

GUIDELINES

to AmpliSens[®] *Listeria monocytogenes*- screen/monitor-FRT PCR kit

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

AmpliSens[®]



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INTENDED USE

The guidelines describe the procedure of using **AmpliSens® *Listeria monocytogenes*-screen/monitor-FRT** PCR kit for detection and quantitation of DNA of *L. monocytogenes* in the biological material (whole blood, umbilical cord blood, cerebro-spinal fluid (CSF), node aspirates, swabs from the respiratory tract, swabs from the eye conjunctiva, amniotic fluid, placenta, swabs from vagina mucous membrane, urine, breast milk, meconium, faeces, autopsy material, environmental samples (concentrated water samples (wastewater, potable water, surface water)), liquid medium for primary enrichment of food product) by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using the following instruments:

- Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia);
- Rotor-Gene Q (QIAGEN, Germany);
- CFX96 (Bio-Rad, USA);
- iCycler iQ, iCycler iQ5 (Bio-Rad, USA).

Nucleic acid extraction kits recommended for DNA extraction

The Federal Budget Institute of Science “Central Research Institute for Epidemiology” recommends the following nucleic acid extraction kits for DNA extraction and further PCR analysis with the use of **AmpliSens® *Listeria monocytogenes*-screen/monitor-FRT** PCR kit:

- **RIBO-prep** for DNA extraction from whole blood, umbilical cord blood, cerebro-spinal fluid (CSF), node aspirates, swabs from the respiratory tract, swabs from the eye conjunctiva, amniotic fluid, swabs from vagina mucous membrane, urine, breast milk, meconium, faeces, environmental samples (concentrated water samples (wastewater, potable water, surface water)), liquid medium for primary enrichment of food product;
- **DNA-sorb-C** for DNA extraction from placenta and autopsy material.

Correspondence of targets and detection channels

Channel for the fluorophore	FAM	JOE	ROX
DNA target	Internal Control STI-87 (IC) DNA	<i>L. monocytogenes</i> DNA	DNA fragment of β -globin gene (IC Glob)
Target gene	Internal Control STI-87 (IC)	listeriolysin O (<i>hly</i>) gene	β -globin gene

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

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

When working with Rotor-Gene 3000 one should use the Rotor-Gene version 6.1 and higher software and the Rotor-Gene 6000 versions 1.7 (build 67) software or higher for Rotor-Gene 6000 and Rotor-Gene Q instruments.

Hereinafter, all the terms corresponding to different instruments and software are indicated in the following order: for Rotor-Gene 3000 / for Rotor-Gene 6000/Q.

Carry out the sample pretreatment and reaction mixture preparation stages according to the PCR kit *Instruction Manual*. When carrying out the amplification it is recommended to use 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).

Programming the thermocycler

1. Turn on the instrument, run the Rotor-Gene software.
2. Insert the tubes or strips into the rotor of the Rotor-Gene 3000/6000/Q instrument beginning from the first well (the rotor wells are numbered, the numbers are used for the further programming of the samples' order in the thermocycler). Program the instrument.

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

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 (or 72-Well Rotor)** and 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

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

the **15 µl oil layer volume** option. Click the **Next** button.

- 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 unified amplification program

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
1	50	15 min	-	1
2	95	15 min	-	1
3	95	10 s	-	45
	60	20 s	FAM, JOE, ROX	

NOTE: Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program. If in one instrument only the tests for the pathogen DNA detection are carried out simultaneously, the first step of reverse transcription (50 °C – 15 min) can be omitted for time saving.

Note – The Cy5/Red and Cy5.5/Crimson channels are enabled when required if the “multiprime” format tests are performed.

- Задать параметры калибрования (активировать **Calibrate/Gain Optimisation.../Опт.уровня сигн.** в мастере нового эксперимента):

- perform the fluorescence detection in the 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 the first detection (tick the **Perform Calibration Before 1st Acquisition/ Perform Optimisation Before 1st Acquisition** option);
- set calibration from **5FI** to **10FI** for all the channels (FAM/Green, JOE/Yellow, ROX/Orange). To do this click the **Edit...** button in the **Auto gain calibration channel settings** window.

- Click the **Next** button. . For saving the programmed template it is necessary to click the **Save Template** button and enter the template file name, corresponding to the amplification program - **AmpliSens® Listeria monocytogenes-screen-monitor-FRT**. Save the file into a proposed folder: **Templates\Quick Start Templates**; close the **New Run Wizard window**. After that the programmed template will appear in the template list in the **New Run** window.

Using the ready template for the run

- Click the **New** button in the software main menu. In the opened **New Run** window select the **Advanced** tab. Then select the **AmpliSens® Listeria monocytogenes-screen-monitor-FRT** template (which is programmed as described in the “**Creating the template for the run**” section) in the template list.

2. 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. Click the **Next** button.
3. In the opened window check that the **reaction volume** is **25 µl** and the **15 µl oil layer volume** option is activated. Click the **Next** button.
4. In the next window the correctness of the amplification 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).
5. 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 **Standard** for DNA-calibrators C1 and C2 and enter the values from the *Important product information bulletin* enclosed to the given lot of the PCR kit into the **Given conc.** column. Set the type **None** for the cells matching with the corresponding empty tubes.

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

Note – To edit the table of samples 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.

Data analysis:

The obtained results are analyzed by the Rotor-Gene software. 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 *C_t* (threshold cycle) value of the DNA sample in the corresponding column of the results table. The calibration curve plotting and calculation of the *L. monocytogenes* DNA concentration are performed automatically in accordance with the *C_t* values.

Amplification data analysis in the FAM/Green channel:

1. Activate the button **Analysis** in the menu, select the mode of the analysis **Quantitation**, activate the buttons **Cycling A. FAM/Cycling A. Green, Show**.
2. Cancel the automatic choice of the threshold line level **Threshold**.

3. Select the **Linear scale**.
4. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of 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 (the **Quantitation Results** window) one will be able to see the *Ct* values and DNA concentration values (**Calc Conc (copies/reaction)**).

Results analysis in the JOE/Yellow and ROX/Orange is carried out similarly to results analysis in the FAM/Green channel in accordance with the settings in the table below.

Channel	Threshold	More Settings / Outlier Removal	Slope Correct
FAM/Green	0.03	10%	on
JOE/Yellow	0.03	10%	on
ROX/Orange	0.03	10%	on

If the fluorescence curves in the FAM/Green, JOE/Yellow channels do not correspond to the exponential growth, then **NOTE:** **NTC threshold** value can be increased up to 20 %.

Results interpretation

The result of the PCR analysis is considered reliable only if the results for the controls of the amplification and the 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.

AMPLIFICATION AND DATA ANALYSIS USING 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 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).

Programming the thermocycler


1. Turn on the instrument and the power supply unit of the optical block of the instrument.

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

2. Start the program iCycler iQ5.
3. Insert the tubes or strips into the reaction module of the thermocycler and program the instrument according to the *Instruction Manual* given by the manufacturer of 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 tubes (strips) upside down while inserting them into the instrument.

Creating the template for the run

1. Set the plate setup (set the order of the tubes in the reaction chamber and the detection of fluorescent signal):
 - click the **Create New** button in the **Selected Plate Setup** window of the **Workshop** module;
 - in the opened window click the **Whole Plate loading** button and set the plate setup using the buttons of the upper toolbar. Enter the samples' names in the **Identifier/Condition** column in the bar appeared in the screen bottom. Select the fluorescent signal detection in the FAM, HEX, ROX channels. Click the **Select/Add Fluorophores** button, select the fluorophore and tick it in the **Selected** column. Click **OK**. The fluorophore name will appear in the **Fluorophore** window. For addition of fluorescence signal measuring for each sample it is necessary to click the fluorophore (activate it) and select the samples on the plate using the **Fluorophore loading in whole Plate mode**  button under the scheme;
 - set the reaction volume (**Sample Volume**) as **25 µl**, the caps type (**Seal Type**) as **Domed Cap**, and the tubes type (**Vessel Type**) as **Tubes**;
 - Save the set plate setup by clicking the **Save&Exit Plate Editing** button. Enter the file name and click **Save**.
2. Set all the biological samples as **Unknown**, positive controls as «+», and negative controls as «-».

Set the calibrators C1 and C2 in the test channels as **Std (Standard)**. Enter the values specified in the *Important Product Information Bulletin* enclosed to the given lot of the PCR kit in the **Quantity** column.
3. Set the amplification program. To do this, in the **Selected Protocol** window of the **Workshop** module click the **Create New** button. Set the amplification parameters and

save the protocol by activating the **Save&Exit Protocol Editing** button. Enter the name of the file and then click **Save**.

Table 2

AmpliSens unified amplification program

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
1	50	15 min	-	1
2	95	15 min	-	1
3	95	10 s	-	45
	60	20 s	FAM, JOE, ROX	

NOTE: Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program. If only the tests for pathogen agent DNA detection are performed in one instrument then the first step of reverse transcription (50 °C – 15 minutes) can be deleted from the program for time saving.

Note – The **Cy5** channel is enabled when required if the “multiprime” format tests are performed.

4. Before a run it is obligatory to check if the selected protocol (**Selected Protocol**) and the plate scheme (**Selected Plate Setup**) are correct. To begin a run click the **Run** button. For the well factors measurement the **Use Persistent Well Factors** type is selected by default. Click the **Begin Run** button, save the experiment (the results of this experiment will be automatically saved in this file) and click **OK**.
5. At the end of the run it is necessary to close the software and turn off the instrument (the thermocycler and the optical block).

Using the ready template for the run

The test parameters and the plate setup set earlier can be used for the further runs. To do this:

- select the needed file with the run in the upper left window of the **Workshop** module,
- click the **Edit** button in the **Selected Plate Setup** area of the **Workshop** module and edit the plate setup (the files of protocols are saved in the **SampleFiles** folder by default);
- click the **Edit** button in the **Selected Protocol** area of the **Workshop** module and check the correctness of the selected protocol (the files of protocols are saved in the **Users** folder by default).

Data analysis:

The obtained results are analyzed by the iCycler iQ5 / iQ software. 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 in the corresponding column of the results table. The calibration curve plotting and calculation of the *L. monocytogenes* DNA concentration are performed automatically in accordance with the *Ct* values.

1. Start the software and open the needed file with data of the analysis in the **Data File** window of the **Workshop** module. Click the **Analyze** button. Change to the **Data Analysis** mode.
2. Select the **Analysis Mode: PCR Base Line Subtracted Curve Fit** (is set by default).
3. Browse the data separately for each channel.
4. For each channel at a time set the threshold line at the specific level in accordance with the Table 3. To do this, click the **Log View** button (switching to logarithmic view). The threshold line is set as 10-20 % of maximum fluorescence obtained in the corresponding channel for the Positive control of extraction (PCE) in the last amplification cycle. For threshold line setting at the specific level drag it with a cursor while pressing the left mouse button.

Table 3

Channel	Threshold line level, % of maximum fluorescence obtained for the C+ sample
FAM	10-20
JOE/HEX	10-20
ROX	10-20

5. In the results grid (the **Quantitation Results** window) one will be able to see the *Ct* values.
6. In order to analyze the results click the **Results** button which is situated under the buttons with the fluorophores' names.

Results interpretation

The result of the PCR analysis is considered reliable only if the results for the controls of the amplification and the 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.

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, or tubes

(0.2 ml) with transparent caps from the eight-pieces-strips (e.g. Axygen, USA) (detection through the cap of the tube).

Programming the thermocycler

1. Turn on the instrument and start the **Bio-Rad CFX Manager** program.
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 the **Create a new Run/Experiment** (or select **New** in the **File** menu and then select **Run.../Experiment...**). Click **OK**.
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:

Table 4

AmpliSens unified amplification program

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
1	50	15 min	-	1
2	95	15 min	-	1
3	95	10 s	-	45
	60	20 s	FAM, JOE, ROX	

Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program. If only the tests for pathogen agent DNA detection are performed in one instrument then the first step of reverse transcription (50 °C – 15 minutes) can be deleted from the program for time saving.

NOTE:

Note – The Cy5 and Cy5.5 channels are enabled when required if the “multiprime” format tests are performed.

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	50,0	C for 15:00	
		Slow Ramp Rate to 2,5	C per second
2	95,0	C for 15:00	
		Slow Ramp Rate to 2,5	C per second
3	95,0	C for 0:10	
		Slow Ramp Rate to 2,5	C per second
4	60,0	C for 0:20	
		+ Plate Read	
		Slow Ramp Rate to 2,5	C per second
5	GOTO 3	, 45	more times
		END	

3. Set **Sample Volume – 25 µl**.

4. Save the protocol: in the **Protocol Editor New** window select **File**, then **Save As**, name the file and click **Save**.
5. 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** fluorophores and click **OK**. In the **Sample type** menu select **Unknown** for all the samples, except for the DNA-calibrators. 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.
Set the calibrators **C1 LIM** and **C2 LIM** for all the channels as **Sample type – Standard**. Enter a concentration in the **Concentration** box according to the *Important Product Information Bulletin* enclosed to the PCR kit, moreover the **Load** function is to be ticked.
6. Save the plate setup: select **File** and then **Save as** in the **Plate Editor New** window. Enter the file name, click **Save**.
7. 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 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.

8. 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**.

Using the ready template for the run

The test parameters and the plate setup set earlier can be used for the further runs. To do this:

- click the **Select Existing...** button in the **Run Setup** window of the **Protocol** tab. Select the needed file with the amplification program in the **Select Protocol** window. Click **Open**.
- go to the **Plate** tab in the **Run Setup** window. Click the **Select Existing...** button. Select the needed file with the plate setup in the **Select Plate** window. Click **Open**. Click the **Edit selected** button to edit the plate setup.

Data analysis

The obtained results are analyzed by the software of the CFX96 instrument. 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 in the corresponding column of the results table. The calibration curve plotting and calculation of the *L.monocytogenes* DNA concentration are performed automatically in accordance with the *Ct* values.

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. The fluorescence curves, the tube order in the plate and the table with the *Ct* values are represented in the **Data Analysis** window of the **Quantification** tab.
3. For each channel at a time set the threshold line at the specific level in accordance with the Table 5. The threshold line is set as **10-20** % of maximum fluorescence obtained in the corresponding channel for the Positive control of extraction (PCE) in the last amplification cycle. For threshold line setting at the specific level drag it with a cursor while pressing the left mouse button (the fluorescence level is equal to the proximate point marked with number).

Table 5

Channel	Threshold line level, % of maximum fluorescence obtained for the PCE sample
FAM	10-20
HEX	10-20
ROX	10-20

4. Click the **View/Edit Plate...** button on the toolbar and set the samples names and calibrators concentrations in the opened window.
5. To generate report of the run in **.pdf** format it is necessary to select the **Tools** on the toolbar, then select **Reports...** Save the generated report: select **File** and then **Save as**, name the file and click **Save**.

Results interpretation

The result of the PCR analysis is considered reliable only if the results for the controls of the amplification and the 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.

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
13.07.23 EM	Footer	REF H-2161-1-1-CE was added