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GUIDELINES

to AmpliSens® All-screen-FRT PCR kit

for qualitative detection and differentiation of RNA/DNA of *Shigella* spp., enteroinvasive *E.coli* (*EIEC*), *Salmonella* spp., thermophilic *Campylobacter* spp., group F *Adenoviruses* and group A *Rotaviruses*, *Norovirus* genotype 2, and *Astroviruses* in the biological material and environmental samples by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

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INTENDED USE

Guidelines describe the procedure of the use of **AmpliSens® All-screen-FRT** PCR kit for detection and differentiation of RNA/DNA of *Shigella* spp.*,* enteroinvasive *E.coli* (*EIEC*), *Salmonella* spp., thermophilic *Campylobacter* spp., group F *Adenoviruses* and group A *Rotaviruses*, *Norovirus* genotype 2, and *Astroviruses* 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) 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 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 transparent 0.2-ml PCR tubes with flat caps (detection through the bottom of the tube) or 0.1-ml tubes.

Programming the thermocycler

1. Switch on the instrument.
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 loaded with a test tube. If the tubes containing different PCR-mixes are placed in the rotor simultaneously, the first two wells should be loaded with the tubes containing PCR-mix-1-FEP/FRT *Shigella* spp. / *Salmonella* spp.

3. Click the ***New*** button in the program main menu.
4. Select the reaction volume and the rotor-type:

   Reaction volume is **25 µl**;

   Rotor type is ***36-Well Rotor*** or ***72-Well Rotor***.

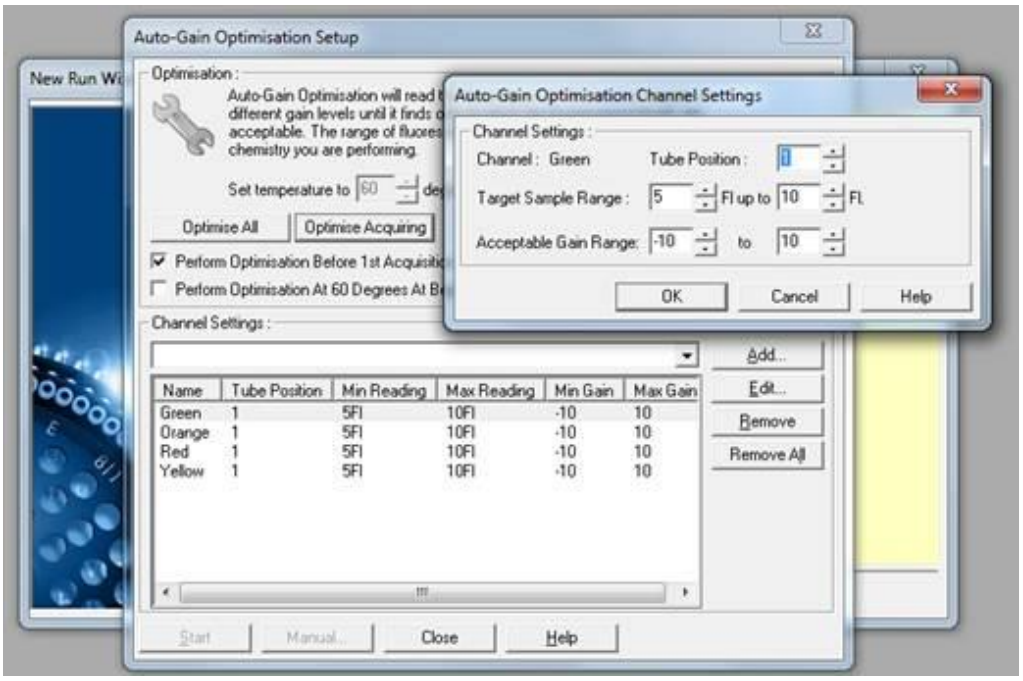
5. Set the amplification program. To do this, click the ***Edit profile*** button.

Amplification program for rotor-type instruments

Step	*Temperature, °C*	*Time*	*Cycles*
1	**50**	30 min	1
2	**95**	15 min	1
3	**95**	10 s	45
	60	25 s Fluorescent signal detection	
	72	10 s	

Detection of fluorescent signal is enabled in the FAM/Green and JOE/Yellow channels (if different tests are carried out simultaneously in the same run, other channels can be used as well).

6. Set calibration parameters (activate ***Calibrate/Gain Optimisation…*** in a new experiment module):
   - perform the fluorescence detection in FAM/Green and JOE/Yellow channels (activate ***Calibrate Acquiring/Optimise Acquiring*** button);
   - perform calibration before the first detection in FAM/Green and JOE/Yellow channels (activate ***Perform Calibration Before 1st Acquisition/Perform Optimisation Before 1st Acquisition***);
   - set calibration for FAM/Green channel from **5Fl** to **10Fl**, specify the number of tube with PCR-mix-1-FEP/FRT *Shigella* spp. / *Salmonella* spp. in ***Tube Position*** (activate the ***Edit…*** button, ***Auto gain calibration channel settings*** window);
   - set calibration for JOE/Yellow channel from **5Fl** to **10Fl**, specify the number of tube with PCR-mix-1-FEP/FRT *Rotavirus* / *Astrovirus* in ***Tube Position*** (activate the ***Edit…*** button, ***Auto gain calibration channel settings*** window).



7. Start the amplification program by activating the ***Start Run*** button***,*** and name the experiment.
8. During or after amplification, it is necessary to program the order of test samples, negative control of extraction, positive and negative controls of amplification. Enter the data in the grid of samples (it opens automatically after the amplification has been started). Indicate the names/numbers of tested samples in the ***Name*** column. Indicate Positive Control of amplification as ***C+***, Negative Control of amplification as ***NCA***. 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*** and ***Cycling A. FAM/Cycling A. Green***, ***Show***.
2. Cancel the ***Threshold*** automatic choice for each opened main window (FAM/Green and JOE/Yellow).
3. Activate the ***Dynamic tube*** and ***Slope Correct*** in the menu of each main window (***Quantitation analysis***). Set ***More settings*** as ***5 – 10 %*** (for the FAM/Green channel) and ***10 %*** (for the JOE/Yellow channel).
4. In the ***CT Calculation*** menu, set ***Threshold = 0.05.***
5. In case the tubes containing different types of reaction mixture are loaded in the rotor simultaneously, amplification data are analyzed separately for each type of the reaction mixture.
6. In the results grid (***Quant. Results*** window), *Ct* values for each test sample will appear. *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 Instruction Manual and *Ct* values specified in the *Important Product Information Bulletin* for the PCR kit.
7. Data for the test samples are analyzed according to Instruction Manual and *Ct* values specified in *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 transparent 0.2-ml domed-cap PCR tubes (detection through the cap of the tube).

Programming the instrument

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

Amplification program for plate-type instruments

Step	***Temperature, °C***	***Time***	***Cycles***
1	**50**	30 min	1
2	**95**	15 min	1
3	**95**	10 s	45
	60	25 s Fluorescent signal detection	
	72	10 s	

Detection of fluorescent signal is enabled in the FAM and JOE/HEX channels (if different tests are carried out simultaneously in the same run, other channels can be used as well).

4. Set the order of 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 pressing the ***Save/Exit Plate Editing*** button.
5. Place the tubes in the instrument strictly in accordance with the created plate setup.
6. Press the ***Run*** button, select ***Run Persistent Plate*** and save the experiment.

Data analysis

1. Data are analyzed in the FAM and JOE/HEX channels.
2. Activate the ***Data Analysis*** button in the menu window.
3. Select the ***Base Line*** in the range of **20-25** in the ***Crossing Threshold User Defined*** menu. Normally, the threshold line should cross only sigmoid curves of signal accumulation of positive samples and controls and should not cross curves of other form. Otherwise, the threshold level should be raised.
4. *Ct* values for test samples are taken into account only if results obtained for control samples C–, C+, and NCA are correct according to the 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 (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. Set ***Sample Volume* – 25 µl.**

Amplification program for plate-type instruments

Step	***Temperature, °C***	***Time***	***Cycles***
1	**50**	30 min	1
2	**95**	15 min	1
3	**95**	10 s	45
	60	25 s Fluorescent signal detection	
	72	10 s	

Detection of fluorescent signal is enabled in the FAM and HEX channels (if different tests are carried out simultaneously in the same run, other channels can be used as well).

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 FAM and HEX 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.
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+ samples 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+, and NCA are correct according to the Instruction Manual and the *Information Product Important Bulletin* for the PCR kit. The sample is considered to be positive, the *Ct* values for the sample does not exceed the *Ct* value specified in the *Important Product Information Bulletin.*
5. Results obtained for the test samples should be interpreted in accordance with the Instruction Manual and the *Important Product Information Bulletin*.
6. Click ***Tools*** on the toolbar, then ***Reports…***, and then save the generated report.

TROUBLESHOOTING

If *Ct* value was defined in the results grid for the sample, but the form of fluorescence curve for this channel was incorrect without the typical exponential growth phase (the curve is linear), the result is incorrect. Such a result should not be considered as positive. Such a result may suggest incorrect setting of the threshold line (or other settings). Once the threshold line (and other settings) has been set correctly, PCR analysis of this sample should be repeated starting from the RNA extraction.

If *Ct* value was defined in the results grid for the Negative Control, but the form of fluorescence curve for this channel was incorrect without the typical exponential growth phase (the curve is linear), the result is incorrect. Such a result should not be considered as negative.