

GUIDELINES

to **AmpliSens® MTC-FRT** PCR kit

for qualitative detection of *Mycobacterium tuberculosis* (MBT) DNA – *Mycobacterium tuberculosis complex* (MTC) in biological material, cultures of microorganisms and environmental objects 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® MTC-FRT** PCR kit for qualitative detection of *Mycobacterium tuberculosis* (MBT) DNA – *Mycobacterium tuberculosis complex* (MTC), including *M.tuberculosis*, *M.bovis*, *M.africanum*, *M.microti*, *M.canetti*, *M.pinipedii* in biological material, cultures of microorganisms and environmental objects 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);
- SmartCycler II (Cephied, USA);
- Mx3000P, Mx3005P (Stratagene, USA);
- CFX96, CFX96 Touch (Bio-Rad, USA).

Correspondence of names of fluorophores and detection channels

Channel for the fluorophore	The detection channels' names for different instruments ¹⁾
FAM	FAM/Green
JOE	JOE/HEX/R6G/Yellow/Cy3

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

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 6.1 and higher software 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 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. Turn on the instrument, run the Rotor-Gene software.
2. Insert the tubes or strips into the rotor of the Rotor-Gene 3000/6000/Q instrument beginning from the first well (the rotor wells are numbered, the numbers are used for the further programming of the samples' order in the thermocycler). Insert the rotor into the instrument, close the lid.

NOTE: Well 1 must be filled with any test tube except for an empty one. If the rotor is loaded with tubes from different PCR kits the No. 1 well should contain the tube with the largest number of fluorophores. For example, for simultaneous detection, quantitation, or differentiation of *Mycobacterium tuberculosis complex*, the tubes for *MTC* quantitation or differentiation should be placed in the rotor first.

Creating the template for the run

1. Click the **New** button in the software main menu. To create the template select the **Advanced** tab in the opened window **New run**.
2. Select the **TwoStep/Hidrolisis Probes** template in the tab for edition and click The **New** button.
3. In the opened window select the **36-Well Rotor** and tick the **No Domed 0.2ml Tubes/Locking Ring Attached** option. Click the **Next** button.
4. In the opened window enter the operator name, select the **Reaction volume – 25 µl**. Tick the **15 µl oil layer volume** option. Click the **Next** button.
5. In the **New Run Wizard** window set the temperature profile of the experiment. To do this click the **Edit profile** button and set the amplification program:

95-65-72 MTC amplification program for rotor-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
Hold	95	15 min	–	1
Cycling 1	95	15 s	–	5
	65	30 s	–	
	72	15 s	–	
Cycling 2	95	15 s	–	40
	65	30 s	FAM/Green, JOE/Yellow	
	72	15 s	–	

- After setting up the temperature profile click the **OK** button.
- Click the **Calibrate/Gain Optimisation...** button in the **New Run Wizard** window. In the opened window for signal measurement optimisation for the selected channels set calibration from **5FI** to **10FI** for the FAM/Green, JOE/Yellow channels. To do this, click the **Calibrate Acquiring/Optimise Acquiring** button. In the opened window for first channel (**Auto Gain Optimisation Channel Settings/Auto Gain Calibration Channel Settings**) indicate the values of minimum and maximum signal in the **Target Sample Range** line. Click the **OK** button. The window for the next channel will open automatically. The selected values for all the channels can be checked in the **Min Reading, Max Reading** boxes.
- Click the **Next** button. For saving the programmed template it is necessary to click the **Save Template** button and enter the template file name, corresponding to the amplification program – **95-65-72 MTC**. Save the file into a proposed folder: **Templates\Quick Start Templates**; close the **New Run Wizard window**. After that the programmed template will appear in the template list in the **New Run** window.

Using the ready template for the run

- Click the **New** button in the software main menu. In the opened **New Run** window select the **Advanced** tab. Then select the template (which is programmed as described in the “Creating the template for the run” section) in the template list.
- In the opened window select the **36-Well Rotor** and tick the **No Domed 0,2ml Tubes / Locking Ring Attached** option. Click the **Next** button.
- In the opened window check that the reaction volume is **25 µl** and the **15 µl oil layer volume** option is activated. Click the **Next** button.
- In the next window the correctness of the amplification program and signal level auto-optimisation parameters can be checked. Go to the next window clicking the **Next** button. Start the amplification by the **Start run** button. Herewith, the rotor with the samples should be already fixed and the lid should be closed. Name the experiment

and save it to the disc (the results of the experiment will be automatically saved in this file).

5. Enter the data into the grid of the samples (it opens automatically after the amplification has been started). Enter the names/numbers of the test samples in the **Name** column. Define the Negative control of amplification as NCA, the Positive control of amplification as C+. Set the type **Unknown** opposite all the test samples, the type **Positive control** – for the Positive control of amplification, the type **Negative control** – for the Negative control of amplification. Set the type **None** for the cells matching with the corresponding empty tubes. Click the **Finish/OK** button.

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

Note – To edit the table of samples before the start it is needed previously to select the **Edit Samples Before Run Started** option in the **User preferences** submenu of the **File** menu.

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

Amplification data analysis in the FAM/Green channel:

1. Activate the **Analysis** button in the menu, then select the mode of the analysis **Quantitation**, activate the buttons **Cycling A. FAM/Cycling A. Green, Show**.
2. Cancel the automatic choice of the threshold line level **Threshold**.
3. Select the **Linear scale**.
4. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of main window (**Quantitation analysis**).
5. In **CT Calculation** menu (in the right part of the window) indicate the threshold line level **0.03** 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-Cycling A. FAM/Quant. Results-Cycling A. Green** window) one will be able to see the **C_t** values.

Results analysis in the JOE/Yellow channel is carried out similarly to results analysis in the FAM/Green channel in accordance with the settings in the table below.

Channel	Threshold	Dynamic tube	Slope Correct	More Settings/Outlier Removal
FAM/Green	0.03	on	on	10%
JOE/Yellow	0.05	on	off	15%

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.

AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ and 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 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).

Programming the thermocycler

1. Turn on the instrument and the power supply unit of the optical block of the instrument.

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

2. Start the program iCycler iQ5 / iQ.


3. Insert the tubes or strips into the reaction module of the thermocycler and program the instrument according to the *Instruction Manual* given by the manufacturer of 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.

Creating the template for the run

1. Set the plate setup (set the order of the tubes in the reaction chamber and the detection of fluorescent signal).

- For **iCycler iQ**: edit the plate setup in the **Edit Plate Setup** window of the **Workshop** module. To do this, set the order of the tubes in the reaction chamber in the **Samples: Whole Plate Loading** option and indicate the name of each sample in the **Sample Identifier** window. Set the fluorescence signal detection in all the tubes through **FAM-490** and **JOE-530** channels. 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** (a 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.
- For **iCycler iQ5**: click the **Create New** or **Edit** button in the **Selected Plate Setup** window of the **Workshop** module. Edit plate setup in the **Whole Plate loading** mode. Enter the samples' names in the **Identifier/Condition** column in the bar appeared in

the screen bottom. Select the fluorescent signal detection in the FAM, JOE/HEX channels. Click the **Select/Add Fluorophores** button, select the fluorophore and tick it in the **Selected** column. Click **OK**. The fluorophore name will appear in the **Fluorophore** window. For addition of fluorescence signal measuring for each sample it is necessary to click the fluorophore (activate it) and select the samples on the plate using the **Fluorophore loading in whole Plate mode**  button under the scheme. 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**. Save the set plate setup by clicking the **Save&Exit Plate Editing** button. Enter the file name and click **Save**.

2. Set all the biological samples as **Unknown**, positive controls as «+», and negative controls as «-».
3. Set the amplification program. To do this, in the **Selected Protocol** window of the **Workshop** module click the **Create New** button. Set the amplification parameters and save the protocol by activating the **Save&Exit Protocol Editing** button. Enter the name of the file and then click **Save**.

95-65-72 MTC amplification program

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	95	15 min	–	1
2	95	15 s	–	5
	65	30 s	–	
	72	15 s	–	
3	95	15 s	–	40
	65	30 s	FAM, JOE/HEX	
	72	15 s	–	

4. Start the selected program with the required plate setup.
 - For **iCycler iQ**. Ensure the correctness of the selected protocol and plate setup in the **Run Prep** window. For the detection of the well factor select **Experimental Plate** under the **Select well factor source** line. Set the reaction mix volume as **25 µl**. Press **Begin Run** to start. Name the experiment (this file will automatically save the results for this experiment) and click **OK** button
 - For **iCycler iQ5**. Ensure the correctness of the **Selected Protocol** and **Selected Plate Setup** before starting the program. To start the program click **Run**. For detection of the well factor select **Use Persistent Well Factor** (set by default). Click the **Begin Run** button. Name the experiment (the results of the experiment will be automatically saved in this file) and click **OK**.

5. At the end of the run it is necessary to close the software and turn off the instrument (the thermocycler and the optical block).

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:

- select the needed file with the run in the upper left window of the **Workshop** module;
- click the **Edit** button in the **Selected Plate Setup** area of the **Workshop** module and edit the plate setup (the files of protocols are saved in the **SampleFiles** folder by default);
- click the **Edit** button in the **Selected Protocol** area of the **Workshop** module and check the correctness of the selected protocol (the files of protocols are saved in the **Users** folder by default).

Data analysis:

The obtained results are analyzed by the iCycler iQ5 / iQ 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 *C_t* (threshold cycle) value in the corresponding column of the results table.

1. Start the software and open the needed file with data of the analysis in the **Data File** window of the **Workshop** module. Click the **Analyze** button.
 - For **iCycler iQ**. Select the icon of the required channel in the **Select a Reporter** menu. Make sure that **PCR Base Line Subtracted Curve Fit** (set by default) is activated. Set the threshold line level: enter **30** for FAM-490 and **45** for JOE-530 channel in the **Threshold Position** field. Click the **PCR Quant** button. *C_t* values of the analyzed channel will appear in the **Quant. Results** table.
 - For **iCycler iQ5**. Select the icon of the required channel in the module window. Make sure that **PCR Base Line Subtracted Curve Fit** (set by default) is activated. Set the threshold line at the level corresponding to 10% maximum fluorescence obtained for **Positive Control DNA MTC / STI** in the last cycle of amplification for all channels. Click the **PCR Quant** button. *C_t* values of the analyzed channel will appear in the **Quant. Results** table. The level of fluorescence is equal to the nearest digit scale mark. Moreover, the fluorescence curve of the Positive Control DNA MTC / STI should be sigmoid. The threshold line can be set by default if falls within the specified range. To select the graph of the sample, use the **Display Wells** button or cursor the required graph

and double click. To change the threshold line level, track it by cursor holding the left mouse button or select the **Baseline Threshold** menu (from drop-down menu that displayed when fluorescence graph window is double clicked), then select **User Defined** option, and then enter the required value in the **Threshold Position** field. To view the results grid, click the **Results** button.

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.

AMPLIFICATION AND DATA ANALYSIS USING SmartCycler II (Cepheid, 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 disposable polypropylene 0.025-ml tubes.

Programming the thermocycler

1. Insert the tubes into the thermocycler sites. Close the sites' lids.

Before placing the tubes into the instrument it is necessary to sediment the reaction mixture to the bottom of the tubes using the microcentrifuge of SmartCycler II instrument.

2. Program the instrument according to the *Instruction Manual* provided by the manufacturer.
3. Select **Define Protocols** in the main program menu. Select the **New Protocol** button at the right bottom part of the opened window. Name the protocol as **95-65-72 MTC** and set the **95-65-72 MTC** amplification program:

95-65-72 MTC amplification program for SmartCycler II (Cepheid, USA)

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	95	15 min	–	1
2	95	20 s	–	45
	65	50 s	FAM, Cy3	
	72	20 s	–	

Note – Enable the signal detection by clicking the **Optics** cell of the second step and selecting **ON**.

4. Click the **Save Protocol** button at the bottom of the window.
5. Select **Create Run** in main menu. Enter the file name to save the experiment data in the **Run Name** window. Select **Dye set** in the middle of the left panel of the monitor and select the **FCTC25** dye combination from the drop-down menu.
6. Click the **Add/Remove Sites** button at the center of the monitor. In the newly opened window select the required protocol (program) and select the analyzed wells. Click **OK**.
7. All settings of this experiment are given in the table in the upper part of the window. All samples are indicated (by default) as **UNKN** (unknown) in the **Sample Type** column of this table. Each sample should be indicated in the **Sample ID** column.
8. Click **Start Run** button at the bottom of the monitor to start the run.

Data analysis

1. Select **Analysis settings** in the menu. Set the threshold line level to **30** for **FAM** and **Cy3** channels.

2. In the **Results Table** window *Ct* values will appear for the **FAM** and **Cy3** channels.
3. The results can be interpreted using the automatic evaluation, reflected in the results table (**Results**), or by visual analysis of the fluorescence curves for the **FAM** and **Cy3** channels.

Results interpretation

1. If the result displayed in the results grid in Std/Res FAM line is POS (the fluorescence curve of the sample crosses the threshold line ($Ct_{FAM} \neq 0$) in the FAM data graph); whereas, the result displayed in the results grid in Std/Res Cy3 line is POS ($Ct_{Cy3} \neq 0$) or NEG ($Ct_{Cy3} = 0$), the sample is positive, *Mycobacterium tuberculosis* DNA **is detected**.
2. If the result displayed in the results grid in Std/Res FAM line is NEG (the fluorescence curve of the sample does not cross the threshold line ($Ct_{FAM} = 0$) in the FAM data graph); whereas, the result displayed in the results grid in Std/Res Cy3 line is POS ($Ct_{Cy3} \neq 0$), the sample is negative, *Mycobacterium tuberculosis* DNA **is not detected**.
3. If the results displayed in the Std/Res FAM and Std/Res Cy3 lines are NEG ($Ct_{FAM} = 0$ and $Ct_{Cy3} = 0$), it is necessary to repeat amplification. If the result is the same, repeat DNA extraction. If the result is the same again, it is considered to be **invalid**. In this case, it is recommended to repeat material sampling.

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 round or optically transparent 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).

Programming the thermocycler

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.

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.

4. Select **Optics Configuration** in the **Options** menu and in the **Dye Assignment** tab set FAM parameter next to the **FAM filter set** item and JOE parameter next to the **HEX/JOE filter set**.
5. Set the fluorescence detection parameters in the **Plate Setup** menu. To do this, select all the cells with the test tubes and mark them as **Unknown** in the **Well type** field. Select **FAM** and **JOE** fluorophores in the **Collect fluorescence data** option.
6. Open the **Well Information** window by double clicking the left mouse button on required sample well. Name each sample.
7. Select the **Thermal Profile Setup** menu and set the amplification program:

95-65-72 MTC amplification program for plate-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	95	15 min	–	1
2	95	15 s	–	5
	65	30 s	–	
	72	15 s	–	
3	95	15 s	–	40
	65	30 s	FAM, JOE/HEX	
	72	15 s	–	

8. Select **Run** in the menu. Check the correctness of set amplification program. Activate the **Start** button and check **Turn lamp off at end of run**. Save the experiment.

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. Check the correctness of the automatically chosen threshold line for each channel. 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. To do this in the **Dyes shown** panel activate the reflection for each channel, view the threshold line position and change it, if it is necessary.
5. Select **Text report** in the **Area to analyze** block.

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.

AMPLIFICATION AND DATA ANALYSIS USING CFX96, CFX96 Touch (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**.

95-65-72 MTC amplification program

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	95	15 min	–	1
2	95	15 s	–	5
	65	30 s	–	
	72	15 s	–	
3	95	15 s	–	40
	65	30 s	FAM, HEX	
	72	15 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
→ 2	95,0 C for 0:15
	Slow Ramp Rate to 2,5 C per second
3	65,0 C for 0:30
	Slow Ramp Rate to 2,5 C per second
4	72,0 C for 0:15
	Slow Ramp Rate to 2,5 C per second
5	GOTO 2, 4 more times
→ 6	95,0 C for 0:15
	Slow Ramp Rate to 2,5 C per second
7	65,0 C for 0:30
	+ Plate Read
	Slow Ramp Rate to 2,5 C per second
8	72,0 C for 0:15
	Slow Ramp Rate to 2,5 C per second
9	GOTO 6, 39 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 and JOE 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 fluorescence curve with the threshold line set at the specific level, that corresponds to the presence (or absence) of the *C_t* (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 *Ct* values are represented in the **Data Analysis** window of the **Quantification** tab.

Variant 1

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 Positive Controls in the last amplification cycle. Make sure that fluorescence curve of the Positive Control crosses the threshold line in the area of exponential growth of fluorescence passing onto linear growth.

Variant 2

For each channel indicate **Log Scale** with a checkmark. Set the threshold line at the level where fluorescence curves are linear (use the left mouse button).

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


Click **Tools** on the toolbar, then **Reports...**, and then save the generated report choosing **File** and **Save As** and name the file.

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.

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
06.06.11 VV	Catalogue number	REF R-B57(RG,iQ,SC,Dt)-CE-B was added
01.07.11 RT	Cover page	The name of Institute was changed to Federal Budget Institute of Science "Central Research Institute for Epidemiology"
14.06.13 PE	Footer	Catalogue number R-B57(RG,iQ,SC,Dt)-CE-B was deleted
07.05.15 PM	Text	Text was corrected in accordance with the template
	Intended use	The CFX96 (Bio-Rad, USA) and Mx3000P, Mx3005P (Stratagene, USA) instruments were added
	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) AND Rotor-Gene Q (QIAGEN, Germany) instruments, Amplification and data analysis with the use of iCycler iQ and iCycler iQ5 (Bio-Rad, USA) instruments, Amplification and data analysis using SmartCycler (Cepheid, USA)	The amplification programs were added
	Amplification and data analysis using Mx3000P, Mx3005P (Stratagene, USA)	The chapters were added
	Amplification and data analysis using CFX96 (Bio-Rad, USA)	
29.05.15 PM	Amplification and data analysis using Mx3000P, Mx3005P (Stratagene, USA)	The subchapter Results interpretation was rewritten.
	Amplification and data analysis using CFX96 (Bio-Rad, USA)	The subchapter Results interpretation was added
10.06.21 EM	Through the text	The symbol  was changed to NOTE:
	Cover page	The phrase "For research use only. Not for diagnostic procedures" was added
	Amplification and data analysis with the use of iCycler iQ and iCycler iQ5 (Bio-Rad, USA) instruments	10% of maximum level of fluorescence was changed to 15%
01.07.21 EM	Through the text	All sections were updated according to the template; the information about ROX and Cy5 channels was deleted from the amplification programs; threshold line settings were changed from 15 to 10 % for iCycler
13.07.23 EM	Footer	REF R-B57(RG,iQ,SC,Dt)-CE was added