

GUIDELINES

to AmpliSens[®] *Shigella* spp. and *EIEC* / *Salmonella* spp. / *Campylobacter* spp.-FRT PCR kit

for qualitative detection and differentiation of *Shigella* species (*Shigella* spp.) and enteroinvasive *E.coli* (*EIEC*), *Salmonella* species (*Salmonella* spp.), and thermophilic *Campylobacter* species (*Campylobacter* spp.) DNA in environmental samples and biological material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

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INTENDED USE

The guidelines describe the procedure of using **AmpliSens® *Shigella* spp. and *EIEC* / *Salmonella* spp. / *Campylobacter* spp.-FRT** PCR kit for detection and differentiation of DNA of *Shigella* spp., enteroinvasive *E.coli* (*EIEC*), *Salmonella* spp., and thermophilic *Campylobacter* spp. in the biological material and environmental samples by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using the following instruments:

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

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

When working with the 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 terms corresponding to different instruments and software are indicated in the following order: for Rotor-Gene 3000 / for Rotor-Gene 6000.

Pretreat samples and prepare reaction mixtures according to the instruction manual. When working with the Rotor-Gene 3000 and Rotor-Gene 6000 instruments, it is recommended to use 0.2-ml clear tubes with flat caps (detection through the bottom of the tube) or 0.1-ml tubes.

Programming the thermocycler

1. Switch the instrument on.
2. Insert the tubes into the carousel of the Rotor-Gene 3000/6000/Q instrument (the carousel cells are numbered, the numbers are used for the further programming of the samples' position in the thermocycler). Program the instrument.

NOTE: Well No. 1 must be filled with a test tube.

3. Click **New** in the main program menu.
4. Choose the reaction mixture volume and the rotor type:

Reaction volume – 25 µl

Rotor type-36-Well Rotor or **72-Well Rotor** depending on the used rotor type.

5. Set the amplification program by pressing the **Edit profile** button

Amplification program for rotor-type instruments

Step	Temperature, °C	Time	Number of cycles
1	95	15 min	1
2	95	10 s	45
	60	25 s <i>fluorescent signal detection</i>	
	72	10 s	

Detection of fluorescent signal is enabled in FAM/Green and JOE/Yellow channels (detection is enabled in other channels if several tests are carried out simultaneously).

6. Set calibration parameters (activate **Calibrate/Gain Optimisation...** in a new experiment module):
 - Perform fluorescence measurement in FAM/Green and JOE/Yellow channels (activate **Calibrate Acquiring/Optimise Acquiring**);
 - Perform calibration before the first measurement in FAM/Green and JOE/Yellow channels (activate **Perform Calibration Before 1st Acquisition/Perform Optimisation Before 1st Acquisition**);
 - Set channel calibrations: for FAM/Green channel from 5FI to 10FI; for JOE/Yellow channel from 5 FI to 10FI (activate the **Edit...** button, **Auto gain calibration channel settings** window);
7. Select the **Start run** button for amplification run and name the experiment.
8. During or after amplification, it is necessary to program the placement of test samples, negative control of extraction, positive and negative controls of DNA amplification. Enter the data in the table of samples (opens automatically after the thermocycling process starts). Indicate the names/numbers of test samples in the **Name** column. Indicate Positive Control of amplification as **C+**, Negative Control of amplification as **C-**. Set the **Unknown** type next to all test samples, the **Positive Control** type next to the Positive Controls, the **Negative Control** type next to the Negative Control of Extraction, and the **NTC** type next to the Negative Control of amplification. For empty wells, indicate **None**.

Data analysis

1. Activate the button **Analysis** in the menu, select the mode of the analysis **Quantitation**, activate the buttons **Cycling A. JOE/Cycling A. Yellow, Show, Cycling A. FAM/Cycling A. Green, Show**.
2. Cancel the automatic choice of the threshold line level for each of the main open windows (FAM/Green, JOE/Yellow) by activating the **Threshold** button.

3. Activate the **Dynamic tube** and **Slope Correct** buttons in the main window menu (**Quantitation analysis**).
4. In the **CT Calculation** menu (in the right part of window), set **Threshold = 0.05** and **More settings** as **10 %**.
5. In the results grid (**Quant. Results** window), one will be able to see the *Ct* values.
6. *Ct* values for test samples are taken into account only if the results obtained for control samples C-, C+ *Shigella / Salmonella*, C+ *Campylobacter / STI*, and NCA are correct according to instruction manual and *Ct* values specified in the *Important Product Information Bulletin* for the PCR kit.
7. Data for the test samples are analyzed separately for each type of PCR-mix-1 according to instruction manual and *Important Product Information Bulletin* for the PCR kit.

AMPLIFICATION AND DATA ANALYSIS USING iCycler 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 PCR tubes (0.2 ml) with domed caps (e.g. Axygen, USA).

Programming the thermocycler

1. Turn on the instrument and optical module at least 20–30 min before the measurement.
2. Click the **Create new** button in the **Workshop** module.
3. Set the amplification program (**Protocol**).

Table 2

Amplification program for plate-type instruments

Step	Temperature, °C	Time	Number of cycles
1	95	15 min	1
2	95	10 s	45
	60	25 s <i>Fluorescent signal detection</i>	
	72	10 s	

Detection of fluorescent signal is enabled in FAM and JOE/HEX channels (detection is enabled in other channels if several tests are carried out simultaneously).

4. Name and save the new protocol.
5. Set the order of the samples in the plate (**Plate**), select channels for fluorophores FAM and JOE/HEX (**Select/add fluorophores**), activate fluorophores for the samples in the created protocol by the **Fluorophore loading in Whole Plate mode** button. Set **Sample Volume – 25 µl**, **Seal Type – Domed cap**, **Vessel Type – Tubes**. Save the

created protocol by the **Save/Exit Plate Editing** button.

6. Add the amplification reagents and DNA samples to the tubes and place them in the instrument.
7. Start the instrument (**Run**), select **Use Persistent Well Factors**, and save the experiment.

Data analysis

1. Data are analyzed in the channels for the FAM and JOE/HEX fluorophores.
2. Activate the **Data Analysis** button in the menu window.
3. Select a range of 20–25 for **Base Line** in the **Crossing Threshold User Defined** menu. Normally, the threshold line should cross only the sigmoid curves of signal accumulation of positive samples and controls and should not cross curves of other shape. Otherwise, the threshold level should be raised.
4. *Ct* values for the test samples are taken into account only if results obtained for control samples C–, C+*Shigella* / *Salmonella*, C+*Campylobacter* / STI, and NCA are correct according to instruction manual and the *Information Product Important Bulletin* for the PCR kit.
5. Results obtained for the test samples should be interpreted in accordance with the instruction manual and the *Important Product Information Bulletin*.

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 optically transparent domed or flat 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).

Program the instrument according to the *Instruction Manual* provided by the manufacturer.

1. Turn on the instrument and start the **Bio-Rad CFX Manager** program.
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**.

Amplification program for plate-type instruments

Step	Temperature, °C	Time	Number of cycles
1	95	15 min	1
2	95	10 s	45
	60	25 s <i>Fluorescent signal detection</i>	
	72	10 s	

Fluorescent signal is detected in the FAM and HEX channels (if other tests are simultaneously carried out then other channels are enabled).

NOTE! Set **Ramp Rate 2,5 °C/s** 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.

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 indicate the required fluorophores with a checkmark; click **OK**; then indicate with a checkmark the fluorescence signal acquiring for the selected wells in the required channels. Define sample names in the **Sample name** window, confirming the names by the **Load** button.

NOTE! If other tests are simultaneously carried out then other channels are enabled.

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.

Data analysis

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).

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

2. For each channel at a time 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 C+ in the last amplification cycle. Make sure that fluorescence curve of the C+ crosses the threshold line at the zone of exponential growth of fluorescence passing onto linear growth.

3. Click the **View/Edit Plate...** button on the toolbar and enter sample names in the opened window, if required.
4. *Ct* values for test samples are taken into account only if results obtained for control samples C–, C+*Shigella / Salmonella*, C+*Campylobacter / STI*, and NCA are correct in accordance with instruction manual and the *Information Product Important Bulletin* for the PCR kit.
5. Data for the test samples are analyzed separately for each type of PCR-mix-1 according to instruction manual and *Important Product Information Bulletin* for the PCR kit.
6. Click **Tools** on the toolbar, then **Reports...**, and then save the generated report.