

## **GUIDELINES**

to **AmpliSens<sup>®</sup> CCHFV-FRT** PCR kit

for qualitative detection of *Crimean-Congo hemorrhagic fever virus (CCHFV)* RNA in biological material (blood plasma and serum) and ticks by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

**AmpliSens<sup>®</sup>**



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## INTENDED USE

The guidelines describe the procedure of using **AmpliSens® CCHFV-FRT** PCR kit for qualitative detection of *Crimean-Congo hemorrhagic fever virus* RNA in biological material (blood plasma and serum) and ticks 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);
- Mx3000P (Stratagene, USA).

Table 1

**Correspondence between targets and detection channels**

PCR-mix-1	Detection in channel	
	FAM/Green	JOE/HEX/Yellow
RT-PCR-mix-1-FRT <i>CCHFV</i>	IC	<i>CCHFV</i>

## 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 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.**

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 Rotor-Gene 3000/6000/Q instrument beginning from well No.1, place the rotor into the instrument, and close the lid (all wells have their own numbers, which are used for programming sample placement in the thermocycler).
3. Click the **New** button in the program main menu.
4. In the opened window, select **Advanced** and click **Dual Labeled Probe** or **Hydrolysis probes** for Rotor-Gene 6000 instrument. Activate the **New** button.

5. Select **36-Well Rotor** and **No Domed 0.2 ml Tubes** for Rotor-Gene 3000 or **Locking ring attached** for Rotor-Gene 6000 instrument. Click **Next**.
6. Reaction volume is **25 µl**. Enter an operator.
7. Activate for Rotor- Gene 6000 **15 µl oil layer volume** button. Click **Next**.
8. In the opened window click the **Edit profile** button and set the amplification program (see Table 2).

Table 2

**Amplification program for rotor-type instruments**

<b>Step</b>	<b>Temperature, °C</b>	<b>Time</b>	<b>Fluorescence detection</b>	<b>Cycles</b>
1	50	30 min	–	1
2	95	15 min	–	1
3	95	10 s	–	5
	54	25 s	–	
	72	15 s	–	
4	95	10 s	–	45
	50	25 s	FAM/Green, JOE/Yellow	
	72	15 s	–	

9. Click **OK**.
10. In the **New Run Wizard** window click the **Calibrate** button or **Gain Optimisation** for Rotor-Gene 6000 instrument.
  - for calibration in FAM/Green and JOE/Yellow channels select **Calibrate Acquiring** or **Optimise Acquiring** for Rotor-Gene 6000 instrument;
  - select **Perform Calibration Before 1<sup>st</sup> Acquisition** or **Perform Optimisation Before 1<sup>st</sup> Acquisition** for Rotor-Gene 6000 instrument;
  - Set channel calibrations: for FAM/Green channel by the first tube and from **5FI** to **10FI**; for JOE/Yellow channel, from **5 FI** to **10FI** (activate the **Edit...** button, **Auto gain calibration channel settings** window);
11. Click **Next**. Select the **Start run** button for amplification to run.
12. Name the experiment and save it to a disk (results of the run will be automatically saved in this file).
13. Set the data in the table of samples (opens by default when run begins). Define names/numbers of test and control samples in the **Name** column. Enter **None** for empty wells.

### Data analysis

#### Data analysis for the IC (FAM/Green channel)

1. Activate the **Analysis** button, then select the **Quantitation** button, and activate the **Cycling A. FAM/Cycling A. Green**. Click **Show**.

2. Cancel automatic **Threshold** setting.
3. Activate the **Dynamic tube** and **Slope Correct** buttons in the main window menu (**Quantitation analysis**).
4. In **CT Calculation** menu set **Threshold = 0.03**.
5. Select the **More Settings** parameter or **Outlier Removal** for Rotor-Gene 6000 and set **NTC threshold** value **10 %**.
6. Ct values for IC will appear in the results grid (**Quant. Results** window) for each test sample.

Data analysis for CCHFV cDNA/DNA (JOE/Yellow channel)

1. Activate the **Analysis** button, then select the **Quantitation** button, and activate the **Cycling A. JOE /Cycling A. Yellow** .Click **Show**.
2. Cancel automatic **Threshold** setting.
3. Activate the **Dynamic tube** and **Slope Correct** buttons in the main window menu (**Quantitation analysis**).
4. In **CT Calculation** menu set **Threshold = 0.05**
5. Select the **More Settings** parameter or **Outlier Removal** for Rotor-Gene 6000 and set **NTC threshold** value **10 %**.
6. Ct values will appear in the results grid (**Quant. Results** window) for each test sample.

**NOTE:** If fluorescent curves on any channel do not show exponential growth, set **NTC threshold = 15%** for this channel

Interpretation of results for control samples

The result of the analysis is considered reliable only if the results obtained for both positive and negative controls of amplification as well as for both positive and negative controls of RNA extraction are correct (see Table 3).

Table 3

**Results for controls of PCR-analysis (Rotor-Gene 3000/6000/Q)**

Control	Stage for control	Ct value in channel	
		FAM/Green	JOE/Yellow
		IC detection	CCHFV detection
C-	RNA extraction	< 28	absent
PCE	RNA extraction	< 28	< 30
NCA	Amplification	absent	absent
C+	Amplification	< 28	< 28

### Interpretation of results for test samples

- *CCHFV* cDNA is **detected** in a sample if its *Ct* value is defined in the result grid in the JOE/Yellow channel (it should be less than the *Ct* value specified in Table 4). Moreover, the fluorescence curve should cross the threshold line in the area of exponential fluorescence growth.
- *CCHFV* cDNA is **not detected** in a sample if its *Ct* value defined in the result grid in the FAM/Green channel is less than the specified boundary value (see Table 4), whereas the *Ct* value in the JOE/Yellow channel is not defined.
- The result is **invalid** if the *Ct* value of a sample in the JOE/Yellow channel is absent whereas the *Ct* value in the FAM/Green channel is either absent or greater than the specified boundary value. Repeat the PCR test for such a sample.

Table 4

**Results for test samples (Rotor-Gene 3000/6000/Q)**

Detection channel	Matrix	Boundary <i>Ct</i> values
FAM/Green	IC detection	< 28 (for blood serum and blood plasma)
		< 30 (for homogenates of ticks)
JOE/Yellow	<i>CCHFV</i> detection	< 38

### **Results of analysis are not taken into account in the following cases:**

1. If the negative result is obtained for all samples (except NCA) in all channels, PCR should be repeated. If the same result is obtained again, analysis should be repeated from the extraction stage. Negative result for NCA is normal.
2. If the *Ct* value of the Positive Control of Amplification (C+) detected in the JOE/Yellow channel is absent or greater than the boundary *Ct* value, PCR should be repeated for all samples in which specific cDNA was not detected.
3. If the *Ct* value of the Negative Control of Extraction (C–) in the JOE/Yellow channel and/or Negative Control of Amplification (NCA) in any channel is detected, the PCR should be repeated for all samples in which cDNA was detected.

### **AMPLIFICATION AND DATA ANALYSIS USING 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 optically transparent caps (e.g. Axygen, USA).

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

**NOTE:** The lamp is to be warmed up during 30 min before starting the experiment.

2. Start the iCycler iQ/iQ5 program.
3. Set plate setup (set the order of the tubes in the reaction chamber and the detection of fluorescent signal in **FAM** and **JOE** channels).
  - 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, JOE/HEX and ROX 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 in all the tubes through **FAM-490**, **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** (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.
4. Set the amplification program (see Table 5).

Table 5

**Amplification program for plate-types instruments**

<b>Step</b>	<b>Temperature, °C</b>	<b>Time</b>	<b>Fluorescence detection</b>	<b>Cycle repeats</b>
1	50	30 min	–	1
2	95	15 min	–	1
3	95	10 s	–	5
	54	30 s	–	
	72	15 s	–	
4	95	10 s	–	45
	50	35 s	FAM-490/FAM, JOE-530/JOE/HEX	
	72	15 s	–	

- For **iCycler iQ5** instrument, to set the protocol press the **Create New** or **Edit** button in the **Selected protocol** window of the **Workshop** module. Set the amplification

program and save the protocol by pressing the **Save&Exit Plate Editing** button. For further amplification runs, this program file in the **Protocol** can be selected (the protocol files are saved in the **Users** folder on default).

- For **iCycler iQ** instrument, set the amplification program by selecting **Edit Protocol** option of the **Workshop** module. To do this, set amplification parameters in the lower window (cycle repeats, time, cycling temperature). Indicate the fluorescent signal detection step: **Cycle 4 - Step 2** in the right window. Save the protocol, enter the file name in the **Protocol Filename** (with .tmo extension) and press the **Save this protocol** button (at the top of the display). For further amplification runs, this program file in the **View Protocol** of the **Library** module can be selected. After selecting or editing the appropriate program, press the **Run with selected plate setup** button to set the use of it.

5. Insert the previously prepared tubes into the module according to the required scheme:

**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.

- For the **iCycler iQ5** instrument, before starting the program, ensure that the protocol (**Selected Protocol**) and the plate setup (**Selected Plate Setup**) were selected correctly. Press the **Run** button to start the program. For the well factor measurement, select the **Use Persistent Well Factors** variant. For amplification use the same type of plastic as for calibration. Press the **Begin Run** button, name the experiment (results of the experiment will be saved in this file automatically), and press the **OK** button. Select the type of the caps **Seal Type – Domed Cap**, and the type of the tubes **Vessel Type – Tubes**.
- For the **iCycler iQ** instrument, before starting the program, ensure that the name of the protocol and the plate setup were selected correct in the **Run Prep** window. For the well factor measurement, select the **Persistent Plate** variant in the **Select well factor source** menu. Set the reaction mix volume in the window **Sample Volume – 25 µl**. Press the **Begin Run** button to start the program, name the experiment (results of the experiment will be saved in this file automatically), and press the **OK** button.

After finishing, start data analysis.

### Data analysis

Data analysis is based on presence or absence of the threshold cycle ( $C_t$ ) value in the corresponding column of the results grid. The fluorescence curve for the analyzed sample

should be S-shaped on the interval of exponential growth and cross the threshold line only once.

### Data processing

- For **iCycler iQ5** instrument, select the required file with data of analysis (in the **Data File** window of the **Workshop** module) and press the **Analyze** button. Select data for the corresponding channel in the module window. Data analysis mode **PCR Base Line Subtracted Curve Fit** should be selected (by default).
- For **iCycler iQ** instrument, activate the **View Post-Run Data** window in the **Library** module. In the **Data Files** window, select the required file with data of analysis and press the **Analyze Data** button. In the **PCR Quantification** option (**Select a Reporter** menu), select the icon of the corresponding channel. The **PCR Base Line Subtracted Curve Fit** data analysis mode should be selected (by default). In the **Threshold Cycle Calculation** menu, select manual setting the threshold line and automatic baseline calculation. Select the **Auto Calculated** in the **Baseline Cycles** submenu, select **User Defined** in the **Threshold Position** submenu. To set the threshold line level, click on it and drag it by cursor holding the left mouse button. Press the **Recalculate Threshold Cycles** button. **Ct** values will appear in the results grid.

#### Amplification data analysis for IC cDNA

1. Press the **FAM** button in the **Data Analysis** menu.
2. Select the **Baseline Threshold** option by pressing the right mouse button on the plot of fluorescence accumulation curves.
3. Select **User Defined, Select all, Edit Range** and set **Start Cycle = 2, Ending Cycle = 20** in the **Base Line Cycles** menu. Select **User Defined** and set **Threshold position = 50** in the **Crossing Threshold** menu. Press the **OK** button.
4. In the results grid (**Results** window), **Ct** values will appear.

#### Amplification data analysis for CCHFV cDNA

1. Press the **JOE** button in the **Data Analysis** menu.
2. Select the **Baseline Threshold** option by pressing the right mouse button on the plot of fluorescence accumulation curves.
3. Select **User Defined, Select all, Edit Range** and set **Start Cycle = 2, Ending Cycle = 20** in the **Base Line Cycles** menu. Select **User Defined** and set **Threshold position = 200** in the **Crossing Threshold** menu. Press the **OK** button.
4. In the results grid (**Results** window), **Ct** values will appear.

### Interpretation of results for control samples

The result of the analysis is considered reliable only if the results obtained for positive and negative controls of amplification as well as for the positive and negative controls of RNA extraction are correct. Results for positive and negative control samples should not exceed the *Ct* values specified in Table 6 (for iCycler iQ and iQ5 instruments (Bio-Rad, USA)).

Table 6

#### Results for controls (for iCycler iQ and iQ5 instruments)

Control	Stage for control	<i>Ct</i> in channel	
		FAM-490/FAM	JOE-530/JOE/HEX
		IC detection	CCHFV detection
C-	RNA extraction	< 31	absent
PCE	RNA extraction	< 31	< 33
NCA	Amplification	absent	absent
C+	Amplification	< 31	< 31

### Interpretation of results for test samples

- CCHFV cDNA is **detected** in a sample if its *Ct* value is defined in the result grid in the JOE-530/JOE/HEX channel (it should be less than *Ct* value specified in Table 7). Moreover, the fluorescence curve should cross the threshold line in the area of exponential fluorescence growth.
- CCHFV cDNA is **not detected** in a sample if its *Ct* value defined in the result grid in the FAM-490/FAM channel is less than the specified boundary *Ct* value (see Table 7), whereas the *Ct* value in the JOE-530/JOE/HEX channel is not defined.
- The result is **invalid** if the *Ct* value of a sample in the JOE-530/JOE/HEX channel is absent whereas the *Ct* value in the FAM-490/FAM channel is either absent or greater than the specified boundary *Ct* value. It is necessary to repeat the PCR test for such a sample.

Table 7

#### Results for test samples (iCycler iQ and iQ5 instruments)

Detection channel	Matrix	Boundary <i>Ct</i> values
FAM-490/FAM	IC detection	< 31 (for blood serum and blood plasma)
		< 33 (for homogenates of ticks)
JOE-530/JOE/HEX	CCHFV detection	< 39

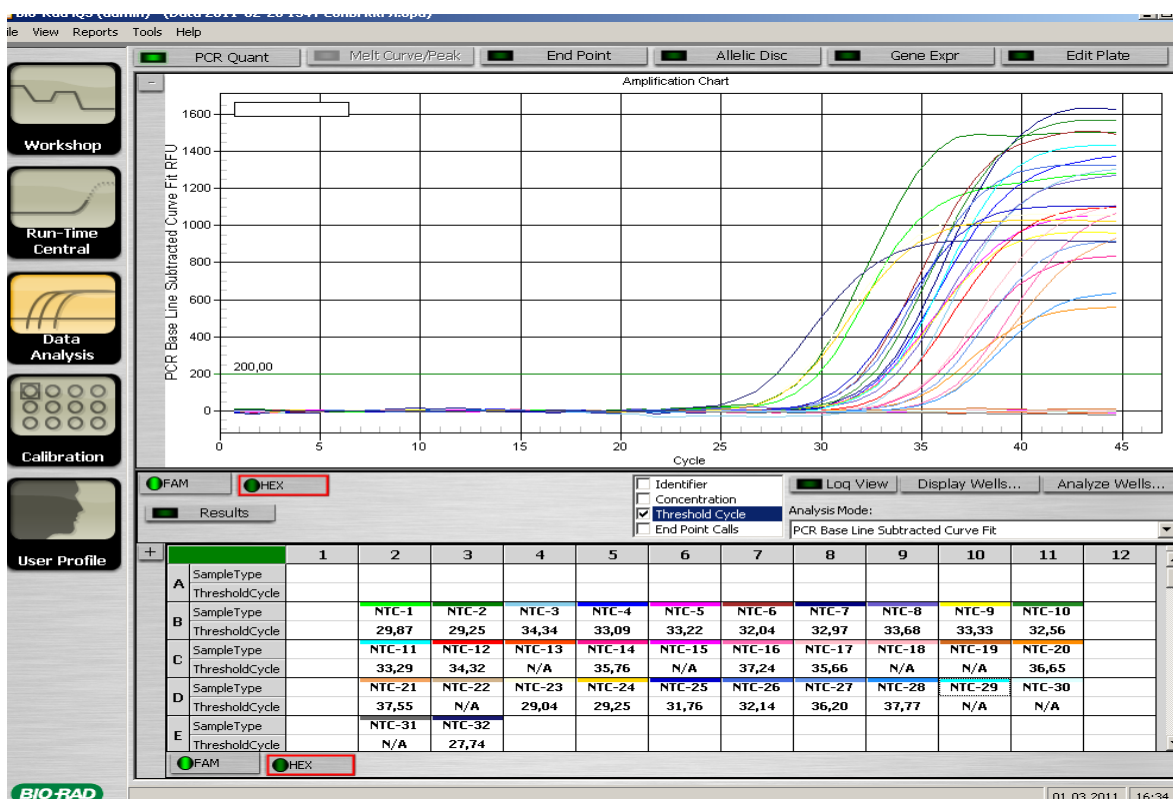
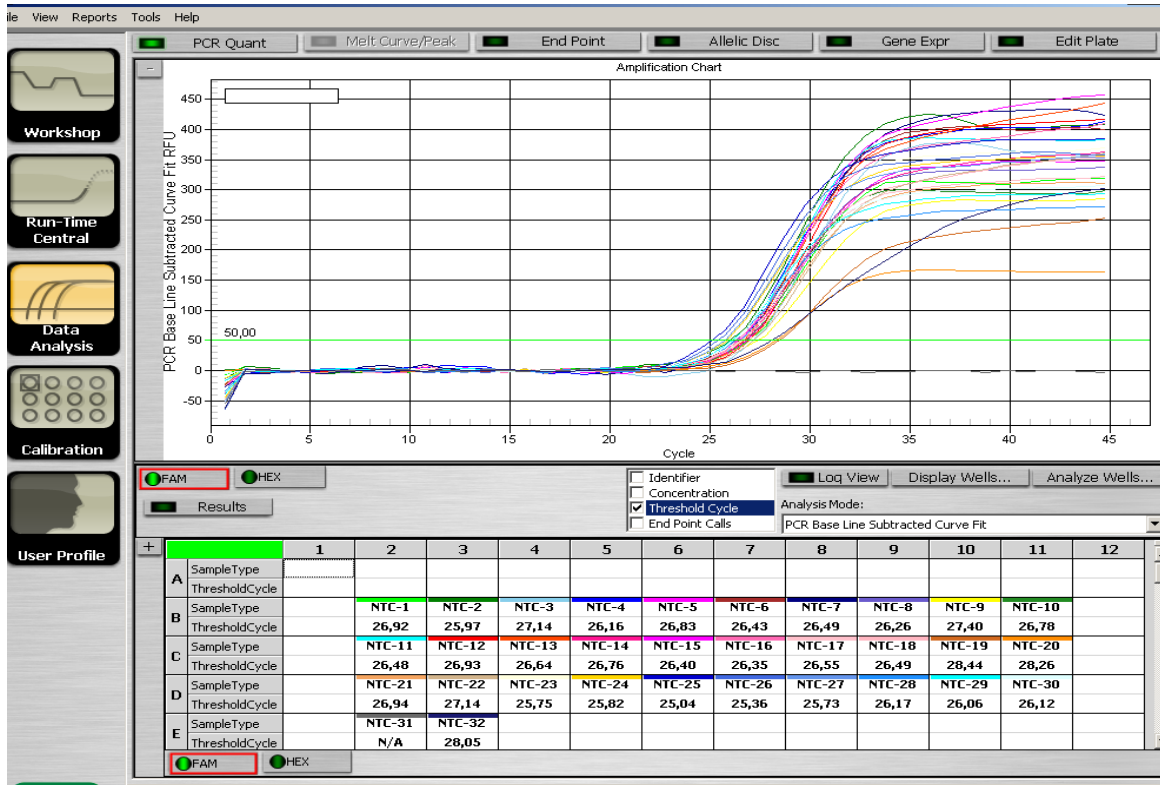
#### Results of analysis are not taken into account in the following cases:

1. If the negative result is obtained for all samples (except NCA), PCR should be repeated. If the same result is obtained again, analysis should be repeated from the extraction stage. Negative result for NCA is normal.
2. If the *Ct* value of the Positive Control of Amplification (C+) detected in the JOE-530/JOE/HEX channel is absent or greater than the boundary *Ct* value, PCR should be

repeated for all samples in which specific cDNA was not detected.

3. If the Ct value of the Negative Control of Extraction (C-) in the JOE-530/JOE/HEX channel and/or Negative Control of Amplification (NCA) in any channel is detected, the PCR should be repeated for all samples in which cDNA was detected.

### Example of obtained results for iCycler iQ5 instrument



## AMPLIFICATION AND DATA ANALYSIS USING Mx3000P (Stratagene, USA)

### Programming Mx3000P 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 optically transparent caps (e.g. Axygen, USA).

Program the thermocycler according to manufacturer's manual.

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 parameter FAM next to the **FAM filter set** item and parameter JOE 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. Name each sample in the **Well Information** window.
7. Set the parameters for the fluorescence detection of the tubes in the **Plate Setup** tab. Select all the wells with tested tubes, press the **Unknown** and the **Collect fluorescence data** buttons in **Well type** menu. Select fluorophores FAM and JOE.
8. Set amplification program in **Thermal Profile Setup** (See Table 8)

Table 8

#### Amplification program for plate-types instruments

Step	Temperature, °C	Time	Fluorescence detection	Cycle repeats
1	50	30 min	–	1
2	95	15 min	–	1
3	95	10 s	–	5
	54	30 s	–	
	72	15 s	–	
4	95	10 s	–	45
	50	35 s	FAM, JOE/HEX	
	72	15 s	–	

9. To start amplification program, click **Run**, check the correctness of the selected amplification program, then click **Start**. Tick the **Turn lamp off at end of run** window. Save the experiment.

## Data analysis

1. Open the saved file and proceed to the **Analysis** mode.
2. Activate the **Results** window in the menu.
3. Select the **Amplification plots** line in the **Area to analyze** block.
4. In **Threshold fluorescence** menu, set the threshold line at a level where the fluorescence curves are linear. It is recommended to select the threshold line level equal to **200** for FAM channel and **500** for JOE/HEX channel. Normally, the threshold line should cross only the sigmoid curves of signal accumulation of positive samples and controls and should not cross the baseline; otherwise, the threshold level should be raised.
5. Select the **Text report** line in the **Area to analyze** block.

## Interpretation of results for control samples

The result of the analysis is considered reliable only if the results obtained for positive and negative controls of amplification as well as for the positive and negative controls of RNA extraction are correct (see Table 9). Results for positive and negative control samples should not exceed the Ct values specified in Table 9 (for **Mx3000P** instrument (Stratagene, USA)).

Table 9

**Results for controls (for Mx3000P instrument)**

Control	Stage for control	Ct in channel	
		FAM	JOE/HEX
		IC detection	CCHFV detection
C-	RNA extraction	< 31	absent
PCE	RNA extraction	< 31	< 33
NCA	Amplification	absent	absent
C+	Amplification	< 31	< 31

## Interpretation of results for test samples

- **CCHFV** cDNA is **detected** in a sample if its Ct value is defined in the result grid in the JOE/HEX channel (it should be less than the Ct value specified in Table 10). Moreover, the fluorescence curve should cross the threshold line in the area of exponential fluorescence growth.
- **CCHFV** cDNA is **not detected** in a sample if its Ct value defined in the result grid in the FAM channel is less than the specified boundary value (see Table 10), whereas the Ct value in the JOE/HEX channel is not defined.
- The result is **invalid** if the Ct value of a sample in the JOE/HEX channel is absent whereas the Ct value in the FAM channel is either absent or greater than the specified

boundary Ct value. It is necessary to repeat the PCR test for such a sample.

Table 10

**Results for test samples (Mx3000P instrument)**

Detection channel	Matrix	Boundary Ct values
FAM	IC detection	< 31 (for blood serum and blood plasma)
		< 33 (for homogenates of ticks)
JOE/HEX	CCHFV detection	< 39

**Results of analysis are not taken into account in the following cases:**

1. If the negative result is obtained for all samples (except NCA), PCR should be repeated. If the same result is obtained again, analysis should be repeated from the extraction stage. Negative result for NCA is normal.
2. If the Ct value of the Positive Control of Amplification (C+) detected in the JOE/HEX channel is absent or greater than the boundary Ct value, PCR should be repeated for all samples in which specific cDNA was not detected.
3. If the Ct value of the Negative Control of Extraction (C-) in the JOE/HEX channel and/or Negative Control of Amplification (NCA) in any channel is detected, the PCR should be repeated for all samples in which cDNA was detected.

**Example of obtained results for Mx3000P instrument**

