes)

8. Mix the content of the tube. Mixture of **NucliSens magnetic silica** and **Internal Control STI-87 (IC)** can be stored not more than 30 min.
9. Place tubes with samples into instrument and start up the DNA extraction program with lysis of samples by selecting **on board**.
10. Wait before NucliSENS easyMAG instrument stop working in **Instrument State-Idle** position (near 15 min).
11. Carefully mix the tube with prepared mixture of **NucliSens magnetic silica** and **Internal Control STI-87 (IC)** by vortex till homogeneous state.
12. Open the cover of instrument and add **20 µl of mixture**. Each tube mix carefully by pipetting with help of multichannel pipettes. Use individual 200 µl filter tips.
13. Continue the DNA extraction program.
14. After the extraction procedure is completed take the tubes out of the instrument. Transfer the tubes with DNA-samples to the amplification area.

AMPLIFICATION AND DATA ANALYSIS USING Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany)

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 (e.g. Axygen, USA), or Rotor-Gene 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).

Programming the thermocycler

1. Switch the instrument on.
2. Insert the tubes into the rotor of the Rotor-Gene 3000/6000/Q instrument so that 1st tube is in Well 1 (the rotor wells are numbered, the numbers are used for the further programming of the samples' position in the thermocycler). Insert the rotor into the instrument, close the lid. Program the instrument.

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. Select the **New** button in the main window.
4. In the opened window select **Advanced** and **Dual Labeled Probe/Hydrolysis probes**. Activate the **New** button.
5. In the opened window select **36-Well Rotor** (or **72-Well Rotor**) and **No Domed 0.2 ml Tubes/Locking ring attached**. Click **Next**.
6. In the opened window set operator and select **Reaction volume** as **25 µl**. Tick the **15 µl oil layer volume** function. Click the **Next** button.
7. In the opened window program the amplification process. To do this select the **Edit profile** button and set corresponding parameters (see Table 1).

Table 1

MRSA amplification program for rotor-type instruments¹

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
Hold	95	15 min	–	1
Cycling 1	95	15 s	–	5
	60	30 s	–	
	72	15 s	–	
Cycling 2	95	15 s	–	40
	55	30 s	FAM/Green, JOE/Yellow, ROX/Orange	
	72	15 s	–	

8. After the amplification program is selected click the **OK** button.
9. Select the **Calibrate/Gain Optimisation...** button in the **New Run Wizard** window:
 - perform the fluorescence detection in FAM/Green, JOE/Yellow and ROX/Orange channels (activate the **Calibrate Acquiring/Optimise Acquiring** button);
 - perform the calibration in FAM/Green, JOE/Yellow and ROX/Orange channels before

¹ For example, Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia), or Rotor-Gene Q (QIAGEN, Germany).

the first detection (activate the **Perform Calibration Before 1st Acquisition/ Perform Optimisation Before 1st Acquisition** button);

- to set channels calibration, indicate **5** in the **Min Reading** box and **10** in the **Max Reading** box.

10. Start the amplification program by activating the **Start Run** button.

11. Name the experiment and save in on a disc (in this file the experiment results will be automatically saved).

12. Enter the data into the grid of the samples (it opens automatically after the amplification has been started). Indicate the names/numbers of the test samples in the box **Name**. Set the type **Unknown** opposite all the test samples, the type **Positive control** – for positive controls, the type **Negative control** – for the negative control of the extraction, and the type **NTC** – for the negative control of amplification. Name calibrators as **Standard** type and set their concentrations in **Given Conc.** column. Concentration values are specified in the *Important product information bulletin*. Name empty tubes as **None**.

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

Data analysis

The results are analyzed by the software of the instrument. The results are interpreted according to the crossing (or not-crossing) of the fluorescence curve with the threshold line that corresponds to the presence (or absence) of the *C_t* (threshold cycle) value in the results grid.

The obtained data – the curves of fluorescence signal accumulation in three channels – are analyzed by the software of the instrument for real-time PCR.

Data analysis of *Staphylococcus aureus* DNA (FAM/Green channel).

1. Check the sample table for presence of the calibrators and their concentrations.
2. Activate the **Analysis** button then select the mode of the analysis **Quantitation** and activate the button **Cycling A. FAM** or **Cycling A. Green**.
3. Cancel **Threshold** automatic choice.
4. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of each main window (**Quantitation analysis**).
5. In the **CT Calculation** menu (in the right part of the window) indicate the threshold line level **0.03** in the **Threshold** box.
6. Choose the parameter **More settings/Outlier Removal** and set **10 %** for the value of negative samples threshold (**NTC/Threshold**).
7. The *C_t* value is absent for the Negative Control of extraction (C–).

8. The *Ct* value is absent for the Negative Control of amplification (NCA) – **DNA-buffer**.
9. The *Ct* value and the concentration value (**Calc Conc (copies/reaction)**) should appear for the DNA-calibrators.
10. The *Ct* values and the concentration value (**Calc Conc (copies/reaction)**) should appear for the Positive Control of extraction (PCE) – **Positive Control DNA MRSA**.

Data analysis of the *mecA* amplification (JOE/Yellow channel).

1. Check the sample table for presence of the calibrators and their concentrations in case of quantitative analysis.
2. Activate the **Analysis** button then select the mode of the analysis **Quantitation** and activate the button **Cycling A. JOE** or **Cycling A. Yellow**. Click **Show**.
3. Cancel **Threshold** automatic choice.
4. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of each main window (**Quantitation analysis**).
5. In the **CT Calculation** menu (in the right part of the window) indicate the threshold line level **0.03** in the **Threshold** box.
6. Choose the parameter **More settings/Outlier Removal** and set **10 %** for the value of negative samples threshold (**NTC/Threshold**).
7. The *Ct* value is absent for the Negative Control of extraction (C–).
8. The *Ct* value is absent for the Negative Control of amplification (NCA) – DNA-buffer.
9. The *Ct* values and the concentration values (**Calc Conc (copies/reaction)**) should appear for the DNA-calibrators.
10. The *Ct* value and the concentration value (**Calc Conc (copies/reaction)**) should appear for the Positive Control of extraction (PCE) – **Positive Control DNA MRSA**.

Data analysis of the Internal Control STI-87 (IC) (ROX/Orange channel).

1. Check the sample table for presence of the calibrators and their concentrations in case of quantitative analysis.
2. Activate the **Analysis** button then select the mode of the analysis **Quantitation** and activate the button **Cycling A. ROX** or **Cycling A. Orange**.
3. Cancel **Threshold** automatic choice.
4. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of each main window (**Quantitation analysis**).
5. In the **CT Calculation** menu (in the right part of the window) indicate the threshold line level **0.03** in the **Threshold** box.
6. Choose the parameter **More settings/Outlier Removal** and set **10 %** for the value of negative samples threshold (**NTC/Threshold**).

7. In the results grid (**Quant. Results** window) the **Ct** values and the concentration values (**Calc Conc (copies/reaction)**) for **Internal Control STI-87 (IC)** for each test sample should appear. The values should be greater than the values specified in the *Important Product Information Bulletin*.
8. The **Ct** value is absent for the Negative Control of amplification (NCA) – **DNA-buffer**.
9. The **Ct** values and the concentration values (**Calc Conc (copies/reaction)**) should appear for the DNA-calibrators.

AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ and iQ5 (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, or tubes (0.2 ml) with transparent caps from the eight-pieces-strips (e.g. Axygen, USA) (detection through the cap of the tube).

1. Switch on the instrument and the power supply unit of the optical part of the instrument.

NOTE: The lamp is to be warmed up during 15 min before starting the experiment.

2. Start the program iCycler iQ/iQ5.
3. Insert the tubes or strips into the reaction module of the amplifier (thermocycler) and program the instrument.

NOTE: Monitor the tubes. There must not be drops left on the walls of the tubes as falling drops during the amplification process may lead to the signal failure and complicate the results analysis. Don't turn the strips upside down while inserting them into the instrument.

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

4. Select **Create new** in **Workshop** module.
5. Set amplification program (see Table 2).

Table 2

MRSA amplification program for plate-type instruments²

Step	Temperature, °C	Time	Fluorescence detection	Cycle repeats
1	95	15 min	–	1
2	95	15 s	–	5
	55	30 s	–	
	72	15 s	–	
3	95	15 s	–	40
	55	30 s	FAM, JOE/HEX, ROX	
	72	15 s	–	

6. Name new protocol and save it.

² For example iCycler iQ, iCycler iQ5 (Bio-Rad, USA), Mx3000P (Stratagene, USA).

7. Set the plate setup (set the order of the tubes in the reaction chamber).
8. In open window mark all test samples as **Unknown**, positive controls as “+”, negative controls as “-”. Set calibrators in JOE/HEX, FAM and ROX as **Standard** and indicate concentration values from *Important product information bulletin*. Then setting calibrators the **Whole Plate Loading** button should be non-activated. Set fluorescence measuring in JOE/HEX, FAM and ROX channels.
9. Name the plate setup and save it.
10. Select **Run**, in open window mark **Use Persistent Well Factors** and select **Begin Run**. Save the experiment.

Data analysis

1. Start the program and open the saved file. To do this select the needed file for the analysis in the **Data File** window of the **Workshop** module. Pass to **Data Analysis** mode.
2. The data for each channel are to be browsed separately.
3. Ensure that automatic selection of threshold level is correct. Normally, the threshold line should cross only sigmoid curves of signal accumulation of positive samples and controls. The threshold line is not to cross the base line. If it happens, it is necessary to raise the threshold level by clicking the **Log View** button and setting the threshold lines level (with the left mouse button) so that the fluorescence curves should be of a linear character and not intersect with the curves of the negative samples. The *Ct* values for analyzed channel will appear in the results grid (**Quant. Results** window).
4. In order to analyze the results click the **PCR Quant** button (for **iCycler iQ**) or activate the **Results** button which is situated under the buttons with the fluorophores' names (for **iCycler iQ5**).

The results interpretation of the test samples is to be performed in accordance with the *Important Product Information Bulletin*.