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For Professional Use Only

GUIDELINES

to AmpliSens® *MTC*-diff-FRT PCR kit

for differentiation of the DNA of *Mycobacterium tuberculosis complex* (*MTC*) including the human (*M.tuberculosis*), the bovine (*M.bovis*) and also the vaccine strain (*M.bovis* BCG) in the biological material and microorganism cultures by the polymerase chain reaction (PCR) with real-time fluorescence hybridization detection

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INTENDED USE

The guidelines describe the procedure of using **AmpliSens® *MTC*-diff-FRT** PCR kit for differentiation of the DNA of *Mycobacterium tuberculosis complex* (*MTC*) including the human (*M.tuberculosis*), the bovine (*M.bovis*) and also the vaccine (*M.bovis* BCG) strains in the biological material and microorganism cultures 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 iQ5 (Bio-Rad, USA);
- Smart Cycler II (Cepheid, USA)
- CFX96, CFX96 Touch (Bio-Rad, USA).

Table 1

Compliance of the fluorophore channels with the detection channels names

Fluorophore channel	Detection channels names for different instrument models[1]
FAM	FAM/Green
JOE	JOE/HEX/R6G/Yellow/Cy3
ROX	ROX/Orange/TxR
Cy5	Cy5/Red

[1] Detection channel name for the instrument in use is given in the respective section of the Guidelines for this reagents kit.

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 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 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 sample tubes loaded into the rotor are being analyzed by different reagents kits, then the first well must be filled with a sample of highest fluorophore level. For example, in a simultaneous rotor loading of tubes tested for presence of *Mycobacterium tuberculosis complex* for quantitative analysis or differentiation, the first well must be filled with tubes containing quantitative detection reagents or *Mycobacterium tuberculosis complex* differentiation reagents.

3. Program the instrument according to the *Instruction Manual* given by the manufacturer of the instrument.

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/Hidrolysis 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

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, ROX/Orange, Cy5/Red	
	72	15 s	–	

6. After setting up the temperature profile click the ***OK*** button.
7. Click the ***Calibrate/Gain Optimisation…*** button in the ***New Run Wizard*** window. In the opened window:
   a) for signal measurement optimisation for the selected channels set calibration from **5FI** to **10FI** for all the channels (FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red). 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.
8. 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.

<u>Using the ready template for the run</u>

1. 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.
2. 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.
3. In the opened window check that the reaction volume is 2**5 µl** and the ***15 µl oil layer volume*** option is activated. Click the ***Next*** button.
4. 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. 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 *Ct* (threshold cycle) value in the corresponding column of the results table.

Amplification data analysis in the FAM/Green channel:

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***.
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 the ***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 ***Quantitation Results*** window) one will be able to see the ***Ct*** values.

Results analysis in the JOE/Yellow, ROX/Orange, Cy5/Red is carried out similarly to results analysis in the FAM/Green channel in accordance with the settings in the table below.

Channel	*Threshold*	*Slope Correct*	*Slope Correct*	*More Settings/ Outlier Removal*
FAM/Green	0.03	on	on	10%
JOE/Yellow	0.05	on	on	10%
ROX/Orange	0.05	on	on	10%
Cy5/Red	0.05	on	on	30%

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 iQ5 (Bio-Rad, USA) INSTRUMENT

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 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 should be warmed up during 15 min before starting the experiment.

2. Start the program **iCycler iQ5**.
3. Insert the tubes or strips or plate 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, so that no drops are left on the walls of the tubes, if the drops fall during the amplification process it may lead to the signal failure and complicate the results analysis. Don't turn the strips/plates 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).
   - click the ***Create New*** button in the ***Selected Plate Setup*** window of the ***Workshop*** module;
   - in the opened window click the ***Whole Plate loading*** button and set the plate setup using the buttons of the upper toolbar. 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, ROX, Cy5 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*	*Number of 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, ROX, Cy5	
	72	15 s	–	

4. Before the run start it is obligatory to check if the selected protocol (***Selected Protocol***) and the plate scheme (***Selected Plate Setup***) are correct. To begin a run click the ***Run*** button. For the well factors measurement the ***Use Persistent Well Factors*** type is selected by default. Click the ***Begin Run*** button, save the experiment (the results of this 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 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. 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.
2. In the module window select the data for the analysed channel. Herewith the data analysis mode must be ***PCR Base Line Subtracted Curve Fit*** (selected by default).
3. Set the threshold line level respective to 10 % of the maximum fluorescence level, obtained for **Positive Control DNA *MTC*-diff / STI (C+ *MTC*-diff / STI)** in the last cycle of amplification for all channels. The fluorescence level of a sample is believed to be equal to the closest upper division of the scale, that denotes the numerical value. Herein it is neccessary that the fluorescence curve for **Positive Control DNA *MTC*-diff / STI (C+ *MTC*-diff / STI)** resembles the characteristic sigmoid shape. It is possible to use the automatically set threshold line level, given that it falls within the defined region. In order to highlight the sample curve use the ***Display Wells*** button or place your coursor on the curve for that sample and double click on it.
4. In order to change the level of the threshold line, drag it with your left mouse button, or select ***Baseline Threshold*** menu (in the dropdown menu, when you click the right mouse button on the fluorescence graphs), then choose the ***User Defined*** option and enter the correct value in the ***Threshold Position*** field. To see results table click ***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) INSTRUMENT

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.

1. Before loading the thermocycler place the reaction mix into the lower thermocycling part of the tube. To do this, one needs to place the tubes into the rotor of specific Mini-Spin centrifuge (Cepheid, USA) and switch it on for 5-7 s.
2. Place the tubes into the thermocycler wells and close the well lids.

Programming the thermocycler:

1. Open the software for SmartCycler.
2. In the main menu select ***Define Protocols***. In a newly opened window select the ***New Protocol*** button in the bottom left corner and name the protocol ***95-65-72 MTC***, program the instrument to run ***95-65-72 MTC*** setup for SmartCycler (Cepheid, USA).

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

Temperature, °C	*Time*	*Number of cycles*
95	900 s	1
95	20 s	45
65	50 s	
72	20 s	

3. Click the ***Save Protocol*** button at the bottom of the window.
4. Select ***Create Run*** in the main menu. Press ***Dye set*** button in the central left part of the window and select the ***FCTC25*** dye combination.
5. Click the ***Add/Remove Sites*** button at the centre of the window. In the newly opened window select the required protocol (program) and select the analyzed wells. Click ***OK***.
6. Click ***Start Run*** button at the bottom of the window to run the program. In the popup dialogue window enter the file name you want the results to be stored in.
7. All settings of this experiment are given in the table at the top of the window. The table contains a ***Sample Type*** column that indicates each sample as ***UNKN*** (unknown) by default. In the ***Sample ID*** column name each sample.

Data analysis

1. Select ***Analysis settings*** in the menu. Set the threshold line level to **30** for each channel: **FAM, Cy3, Texas Red and Cy5**.
2. In the ***Results Table*** window *Ct* values will appear for **FAM, Cy3, Texas Red** and **Cy5** channels.

The results can be interpreted using the automatic evaluation, reflected in the results table (***Results***), or by visual analysis of the fluorescence curves for **FAM, Cy3, Texas Red** and **Cy5** channels**.**

Interpretation of results for control samples

PCR analysis result is considered reliable if correct results are obtained for both positive and negative controls of amplification, according to the Table 2.

Table 2

Results for controls of amplification using SmartCycler II

Control	Stage for control	Result in channel			
		FAM	Cy3	Texas Red	Cy5
C+	PCR	POS	POS	POS	POS
NCA	PCR	NEG	NEG	NEG	NEG

Interpretation of results for biological material and microorganism cultures

Table 3

Result interpretation for analysed samples using SmartCycler II

DNA of mycobacteria strain	FAM	Cy3	Texas Red	Cy5	Result	Result interpretation
M.tuberculosis	POS	POS / NEG	POS / NEG	POS / NEG	Detected	*M.tuberculosis* DNA (human strain) is detected
	NEG	POS / NEG	POS / NEG	POS	Not detected	*M.tuberculosis* DNA (human strain) is not detected
	NEG	POS / NEG	POS / NEG	NEG	Invalid	*M.tuberculosis* DNA (human strain) – detection result is invalid. Repeat sampling
M.bovis	POS / NEG	POS	POS / NEG	POS / NEG	Detected	*M.bovis* DNA (bovine strain) is detected.
	POS / NEG	NEG	POS / NEG	POS	Not detected	*M.bovis* DNA (bovine strain) is not detected
	POS / NEG	NEG	POS / NEG	NEG	Invalid	*M.bovis* DNA (bovine strain) – detection is invalid. Repeat sampling
M.bovis BCG	POS / NEG	POS / NEG	POS	POS / NEG	Detected	*M.bovis* BCG DNA (vaccine strain) is detected

DNA of mycobacteria strain	FAM	Cy3	Texas Red	Cy5	Result	Result interpretation
	POS / NEG	POS / NEG	NEG	POS	Not detected	ДНК *M.bovis* BCG (vaccine strain) is not detected
	POS / NEG	POS / NEG	NEG	NEG	Invalid	*M.bovis* BCG DNA (vaccine strain) – detection result is invalid. Repeat sampling

Interpretation of results

- ***M.tuberculosis*** **DNA** is **detected** in a sample if the results table for the tested sample in the FAM channel displays a **POS** result ($Ct_{\mathbf{FAM}}$≠0) in **Std/Res FAM** column. Herein the fluorescence curve for the tested sample must intercept the threshold line at the area of characteristic exponential growth of fluorescence.
- ***M.bovis*** **DNA** is **detected** in a sample if the results table for the tested sample displays a **POS** result ($Ct_{\mathbf{Cy3}}$≠0) in **Std/Res Cy3** column. Herein the fluorescence curve for the tested sample must intercept the threshold line at the area of characteristic exponential growth of fluorescence.
- ***M.bovis*** **BCG DNA** is **detected** in a sample if the results table for the tested sample displays a **POS** result ($Ct_{\mathbf{TexasRed}}$≠0) in **Std/Res Texas Red** column. Herein the fluorescence curve for the tested sample must intercept the threshold line at the area of characteristic exponential growth of fluorescence. Due to the fact that the vaccine strain ***M.bovis*** **BCG** is part of the bovine strain MBT (***M.bovis***), the results table may also display a **POS** result ($Ct_{\mathbf{Cy3}}$≠0) in **Std/Res Cy3** column.
- ***M.tuberculosis*** **DNA**, ***M.bovis*** **DNA** and ***M.bovis*** **BCG DNA** are **not detected** in a sample if its fluorescence curve for the **FAM**, **Cy3** and **Texas Red** channels does not intercept the threshold line (the results table displays **NEG** results (Ct_{FAM}=0, Ct_{Cy3}=0, $Ct_{TexasRed}$=0) in respective columns, therewith **Std/Res Cy5** displeys a **POS** result ($Ct_{\mathbf{Cy5}}$≠0) or **NEG** result (Ct_{Cy5}=0).
- The validity of results is interpreted independently. If the results table columns **Std/Res FAM** and **Std/Res Cy5** or **Std/Res Cy3** and **Std/Res Cy5** or **Std/Res Texas Red** and **Std/Res Cy5** display **NEG** results (Ct_{FAM}=0, Ct_{Cy3}=0, $Ct_{TexasRed}$=0, Ct_{Cy5}=0, respectively), then the amplification of that sample needs to be repeated, in case of obtaining an analogous result it is necessary to repeat the sample analysis starting from the DNA/RNA extraction step. If a valid result is not obtained, then differentiation

result of the respective **MBT** strain is interpreted as **invalid**, therewith recommended repetition of the sampling step.

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 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 start the ***Bio-Rad CFX Manager*** program.
2. Program the instrument according to the *Instruction Manual* provided by the manufacturer.

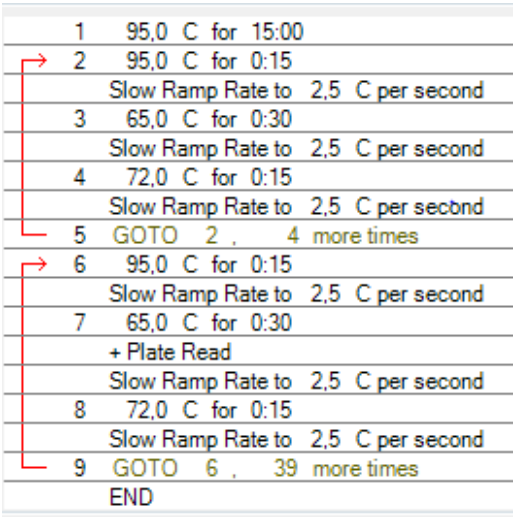
Creating the template for the run

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***…).
2. 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.**

95-65-72 MTC amplification program

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Number of 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, ROX, Cy5	
	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***.



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, Cy5** 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/CFX96 Touch 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.

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 % of maximum fluorescence obtained for the Positive Control in the last amplification cycle. Make sure that fluorescence curve of the Positive Control crosses the threshold line at the zone 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 set the sample names in the opened window.

To generate report of the run it is necessary to select the ***Tools*** on the toolbar, then select ***Reports…*** Save the generated report: select ***File*** and then ***Save as***, name the file and click ***Save***.

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.