

GUIDELINES

to AmpliSens[®] *Poliovirus*-FRT PCR kit

for qualitative detection of *Poliovirus* and *Enterovirus* group C (*HEV-C*) RNA with *Poliovirus* differentiation to strains (Sabin 1, Sabin 2, Sabin 3) 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® Poliovirus-FRT** PCR kit for qualitative detection of *Poliovirus* and *Enterovirus* group C (*HEV-C*) RNA with *Poliovirus* differentiation to strains (*Sabin 1*, *Sabin 2*, *Sabin 3*) in environmental samples and biological material 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);
- iCycler iQ, iCycler iQ5 (Bio-Rad, USA);
- CFX96 (Bio-Rad, USA).

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) INSTRUMENTS

When working with the Rotor-Gene 3000 instrument, use the Rotor-Gene version 6 software. When working with the Rotor-Gene 6000 instrument, use the Rotor-Gene 6000 versions 1.7 (build 67) software or higher.

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 0.2-ml clear tubes with flat caps (detection through the bottom of the tube) or 0.1-ml tubes.

Programming the thermocycler

1. Turn on the instrument.
2. Insert the tubes into the rotor of the Rotor-Gene 3000/6000/Q instrument (the rotor wells are numbered, these numbers are used for further programming of the samples' order in the thermocycler).

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

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

3. In the opened window, select **36-Well Rotor** (or **72-Well Rotor**) and tick the **No Domed 0.2 ml Tubes/ Locking ring attached** option. Click the **Next** button.
4. In the opened window enter the operator name, select the reaction volume: **Reaction volume – 25 µl**. Tick the **15 µl oil layer volume** option. Click the **Next** button.
5. Set the amplification program for rotor-type instruments. To do this, press the **Edit profile** button.

AmpliSens unified amplification program

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
Hold	95	15 min	–	1
Cycling	95	10 s	–	45
	54	20 s	FAM/Green, JOE/Yellow, ROX/Orange	
	72	10 s	–	

6. Set calibration parameters (activate **Calibrate/Gain Optimisation...** in a new experiment module):
 - Perform calibration before the first measurement in FAM/Green and JOE/Yellow channels (activate **Perform Calibration Before 1st Acquisition/Perform Optimisation Before 1st Acquisition**);
 - Measure fluorescence in FAM/Green, JOE/Yellow and ROX/Orange channels. To do this, select channel in the **Channel setting** window and press the **Add** button. Set channel calibrations in **Auto gain calibration channel settings** window (FAM/Green, JOE/Yellow and ROX/Orange – from 5FI to 10FI) and press the **OK** button;
 - Channel calibrations can be changed by pressing the **Edit...** button. Press the **Close** button.
7. Press the **Next** button. Then select the **Start run** button for amplification run and name the experiment.
8. Enter the data into the grid of the samples (it opens automatically after the amplification has been started). Enter the names/numbers of test samples in the **Name** column. Define the Positive Control of Amplification as **C+**; the Negative Control of Amplification as **NCA**. Set the type **Unknown** opposite all the test samples, the type **Positive Control** for the positive controls, the type **Negative Control** for to the Negative Control of Extraction, and the **NTC** type for the Negative Control of Amplification. Set the type **None** for the cells matching with the corresponding empty tubes.

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 Ct (threshold cycle) value of the DNA sample in the corresponding column of the results table.

1. Activate the button **Analysis** in the menu, select the mode of the analysis **Quantitation** and activate the **Cycling A. JOE/Cycling A. Yellow, Show, Cycling A. FAM/Cycling A. Green, Show**, and **Cycling A. ROX/Cycling A. Orange, Show** buttons.
2. Cancel the automatic choice of the threshold line level **Threshold** for each opened main window (FAM/Green, JOE/Yellow and ROX/Orange).
3. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of main window (**Quantitation analysis**).
4. In the **CT Calculation** menu (in the right part of window), set **Threshold = 0.05** and **More settings** as **10 %** for each main window.
5. In the results grid (the **Quantitation Results** window) one will be able to see the Ct values.

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. The samples where Ct values do not exceed the threshold cycle according with the *Important Product Information Bulletin*, are considered positive. If the Ct value of the sample exceeds the value of the threshold cycle, the result is considered doubtful, it is necessary to conduct an additional test of the cDNA sample twice.

Possible problems and special aspects of results analysis with the use of Rotor-Gene 3000/6000/Q software

Possible problems	Reason	Characters	Ways to eliminate
Negative samples are analyzed as positive by Rotor-Gene software	Incorrect mathematical processing of negative samples in the presence of the fluorescence decrease section at the initial cycles (fig.)	A typical positive sample has a characteristic S-shaped curve of fluorescence accumulation (fig.). Incorrectly processed negative samples are viewed as quite straight bottom-up lines (fig.)	One must use the <i>Ignore First</i> by selecting the value 5 cycles. If it does not lead to a proper result, try to increase this value by 1 - 5
	The threshold line crosses descending fluorescence curves at the initial cycles (see figure 7)	The red threshold line crosses (or touches) fluorescence curves at the left (initial cycles) on the chart of processed fluorescence curves (fig.)	Use the <i>Eliminate cycles before ...</i> option by setting 5 (crossing the threshold and the fluorescence curve at the first 5 cycles is ignored)
Sensitivity decrease due to impurity of the instrument lenses	Impurity of lenses leads to reduction of effectiveness of fluorescence excitation and detection. It primarily affects to samples with small quantity of specific DNA, which show low fluorescence growth	Low values of the background signal in all 4 detection channels (<1) at the maximum value of the gain parameter (10)	Clean the horizontal and vertical lenses of the instrument by a dry disposable cotton disc at least once a month
The absence of the fluorescence curves when the fluorescence values are less than 1 or more than 100	The autocalibration parameter from 4FI to 8FI is not set or it is a failure of the first tube in the rotor (tube is absent, DNA sample or reaction mixture is added incorrectly)	Most of fluorescence background signals are less than 1 or greater than 20	Repeat the PCR

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 0.2-ml disposable tubes for PCR with domed caps (for example, Axygen, USA).

Programming the thermocycler

1. Turn on the instrument and optical module at least 20–30 min before measurement.
2. Set the amplification program (**Protocol**) for plate-type instruments.

AmpliSens unified amplification program

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
Hold	95	15 min	–	1
Cycling	95	10 s	–	45
	54	20 s	FAM, JOE/HEX, ROX	
	72	10 s	–	

3. Name the new protocol and save it.
4. Create a new plate of samples **Plate Setup**. Set the order of the tubes in the plate.
5. In opened window, set **Unknown** type for all test samples. Set the fluorescence measurement in three channels (FAM, HEX and ROX) for all samples.
6. Set **Sample Volume – 25 µl**, **Seal Type**, **Vessel Type**. Perform amplification using the same type of plastics as plastics used for calibration. Save the scheme of the plate.
7. Add reagents for amplification and cDNA samples into the tubes and place the latter into 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.

8. Press the **Run** button. In the opened window, set **Use Persistent Well Factors**, press the **Begin Run** button, and save the experiment.

Data analysis

The obtained results are analyzed by the iCycler iQ5 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.

1. Data are analyzed in FAM, JOE/HEX and ROX channels.

2. Start the software and open the needed file with data of the analysis in the **Data File** window of the **Workshop** module. Pass to the **Data Analysis** mode.
3. Check the correctness of threshold line automatic choice for each channel. The threshold line should cross only with S-shaped (sigmoid) curves of signal accumulation of positive samples and should not cross the baseline. If it happens, it is necessary to set the threshold line level for each channel manually. To do this, select **Log View** and set the threshold with the left mouse button at a level where fluorescence curves are linear and do not cross the curves of negative samples.
4. 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. The samples where *Ct* values do not exceed the threshold cycle according with the *Important Product Information Bulletin*, are considered positive. If the *Ct* value of the sample exceeds the value of the threshold cycle, the result is considered doubtful, it is necessary to conduct an additional test of the cDNA sample twice.

Possible problems and special aspects of results analysis with the use of iCycler iQ5 software

Possible problems	Characters	Ways to eliminate
The threshold level is set incorrectly	The threshold line passes with the negative samples or above some or all positive curves (herewith, curves are S-shaped in a linear scale)	Set the threshold line at the level of 10-20 % of maximum fluorescence obtained for the Positive control in the last amplification cycle (in logarithmic scale)
The drops are not sedimented from the walls of the tubes before the run	The appearance of negative or positive "steps" in the curves of the fluorescence accumulation (fig.)	Open the BaseLine Threshold window by clicking the right mouse button on the fluorescence curve. Set the range for calculation of the base line beginning from the first cycle after the step.

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 software.
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. Set **Sample Volume – 25 µl**.

AmpliSens unified amplification program

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
Hold	95	15 min	–	1
Cycling	95	10 s	–	45
	54	20 s	FAM, HEX, ROX	
	72	10 s	–	

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	95,0 C for 15:00	Slow Ramp Rate to 2,5 C per second
2	95,0 C for 0:10	Slow Ramp Rate to 2,5 C per second
3	54,0 C for 0:20	+ Plate Read Slow Ramp Rate to 2,5 C per second
4	72,0 C for 0:10	Slow Ramp Rate to 2,5 C per second
5	GOTO 2, 44 more times	
END		

3. Save the protocol: in the **Protocol Editor New** window select **File**, then **Save As**, name the file and click **Save**.
4. 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. 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.

5. Save the plate setup: select **File** and then **Save as** in the **Plate Editor New** window. Enter the file name, click **Save**.
6. 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.

7. 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.

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.

Check the correctness of threshold line automatic choice for each channel. The

threshold line is to cross only with S-shaped (sigmoid) curves describing the accumulation of the signal detecting positive samples and controls. The threshold line is not to cross the base line. If it happens, it is necessary to set the threshold line level for each channel manually. To do this, tick the **Log Scale** item (logarithmic scale selection) and set (with the left mouse button) the threshold line at the level where the fluorescence curves have a linear character and do not cross with the curves of the negative samples. As a rule, the threshold line is set at the level of 10-20 % of maximum fluorescence obtained for the Positive control in the last amplification cycle. Make sure that the fluorescence curve of the Positive control has the typical exponential growth of fluorescence. To select the curve of C+ sample (or another one appropriate sample), set the cursor to the plate setup or to the results grid.

3. Click the **View/Edit Plate...** button on the toolbar and set the samples names in the opened window.

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. The samples where *Ct* values do not exceed the threshold cycle according with the *Important Product Information Bulletin*, are considered positive. If the *Ct* value of the sample exceeds the value of the threshold cycle, the result is considered doubtful, it is necessary to conduct an additional test of the cDNA sample twice.

Possible problems and special aspects of results analysis with the use of CFX96 for research software

Possible problems	Characters	Ways to eliminate
The threshold level is set incorrectly	The threshold line passes with the negative samples or above some or all positive curves (herewith, curves are S-shaped in a linear scale)	Set the threshold line at the level of 10-20 % of maximum fluorescence obtained for the Positive control in the last amplification cycle (in logarithmic scale)
The drops are not sedimented from the walls of the tubes before the run	The appearance of negative or positive “steps” in the curves of the fluorescence accumulation (fig.)	Repeat the amplification for the sample