GUIDELINES

to **AmpliSens® Escherichioses-FRT** PCR kit

for qualitative detection and differentiation of diarrheagenic

E.coli DNA in environmental samples and clinical material

by the polymerase chain reaction (PCR) with real-time

hybridization-fluorescence detection

AmpliSens®

Not for use in the Russian Federation

TABLE OF CONTENTS

INTENDED USE

The guidelines describe the procedure of using **AmpliSens® Escherichioses-FRT** PCR kit for qualitative detection and differentiation of DNA of different groups of diarrheagenic *E.coli* – enteropathogenic (*EPEC),* enterotoxigenic *(ETEC),* enteroinvasive *(EIEC* (in conjunction with *Shigella* spp.)*),* enterohemorrhagic *(EHEC)*, and enteroaggregative (*EAgEC*), – in environmental samples and clinical material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using the following instruments:

- Rotor-Gene 3000 (four channels), Rotor-Gene 6000 (six channels) (Corbett Research, Australia),
- Rotor-Gene Q (QIAGEN, Germany),
- iCycler iQ5 (Bio-Rad, USA),
- Mx3000P (Stratagene, USA)
- CFX96 (Bio-Rad, USA).

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 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.

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 instrument

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 from the current experiment. If tubes with different types of reaction mixtures are placed in the thermocycler simultaneously, tube with the **PCR-mix-1-FEP/FRT *EIEC / EHEC /* STI** should be placed in the first well.

3. Press the ***New*** button in the main program menu.

4. Select the reaction mixture volume and the rotor type:

Reaction volume – 25 µl

Rotor type – 36-Well Rotor or ***72-Well Rotor*** according to the rotor type.

5. Set the amplification program for the rotor-type instrument (see Table 1). To do this, press the ***Edit profile*** button.

Table 1

The 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 *fluorescent signal detection*	
	72	10 s	

Fluorescent signal is detected in the channels for the FAM, JOE, and ROX fluorophores (if several tests are performed simultaneously, detection in other channels is enabled).

6. Set calibration parameters (activate ***Calibrate/Gain Optimization…*** in a new experiment module):

   – perform the fluorescence detection in FAM/Green, JOE/Yellow, ROX/Orange channels (activate ***Calibrate Acquiring/Optimise Acquiring***);

   – perform the calibration before the first measurement (activate ***Perform Calibration Before 1st Acquisition/Perform Optimization Before 1st Acquisition***);

   – activate ***Edit…, Auto gain calibration channel settings*** window. Set channel calibrations: FAM/Green, from **3Fl** to **7Fl**; JOE/Yellow, from **3Fl** to **7Fl**; ROX/Orange from **5 Fl** до **10Fl**.

7. Start the amplification program by activating the ***Start Run*** button*,* and name the experiment.

8. During the work of the amplifier or after it has stopped working it is necessary to program the position of the test samples, the Negative Control of extraction, the Positive and Negative Controls of Amplification.

To do this, it is necessary to 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 **Name** column. The positive Control of amplification should be indicated as **C+;** the Negative Control of Amplification, as **NCA**. Set the **Unknown** type next to all tested 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. Set the type **None** for the cells matching with the corresponding empty tubes.

Data analysis

1. Press the **Analysis** button in the menu, then select the **Quantitation** analysis mode and press the **Cycling A. FAM/Cycling A. Green, Show, Cycling A. JOE/Cycling A. Yellow**, **Show** or **Cycling A. ROX/Cycling A. Orange, Show** buttons.

2. Cancel the automatic choice of the threshold line level for each of the main open windows (FAM/Green, JOE/Yellow и ROX/Orange) by activating the **Threshold** button.

3. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of each main window (**Quantitation analysis**) and set **More settings** as **10 %**.

4. In the **CT Calculation** menu (in the right part of the window), set **Threshold = 0.05**.

5. Data analysis for each type of reaction mixtures rotor is carried out separately if they were loadied in the rotor simultaneously.

6. In the results grid (the **Quant. Results** window), *Ct* values for each tested sample will appear. The *Ct* values for test samples are taken into account only if the results obtained for control samples C−, C+, and NCA are correct according to boundary *Ct* values specified in the *Important Product Information Bulletin* for the PCR kit.

7. *Ct* values for positive controls of amplification (C+) in appropriate channels should not exceed boundary *Ct* values specified in the *Important Product Information Bulletin* for the PCR kit.

8. *Ct* values should be absent for the Negative Control of extraction (C−) (except for results in the FAM channel when using PCR-mix-1-FEP/FRT *EIEC / EHEC / STI*).

9. Data for the tested samples are analyzed according to the *Istruction manual* and boundary *Ct* values specified in the *Important Product Information Bulletin* for the PCR kit. **Samples are considered positive** if their *Ct* values do not exceed the boundary *Ct* value specified in the *Important Product Information Bulletin* for the PCR kit.

AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ5 (Bio-Rad, USA) INSTRUMENT

Carry out the sample pretreatment and reaction mixture preparation stages according to the PCR kit *Instruction Manual*. When working with the iCycler iQ5 instrument, it is recommended to use 0.2-ml disposable polypropylene tubes for PCR with domed caps (for example, Axygen, USA).

Programming the instrument

1. Switch on the instrument and optical module for at least 20–30 min before measurement.
2. Press the **Create new** button in the **Workshop** module to enter the mode of creating a new amplification protocol.
3. Set the amplification program (**Protocol**):

Table 2

The 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 *fluorescent signal detection*	
	72	10 s	

Name a new protocol and save it.

4. Set the order of tubes in the **Plate**. Select fluorophores (**Select/add fluorophores**) and press **Fluorophore loading in Whole Plate mode** to activate fluorophores for the samples in the created protocol. Set **Sample Volume** (**25 µl), Seal Type** – **Domed Cap**, **Vessel Type** – **Tubes,** and press **Save/Exit Plate Editing** to save the created protocol.
5. Add reagents for amplification and DNA samples into the tubes and place the latter into the instrument.
6. Press the **Run** button. In the opened window, set **Use Persistent Well Factors,** press the **Begin Run** button, and save the experiment.

Data analysis

1. Data are analyzed in FAM, HEX and ROX channels.
2. Press the **Data Analysis** button in the menu to start the data analysis.
3. Select **20 – 25** for **Base line** in **Crossing Threshold User Defined**. Normally, the

threshold line should cross only sigmoid curves of signal accumulation of positive samples and controls and should not cross the curves of different shapes. Otherwise, the threshold level should be raised.

4. *Ct* values for tested samples are taken into account only if the results obtained for control samples C–, C+, and NCA are correct (see the *Important Product Information Bulletin* for the PCR kit).

5. *Ct* values obtained for positive controls (C+) in appropriate channels should not exceed boundary *Ct* values specified in the *Information Product Important Bulletin* enclosed in the PCR kit.

6. *Ct* values should be absent for the Negative Control of extraction (C–) (except for the result in the FAM channel when using PCR-mix-1-FEP/FRT *EIEC / EHEC / STI*).

7. The results of testing of the clinical samples are analyzed according to the *Instruction manual* and boundary *Ct* values listed in the *Important Product Information Bulletin* for the PCR kit. **Samples are considered positive** if their *Ct* values do not exceed the boundary *Ct* values specified in the *Important Product Information Bulletin* for the PCR kit.

AMPLIFICATION AND DATA ANALYSIS USING Mx3000P (Stratagene, USA)

Carry out the sample pretreatment and reaction mixture preparation stages according to the PCR kit *Instruction Manual*. When working with the Mx3000P instrument, it is recommended to use 0.2-ml polypropylene disposable tubes for PCR with domed caps (for example, Axygen, USA).

1. Switch on the instrument. Run the software Stratagene Mx3000P.

2. Select **Quantitative PCR (Multiple Standards)** and **Turn lamp on for warm-up** in the **New Experiment Options** window.

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

3. Insert the tubes into the instrument, and close the fixer and the door of the instrument.

4. Select **Optics Configuration** in **Options** menu and, in the **Dye Assignment** tab, set parameter **FAM** next to the **FAM filter set** item, parameter **JOE** next to the **HEX/JOE filter set** and parameter **ROX**, next to the **ROX filter set**.

5. Set fluorescence detection parameters in the **Plate Setup** menu. To do this:
   – Select all wells with tested tubes or strips;
   – Mark all selected wells as **Unknown** in the **Well type** window. Select **FAM**, **JOE** and **ROX** fluorophores for the **Collect fluorescence data** option.

6. Name each sample in the **Well Information** window.

7. Set amplification program in **Thermal Profile Setup** tab for the plate-type instrument (see the *Instruction manual* for the PCR kit). To do this:
   A. Press the **Import…** button. Navigate to the folder containing the previous experimental file and open it. The required thermocycling profile will appear in the **Thermal Profile** window;
   B. Set the appropriate amplification program in **Thermal Profile Setup** menu.

Table 3

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 fluorescent signal detection	
	72	10 s	

NOTE: If the given PCR kit is used with the kit for setting the parameter of the fluorescence signal detection at the set temperature it is necessary to select the **All points** option for the **Data collection marker for dragging** parameter

and drag it with a mouse from the right part

8. Select the **Run** button, then the **Start** button, and name the file.

Data analysis.

1. Select **Analysis** by clicking the corresponding button of the tool bar.

2. The **Analysis Selection/Setup** tab will open. Make sure that all the test samples are active (the cells corresponding to the samples should be of a different colour).

3. Select the **Results** tab.

4. Make sure that the automatic selection of the threshold level for each channel is correct. Normally, the threshold line should cross only the sigmoid curves of signal accumulation of positive samples and controls and should not cross the baseline. Otherwise, the threshold level should be raised. To do this, activate each channel image separately in the lower **Dyes shown** panel. Check the threshold line location and change it, if necessary.

5. *Ct* values for tested samples are taken into account only if the results obtained for control samples C–, C+, and NCA are correct (see the *Instruction manual* and the *Important Product Information Bulletin* for the PCR kit).

6. *Ct* values obtained for positive controls (C+) in appropriate channels should not exceed boundary *Ct* values specified in the *Information Product Important Bulletin* enclosed in the PCR kit.

7. *Ct* values should be absent for the Negative Control of extraction (C–) (except for the result in the FAM channel when using PCR-mix-1-FEP/FRT *EIEC / EHEC / STI*).

8. The results of testing of the clinical samples are analyzed according to the *Instruction manual* and boundary *Ct* values listed in the *Important Product Information Bulletin* for the PCR kit. **Samples are considered positive** if their *Ct* values do not exceed the boundary *Ct* values specified in the *Important Product Information Bulletin* for 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 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. Switch 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.***

Table 3

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 *fluorescent signal detection*	
	72	10 s	

Fluorescent signal is detected in the FAM, HEX and ROX 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.

<u>**Data analysis**</u>

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 tested samples are taken into account only if the results obtained for control samples C–, C+, and NCA are correct in accordance with instruction manual and *Information Product Important Bulletin*.

5. The results of testing the test samples are analyzed according to the instruction manual and *Important Product Information Bulletin* for the PCR kit. **Sample is considered positive** if the *Ct* value does not exceed the boundary *Ct* value specified in the *Important Product Information Bulletin* for the PCR kit.

6. The *Ct* value should be absent in the Negative Control of Extraction (C-) (except for C– in FAM channel for **PCR-mix-1-FEP/FRT** *EIEC / EHEC / STI*).

7. Click **Tools** on the toolbar, then **Reports...**, and then save the generated report.

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
09.09.11 RT	Tables 1, 2, 3 (amplification program)	Cycles 2 and 3 are combined into one cycle (cycle 2)
01.04.14 SA	Cover page	Address of European representative was added
24.10.14 PM	Text	Corrections according the template and the Russian guidelines
	Intended use	CFX96 (Bio-Rad, USA) instrument was added
	Amplification and data analysis using CFX96 (Bio-Rad, USA)	The section was added
29.12.20 KK	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 EM	Front page	The name, address and contact information for Authorized representative in the European Community was changed