

GUIDELINES

to AmpliSens[®] *Enterovirus 71-FRT* PCR kit

for qualitative detection of RNA of *Enterovirus* type 71 in the biological material (cerebrospinal fluid, fecal samples) and natural environments (concentrated water samples) by the 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® Enterovirus 71-FRT** PCR kit for qualitative detection of RNA of *Enterovirus* type 71 in the biological material and natural environments 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);
- iCycler iQ, iCycler iQ5 (Bio-Rad, USA);
- CFX96 (Bio-Rad, USA);
- Mx3000P, Mx3005P (Stratagene, 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 6 Version 6.1. software or higher 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 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 thermocycler

1. Switch the instrument on.
2. Insert the tubes into the carousel of the Rotor-Gene 3000/6000/Q instrument. First tube should be placed in first well. Insert the carousel into the instrument, close the cap (the carousel cells are numbered, the numbers are used for the further programming of the samples' position in the thermocycler).

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

3. Click **New** button in the main program menu.
4. In the opened window select **Advanced** menu and **Dual Labeled Probe/Hydrolysis probes (TaqMan)**. Click the **New** button.
5. In the opened window select **36-Well Rotor** (or **72-Well Rotor**) and **No Domed Tubes**

(for the **Rotor-Gene 3000** instrument) or **Locking ring attached** (for **Rotor-Gene 6000** instrument). Click the **Next** button.

6. In the opened window select the operator and set the **Reaction volume** as **25 µl**. Set the checkmark opposite **15 µl oil layer volume**. Click **Next**.
7. In the opened window click the **Edit profile** button and set the temperature profile of the experiment as follows (see Table 1):

Table 1

The amplification program for rotor-type instruments

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/Green, JOE/Yellow	

NOTE: Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program.

Note – When several tests are performed simultaneously the detection in all used channels is enabled.

8. Click **OK**.
9. Select the **Calibrate/Gain Optimisation** button in the **New Run Wizard** window:
 - perform the fluorescence detection in FAM/Green and JOE/Yellow channels (activate the **Calibrate Acquiring/Optimise Acquiring** button);
 - perform the calibration in FAM/Green, JOE/Yellow channels before the first detection (activate the **Perform Calibration Before 1st Acquisition/ Perform Optimisation Before 1st Acquisition** button);
 - to set channels calibration, indicate **5** in the **Min Reading** box and **10** in the **Max Reading** box (activate **Edit...**, the window **Auto gain calibration channel settings**). Click **Close**.
10. Click **Next**. Start the amplification program by activating the **Start Run** button.
11. Name the experiment and save it to the disk (results of the run will be automatically saved in this file).
12. 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 box **Name**. Specify the negative control of PCR as C–, positive control of PCR as C+. Set the type **Unknown** opposite all the test samples, the type **Positive control** – for positive controls, the type **Negative control** – for the negative control of the extraction, and the type **NTC** – for the negative control of PCR. Set the type **None** for the cells matching

with the corresponding empty tubes.

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

Data analysis:

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** and **Cycling A. JOE/Cycling A. Yellow, 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. Select **Linear scale**.
4. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of each main window (**Quantitation analysis**).
5. In the **CT Calculation** menu (in the right part of the window) indicate the threshold line level **0.05** 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 **Quant. Results** window) one will be able to see the *Ct* values.
8. The *Ct* values for the test samples are to be registered if the results for the control samples (NCA, C– and C+) are satisfying (in accordance to the *Instruction Manual* and *Important Product Information Bulletin*).
9. The interpretation of the test's results for the test samples is carried out in accordance to the *Important Product Information Bulletin* for the PCR kit. The samples are considered positive if their *Ct* value does not exceed the value of the threshold cycle specified in the *Important Product Information Bulletin*.

AMPLIFICATION AND DATA ANALYSIS WITH THE USE OF iCycler iQ and 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 (0.1 ml) with transparent caps from the eight-pieces-strips (e.g. Axygen, USA) (detection through the cap of the tube).

1. Switch the instrument on and the power supply unit of the optical part of the instrument.

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

2. Start the program iCycler iQ/iQ5.

3. Insert the tubes or strips into the reaction module of the amplifier and program 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 strips upside down while inserting them into the instrument.

Program the thermocycler only according to the *Instruction Manual* given by the manufacturer of the instrument:

1. Set the plate setup (set the order of the tubes in the reaction chamber and the detection of fluorescent signal).
 - For **iCycler iQ5** click the **Create New** or **Edit** buttons in the **Selected Plate Setup** window of the **Workshop** module. One can edit the plate setup in the **Whole Plate loading** mode. 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**. Select the fluorescent signal detection through the FAM and JOE/HEX channels. Save the set plate setup by clicking the **Save&Exit Plate Editing** button.
 - For **iCycler iQ** select the setup of the samples' position in the reaction module by choosing the **Samples: Whole Plate Loading** option in the **Edit Plate Setup** window of the **Workshop** module. Name each sample in the **Sample Identifier** window. Set the fluorescence signal detection for all the tubes on **FAM, JOE** channels in **Select and load Fluorophores** option. Save the plate setup by naming the file in the **Plate Setup Filename** window (with .pts filename suffix) and clicking the **Save this plate setup** button (in the upper part of the screen). One can edit the plate setup which was used before. To do this, choose **View Plate Setup** in the **Library** window, select the needed setup in **Plate Setup** (the file with .pts filename suffix) and click the **Edit** button to the right. It is necessary to save the edited file before using. Set the using of the given plate setup by clicking the **Run with selected protocol** button.
2. Set all the test samples as **Unknown**, positive controls as «+», and negative controls as «-».
3. Set the amplification program (see 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/HEX	

NOTE: Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program.

Note – When several tests are performed simultaneously the detection in all used channels is enabled.

- for **iCycler iQ5** in the **Selected Protocol** window of the **Workshop** module click the **Create New** or **Edit** buttons. Set the amplification parameters and save the protocol by activating the **Save&Exit Protocol Editing** button. Later, for further runs one may select the file containing this program in the **Protocol** box (the protocol files are saved in the **Users** folder on default);
- for **iCycler iQ** select **Edit Protocol** option of the **Workshop** module. To do this set the amplification program in bottom window and select **Cycle 2 – Step 2** in right window. Save the protocol by naming the file in the **Protocol Filename** window (the file with .tmo filename suffix) and clicking the **Save this protocol** button (in the upper part of the screen). For further runs one may select the file with this program in **View Protocol** tab of **Library** module. After selection or editing of necessary program set the use of this program by clicking **Run with selected plate setup** button.

4. Before starting the program execution:

- for **iCycler iQ5** it is **obligatory** to check if the selected protocol (**Selected Protocol**) and the plate scheme (**Selected Plate Setup**) are correct. Start the instrument (**Run**), select **Collect Well Factors from Experimental Plate**. Click **Begin Run** and name the experiment (the results of this experiment will be automatically saved in this file). Click **OK**;
- for **iCycler iQ** it is **obligatory** to check if the selected protocol name and the plate scheme are correct (**Run Prep** window). Select **Experimental Plate** in **Select well factor source** menu. Set the reaction mix volume **25 µl** in **Sample Volume** window. Click **Begin Run** and name the experiment (the results of this experiment will be automatically saved in this file). Click **OK**.

5. Proceed to the results analysis at the end of the program.

Data analysis:

The analysis of the results is carried out for JOE/HEX and FAM. The results are interpreted according to the crossing (or not-crossing) of the S-shaped fluorescence curve with the threshold line (set in the middle of the linear fragment of fluorescence growth of the positive control in the log scale) and shown as the presence (or absence) of the *C_t* (threshold cycle) value in the results grid.

1. Start the program and open the saved file. To do this:
 - for **iCycler iQ5** select the needed file for the analysis in the **Data File** window of the **Workshop** module, and click the **Analyze** button;
 - for **iCycler iQ** activate the **View Post-Run Data** in the **Library** module. Select the needed file with the analysis data and click the **Analyze Data** button.
2. Browse the received data. To do this:
 - for **iCycler iQ5** select **Analysis Mode: PCR Base Line Subtracted Curve Fit** (by default);
 - for **iCycler iQ** select the item of corresponding channel in **PCR Quantification** tab of **Select a Reporter** menu. **PCR Base Line Subtracted Curve Fit** data analysis mode is to be selected (by default).
3. The data for each channel are to be browsed separately.
4. Set the level of the threshold line. To do this:
 - for **iCycler iQ5**: set the **Base Line Cycles – Auto Calculated** parameter in the **Base Line Threshold** window (in case of the curves excursion set this parameter in the **User Defined, 2 through 10 cycles** mode), set the **Crossing Threshold – Auto Calculated** parameter. Normally, the threshold line is to intersect only with S-shaped 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 raise the threshold level by clicking the **Log View** button and setting the threshold lines level (with the left mouse button) so that the fluorescence curves should be of a linear character and not intersect with the curves of the negative samples;
 - for **iCycler iQ** choose the mode of the threshold line automatic setting and the automatic calculation of the base line in the **Threshold Cycle Calculation** menu. To do this, select **Auto Calculated** in the **Baseline Cycles** menu and **Auto Calculated** in the **Threshold Position** menu. Normally, the threshold line is to intersect only with S-shaped 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 choose **User Defined** in the **Threshold Position** submenu and raise the threshold level by clicking the **Log View** button and setting the threshold lines level (with the left mouse button) so that the fluorescence curves should be of a linear character and not intersect with the curves of the negative samples.

5. In order to analyse the results click the **PCR Quant** button (for **iCycler iQ**) or activate the **Results** button which is situated under the buttons with the fluorophores' names (for **iCycler iQ5**).
6. The *Ct* values for the test samples are to be registered if the results for the control samples (NCA, C– and C+) are satisfying (in accordance to the *Instruction Manual* and *Important Product Information Bulletin*).
7. The interpretation of the test's results for the test samples is carried out in accordance to the *Important Product Information Bulletin* for the PCR kit. The samples are considered positive if their *Ct* value does not exceed the value of the threshold cycle specified in the *Important Product Information Bulletin*.

AMPLIFICATION AND DATA ANALYSIS WITH THE USE OF CFX96 (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 (detection through the cap of the tube).

NOTE: Program the thermocycler only according to the *Instruction Manual* given by the manufacturer of the instrument.

Programming the thermocycler

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**.

Table 3

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, HEX	

NOTE: Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program.

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 tubes in the required channels. Define sample names in the **Sample name** window.
Note – When several tests are performed simultaneously the detection in all used channels is 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. Check the correctness of automatic threshold line selection for each channel using one of these methods:
 - a) Set the threshold line level for each channel in turn (drag it by pushing the left mouse button) up to 10–20 % from the maximum fluorescence level of C+ samples in the last amplification cycle. The fluorescence curve of C+ sample should cross the threshold line in the area where the fluorescence typical exponential rise becomes a linear one.

- b) Indicate **Log Scale** with a checkmark for each channel. Set the threshold line at the level where fluorescence curves are linear (use the left mouse button). Deselect checkmark for **Log Scale**.
2. Click the **View/Edit Plate** button on the toolbar and enter sample names in the opened window.
3. The *Ct* values for the test samples are to be registered if the results for the control samples (NCA, C– and C+) are satisfying (in accordance to the *Instruction Manual* and *Important Product Information Bulletin*).
4. The interpretation of the test's results for the test samples is carried out in accordance to the *Important Product Information Bulletin* for the PCR kit. The samples are considered positive if their *Ct* value does not exceed the value of the threshold cycle specified in the *Important Product Information Bulletin*.
5. Click **Tools** on the toolbar, then **Reports...**, and then save the generated report.

AMPLIFICATION AND DATA ANALYSIS USING Mx3000P, Mx3005P (Stratagene, 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).

1. Switch the instrument on, start the program Mx3000P/Mx3005P.
2. Select **Quantitative PCR (Multiple Standards)** in the **New Experiment Options** window and check the **Turn lamp on for warm-up** box.

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

3. Insert the tubes into the instrument, lock the fixing arm and the door of the instrument.
4. Select **Optics Configuration** in the **Options** menu and in the **Dye Assignment** tab set JOE parameter next to the **HEX/JOE filter set** and FAM parameter next to the **FAM filter set** item.

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 strips/plate upside down while inserting them into the instrument.

5. Set the fluorescence detection parameters in the **Plate Setup** menu. To do this, select all the cells with the test tubes or stripes by holding down the **Ctrl** button and selecting the needed range with the mouse and mark them as **Unknown** in the **Well type** field. Select **FAM** and **JOE** fluorophores in the **Collect fluorescence data** option. Then name each

test sample by double click (**Well Information window**). One can name the samples during the amplification or after it returning to **Plate Setup** menu.

Set the amplification program. To do this use one of this method:

Using of the template file for setting the amplification program (is recommended).

Select the **Thermal Profile Setup** tab. Click the **Import...** button which is to the right of the depicted thermocycling profile. Select the fold containing the previous experimental file and open the fold. The needed thermocycling profile will appear in the **Thermal Profile** window.

Individual programming

1. Select all the cells in the **Plate Setup** tab where the test tubes are set. Select the **Thermal Profile Setup** menu and set the amplification program (see Table 4)

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, HEX	

NOTE: Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program.

NOTE: Set **Ramp Rate 2,5 °C/s** by clicking the **Step Options** button for each step of cycling.

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 of the box to the field with the necessary temperature.

2. Start the amplification by clicking the **Run** and **Start** buttons, then name the experiment.

Data analysis:

The results are interpreted according to the crossing (or not-crossing) of the S-shaped fluorescence curve with the threshold line (set in the middle of the linear fragment of fluorescence growth of the positive control in the log scale) and shown as the presence (or absence) of the *Ct* (threshold cycle) value in the results grid.

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). Otherwise select all the test samples by holding down the **Ctrl** button and selecting the needed range with the mouse.

3. Select the **Results** tab.
4. Make sure that two fluorescence channels are active (the **JOE/HEX** and **FAM** buttons are activated in the **Assays Shown** field in the lower part of the program's window).
5. Select the **Threshold fluorescence** field and make sure that tick marks are put against two fluorescence channels: **JOE/HEX** and **FAM**. Check the correctness of the automatically chosen threshold line. Normally, the threshold line is to intersect only with S-shaped 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 raise the threshold level. The curves of the signal accumulation are shown by the instrument in the linear form by default. In order to change the form of the curves and make them logarithmical double click the left mouse button near one of the coordinate axes (X or Y) in the **Graph properties** window for Y axis (**Y axis**) and put the tick mark against the **Log** point in the **Scale** field.
6. Result of the analysis is considered reliable only if the results for both Positive and Negative Controls of amplification and Negative Controls of RNA extraction are correct in accordance with the table of control reactions results analysis (see the Instruction Manual) and the boundary values *Ct* specified in *Important Product Information Bulletin* enclosed to the PCR kit.
7. The interpretation of the test's results for the test samples is carried out in accordance to the *Important Product Information Bulletin* for the PCR kit. The samples are considered positive if their *Ct* value does not exceed the value of the threshold cycle specified in the *Important Product Information Bulletin*.

TROUBLESHOOTING

If the threshold cycle value is detected for the sample in the results grid, but the fluorescence curve is not regular with the specific zone of exponential growth of fluorescence (in particular, the fluorescence curve is linear), this result is incorrect, it cannot be interpreted as a positive result. This result may indicate incorrect setting threshold line level (or other analysis parameters). If this result is received at the correct threshold level (and other parameters), the analysis must be repeated for this sample (samples) beginning from the RNA extraction stage to get the correct result.