

GUIDELINES

to **AmpliSens[®] *Helicobacter pylori*-FRT** PCR kit
for qualitative detection of *Helicobacter pylori* DNA in clinical
material 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 the use of **AmpliSens® *Helicobacter pylori*-FRT** PCR kit for detection of *Helicobacter pylori* DNA in clinical material (biopsy material of gastric mucosa) by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using the following instruments:

- Rotor-Gene 3000 (four channels) (Corbett Research, Australia);
- Rotor-Gene 6000 (five and six channels) (Corbett Research, Australia);
- iCycler iQ (three and more channels), iCycler iQ5 (Bio-Rad, USA);
- CFX96 (Bio-Rad, USA).

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

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 instrument.

Hereinafter, all terms corresponding to different instruments and software are indicated in the following order: for Rotor-Gene 3000 version 6 / for Rotor-Gene 6000.

Carry out the sample pretreatment and reaction mixture preparation stages according to the PCR kit *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).

NOTE: Well No 1 must be filled with a test tube from the current experiment.

3. 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). Press the **Next** button.
4. In the opened window, set the operator and select the reaction mixture volume (**Reaction volume, 25 µl**). Select **15 µl oil layer volume** (for **Rotor-Gene 6000** instrument). Press the **Next** button.

- Set the amplification program for the rotor-type instrument (see Table 1).

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 Fluorescence signal detection	
	72	10 c	

Fluorescent signal is detected in the FAM/Green and JOE/Yellow channels (when several tests are performed simultaneously the detection in other used channels is enabled).

- Click **OK**.
- Select the **Calibrate/Gain Optimisation** button in the **New Run Wizard** window:
 - perform the calibration before the first measurement (activate **Perform Calibration Before 1st Acquisition/Perform Optimisation Before 1st Acquisition**)
 - perform the fluorescence detection in FAM/Green and JOE/Yellow 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 - from 5FI to 10FI, JOE/Yellow – from 5 FI to 10FI) and press the **OK** button;
 - channel calibrations can be changed by pressing the **Edit...** button. Press the **Close** button.
- Press the **Next** button. Then select the **Start run** button for amplification run and name the experiment.
- Enter the data in the grid of samples (it opens automatically after the amplification has been started). Indicate the names/numbers of 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 test samples, the **Positive Control** type next to the Positive Controls, **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

- 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**.
- Cancel the automatic choice of the threshold line level for each of the main open windows (FAM/Green and JOE/Yellow) by activating the **Threshold** button.

3. Activate the **Dynamic tube** and **Slope Correct** in the menu of each main window (**Quantitation analysis**).
4. In the **CT Calculation** menu (in the right part of the window) set **Threshold = 0.05** and **More settings** as **10 %**.
5. In the results grid (the **Quant. Results** window) one will be able to see the *Ct* values.
6. The result of the PCR analysis is considered reliable only if the results for the positive and negative controls of amplification and negative control of extraction are correct in accordance to the table with results for controls (see the *Instruction manual*) and boundary *Ct* values specified in the *Important Product Information Bulletin* enclosed to the PCR kit.
7. 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.

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 working with the iCycler iQ5 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 and the power supply unit of the optical part of the instrument at least 20–30 min before measurement.
2. Set the amplification program (**Protocol**) for plate-type instruments (see Table 2).

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 Fluorescence signal detection	
	72	10 s	

Fluorescent signal is detected in the FAM and JOE/HEX channels (when several tests are performed simultaneously the detection in other used channels is enabled).

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 clinical samples. Set the fluorescence measurement in two channels (JOE/HEX and FAM) for all samples.
6. 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**. Carry out amplification

using the same type of plastics as plastics used for calibration. Save the scheme of the plate.

7. Add reagents for amplification and DNA samples into the tubes and place them 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 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

1. The data are analyzed in JOE/HEX and FAM channels.
2. Start the software and open the saved file. To do this, select **Data file** in the **Workshop** module and select the data file. Pass to the **Data Analysis** mode.
3. Make sure that the automatic selection of the threshold level for each channel is correct. Normally, the threshold line should cross only 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, 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. Activate the **Results** button (under the buttons with fluorophores names) for data analysis.
5. The result of the PCR analysis is considered reliable only if the results for the positive and negative controls of amplification and negative control of extraction are correct in accordance to the table with results for controls (see the *Instruction manual*) and boundary *Ct* values specified in the *Important Product Information Bulletin* enclosed to the PCR kit.
6. 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.

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**.

Table 3

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 Fluorescence signal detection	
	72	10 s	

Fluorescent signal is detected in the FAM and HEX channels (when several tests are performed simultaneously the detection in other used channels is 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 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, confirming the name of each sample by the **Load** button.

NOTE! When several tests are performed simultaneously the detection in other used channels is enabled.


6. In the **Plate Editor New** window select **File**, then **Save As**, name the plate and save. 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 CFX96 PCR instrument 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 and shown as the presence (or absence) of the *C_t* (threshold cycle) value in the results grid.

1. The fluorescence curves, plate setup, and results grid with *C_t* 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+ samples 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 name or rename samples in the opened window.
4. The result of the PCR analysis is considered reliable only if the results for the positive and negative controls of amplification and negative control of extraction are correct in accordance to the table with results for controls (see the *Instruction manual*) and boundary *C_t* values specified in the *Important Product Information Bulletin* enclosed to the PCR kit.
5. 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.
6. 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
14.04.11 RT	Footer	Catalogue number R-B9(RG,iQ)-CE-B for in bulk form was added
19.06.11 RT	Cover page	The name of Institute was changed to Federal Budget Institute of Science “Central Research Institute for Epidemiology”
19.06.13 FN	Footer	Catalogue number REF R-B9(RG,iQ)-CE-B was deleted
02.04.14 SA	Cover page	Address of European representative was added
06.11.14 ME	Intended use	The CFX96 instrument was added, the phrase about pretreatment of clinical material was deleted
	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) Amplification and data analysis using iCycler iQ5 (Bio-Rad, USA)	The amplification programs were added. The items about results interpretation for the control and test samples were corrected
	Amplification and data analysis using CFX96 (Bio-Rad, USA)	The section was added
28.12.20 MM	Through the text	The symbol  was changed to NOTE:
	Cover page	The phrase “Not for use in the Russian Federation” was added
18.03.21 EM	Front page	The name, address and contact information for Authorized representative in the European Community was changed