

GUIDELINES

to AmpliSens® *TBEV, B.burgdorferi sl, A.phagocytophilum, E.chaffeensis / E.muris-FRT* PCR kit

for detection and differentiation of tick-borne infection pathogens:
RNA of *TBEV – tick-borne encephalitis virus, Borrelia burgdorferi sl – Ixodes tick-borne borreliosis (ITB) pathogen, Ehrlichia chaffeensis and Ehrlichia muris – human monocytic ehrlichiosis (HME) pathogens and DNA of Anaplasma phagocytophilum – human granulocytic anaplasmosis (HGA) pathogen in biological 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 using **AmpliSens® TBEV, B.burgdorferi sl, A.phagocytophilum, E.chaffeensis / E.muris-FRT** PCR kit for detection of RNA of *tick-borne encephalitis virus (TBEV)*, *Borrelia burgdorferi sl* (Ixodes tick-borne borreliosis (ITB) pathogen), *Ehrlichia chaffeensis* and *Ehrlichia muris* (human monocytic ehrlichiosis (HME) pathogens) and DNA of *Anaplasma phagocytophilum* (human granulocytic anaplasmosis (HGA) pathogen) in biological material (ticks, blood, cerebrospinal fluid, and autopsy material) 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).

Correspondence of fluorophores and detection channels

Channel for the fluorophore	Detection channel name for different instrument models ¹
FAM	FAM/Green
JOE	JOE/HEX/R6G/Yellow/Cy3
ROX	ROX/Orange/TxR

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 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 (detection through the bottom of the tube). or 0.1 ml PCR tubes.

Programming the thermocycler

1. Turn on the instrument, run the Rotor-Gene software.

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

2. Insert the tubes 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: The tubes with **PCR-mix-1-FRT TBEV, A.ph., E.ch. / E. m.** should be placed into the rotor first if both PCR-mixes-1 are used in the run

Programming the thermocycler

1. Click the **New** button in the software main menu.
2. In the opened window, select **Advanced** tab and click **Dual Labeled Probe/Hydrolysis probes** template. Activate the **New** button.
3. In the opened window select the **36-Well Rotor** (or **72-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, set the **Reaction volume – 25 µl**. Tick the **15 µl oil layer volume** option for Rotor-Gene 6000. Click the **Next** button.
5. In the opened window click the **Edit profile** button and set the temperature profile of the experiment as follows:

Table 1

Amplification program for Rotor-Gene 3000/6000 and Rotor-Gene Q

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
Hold	95	15 min	—	1
Cycling	95	10 s	—	5
	60	30 s	—	
	72	15 s	—	
Cycling2	95	10 s	—	40
	56	30 s	FAM/Green, JOE/Yellow, ROX/Orange	
	72	15 s	—	

6. Click the **OK** button.
7. Click the **Calibrate/Gain Optimisation...** button in the **New Run Wizard** window. In the opened window:
 - perform the calibration in the FAM/Green, JOE/Yellow, and ROX/Orange channels (click the **Calibrate Acquiring/Optimize Acquiring** button);
 - perform the calibration before the first detection (click the **Perform Calibration Before 1st Acquisition/Perform Optimization Before 1st Acquisition** button);
 - set channels calibration from 5FI to 10FI for all channels (the **Edit** button in the **Auto gain calibration channel settings** window). Click the **Close** button.
8. Click the **Next** button. Start the amplification program by activating the **Start Run** button.

9. Name the experiment and save it to the disk (the results of the run will be automatically saved in this file).
10. 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.

NOTE: Samples indicated as **None** will not be analyzed.

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 of the DNA sample 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 **Cycling A. FAM/Cycling A. Green, Show** buttons.
2. Cancel the automatic choice of the threshold line level **Threshold**.
3. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of main window (**Quantitation analysis**).
4. In the **CT Calculation** menu (in the right part of the window) indicate the threshold line level **0.03** in the **Threshold** box.
5. Choose the parameter **More settings/Outlier Removal** and set **5 %** for the value of negative samples threshold (**NTC/Threshold**).
6. In the results grid (the **Quantitation Results** window) one will be able to see the *Ct* values.

Amplification data analysis in the JOE/Yellow channel:

1. Activate the button **Analysis** in the menu, select the mode of the analysis **Quantitation**, activate the **Cycling A JOE/Cycling A Yellow, Show** buttons.
2. Cancel the automatic choice of the threshold line level **Threshold**.
3. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of main window (**Quantitation analysis**).
4. In the **CT Calculation** menu (in the right part of the window) indicate the threshold line level **0.03** in the **Threshold** box.
5. Choose the parameter **More settings/Outlier Removal** and set **5 %** for the value of negative samples threshold (**NTC/Threshold**)

6. In the results grid (the **Quantitation Results** window) one will be able to see the *Ct* values.

Amplification data analysis in the ROX/Orange channel:

1. Select the **Analysis** sign in the main menu, select the **Quantitation** mode. Activate the **Cycling A ROX/Cycling A Orange** button. Click **Show**.
2. Cancel the automatic choice of the threshold line level **Threshold**.
3. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of main window (**Quantitation analysis**).
4. In the **CT Calculation** menu (in the right part of the window) indicate the threshold line level **0.03** in the **Threshold** box.
5. Choose the parameter **More settings/Outlier Removal** and set **5 %** for the value of negative samples threshold (**NTC/Threshold**)
6. In the results grid (the **Quantitation Results** window) one will be able to see the *Ct* values.

NOTE: If the fluorescence curves do not show exponential growth, set **10 %** for the value of negative samples threshold (**NTC/Threshold**)

Results interpretation

The result of the PCR analysis is considered reliable only if the results of Positive and Negative Controls of amplification as well as Negative Control of extraction are correct (Table 2). The results of Positive and Negative controls should not exceed *Ct* values specified in Table 2.

Table 2

Results for controls

Control	Stage for control	Ct value		
		FAM/Green	JOE/Yellow	ROX/Orange
PCR-mix-1-FRT TBEV, A.ph., E.ch. / E.m.				
		Detection of TBEV	Detection of A.phagocytophilum	Detection of E.chaffeensis / E.muris
C-	RNA/DNA extraction	Absent	Absent	Absent
NCA	PCR	Absent	Absent	Absent
C+TBEV, B.b. sl, A.ph., E.ch. / E.m. / STI	PCR	<27	<27	<27
PCR-mix-1-FRT B.b. sl / IC				
		Detection of IC	Detection of B.burgdorferi sl	—
C-	RNA/DNA extraction	<30	Absent	—
NCA	PCR	Absent	Absent	—
C+TBEV, B.b. sl, A.ph., E.ch. / E.m. / STI	PCR	<27	<27	—

Principle of interpretation is the following:

- *TBEV* cDNA is **detected** if the *Ct* value determined in the FAM/Green channel (with the use of **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.***) is less than the boundary *Ct* value specified in the Table 3.
- *A.phagocytophilum* DNA is **detected** if the *Ct* value determined in the JOE/Yellow/HEX channel (with the use of **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.***) is less than the boundary *Ct* value specified in the Table 3.
- *E.chaffeensis / E.muris* cDNA is **detected** if the *Ct* value determined in the ROX/Orange channel (with the use of **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.***) is less than the boundary *Ct* value specified in the Table 3.
- *Borrelia burgdorferi sl.* cDNA is **detected** in a sample if the *Ct* value determined in the JOE/Yellow/HEX channel (with the use of **PCR-mix-1-FRT *B.b. sl / IC***) is less than the boundary *Ct* value specified in the Table 3.

Moreover, the fluorescence curve of the sample should cross the threshold line in the area of typical exponential growth of fluorescence

- cDNA/DNA of the above-mentioned microorganisms **are not detected** if the *Ct* value determined in the FAM/Green channel (with the use of **PCR-mix-1-FRT *B.b. sl / IC***) is less than the boundary *Ct* value specified in Table 3, whereas the *Ct* value is not determined (absent) in the channel assigned for detection of the specific pathogen.
- The result is **invalid** if the *Ct* value is not determined (absent) in the channels for detection of specific signal, whereas the *Ct* value in the channel for the FAM fluorophore (with the use of **PCR-mix-1-FRT *B.b. sl / IC***) is also not determined (absent) or greater than the specified boundary *Ct* value. In such cases, the PCR analysis should be repeated for such samples.

Table 3

Results for test samples obtained with Rotor-Gene 3000/6000 and Rotor-Gene Q

PCR-mix-1-FRT	Signal in channel (<i>Ct</i>)		
	FAM/Green	HEX/Yellow	ROX/Orange
<i>TBEV, A.ph., E.ch. / E.m.</i>	Detection of <i>TBEV</i>	Detection of <i>A.phagocytophilum</i>	Detection of <i>E.chaffeensis / E.muris</i>
	<38	<38	<38
<i>B.b. sl / IC</i>	Detection of IC	Detection of <i>B.burgdorferi sl</i>	—
	<35	<38	—

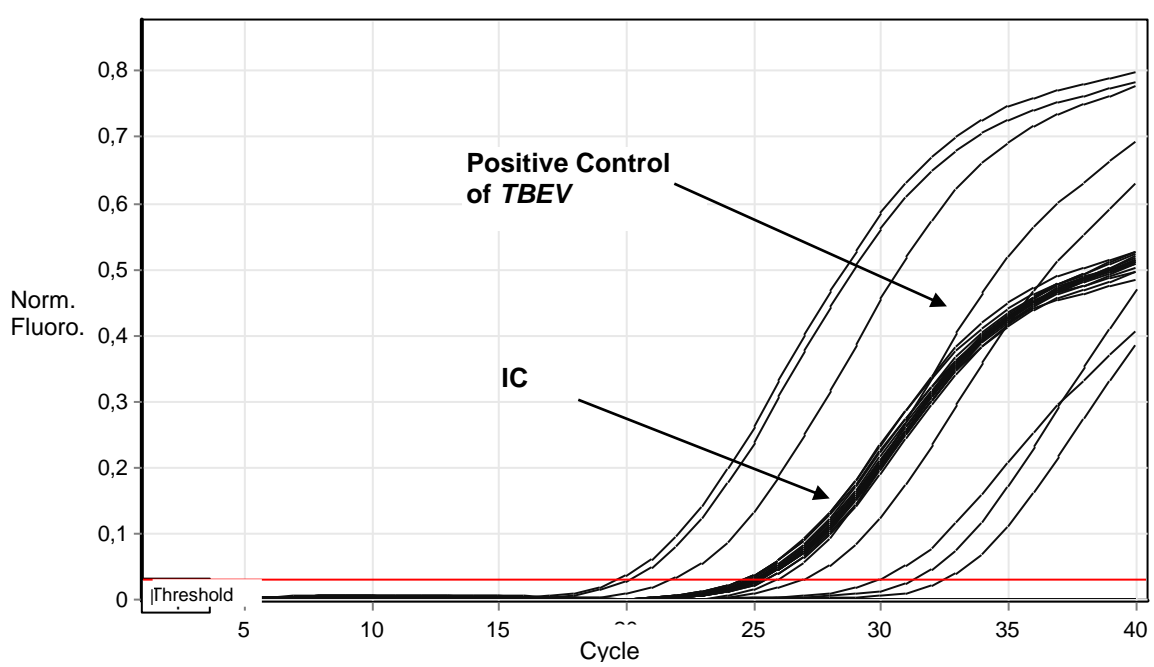
Troubleshooting

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

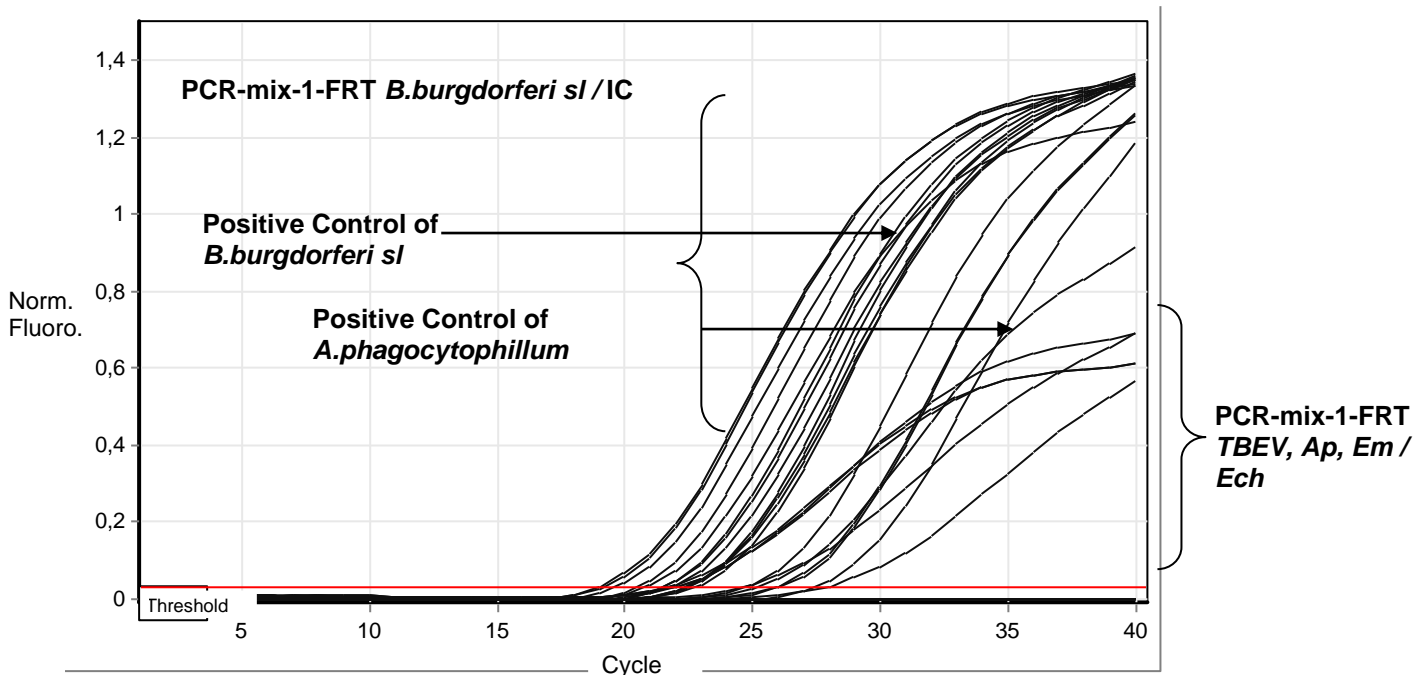
- If the negative result in all channels was obtained for the samples (except for NCA), the amplification and detection should be repeated for such samples. If the same result is obtained once again, repeat the analysis of the sample starting from the extraction stage. The negative result obtained in all channels is accepted as normal for NCA.
- If the *Ct* value determined for the Positive Control of amplification ($C+_{TBEV, B.b. sl, A.ph., E.ch. / E.m. / STI}$) is absent or greater than the specified boundary *Ct* value in the FAM/Green, JOE/Yellow, ROX/Orange channels (with the use of **PCR-mix-1-FRT TBEV, A.ph., E.ch. / E.m.**) or in the FAM/Green and JOE/Yellow channels (with the use of **PCR-mix-1-FRT B.b. sl / IC**), the amplification should be repeated for all samples in which specific cDNA/DNA was not found in the appropriate channel.
- If the *Ct* value for the Negative Control of extraction (C-) in the FAM/Green, JOE/Yellow, ROX/Orange channels (with the use of **PCR-mix-1-FRT TBEV, A.ph., E.ch. / E.m.**) and in the JOE/Yellow channel (with the use of **PCR-mix-1-FRT B.b. sl / IC**) and/or Negative Control of amplification (NCA) (in any channel) is detected in the results grid, PCR analysis should be repeated for all samples in which specific cDNA/DNA was detected in the appropriate channel.

Examples of obtained results

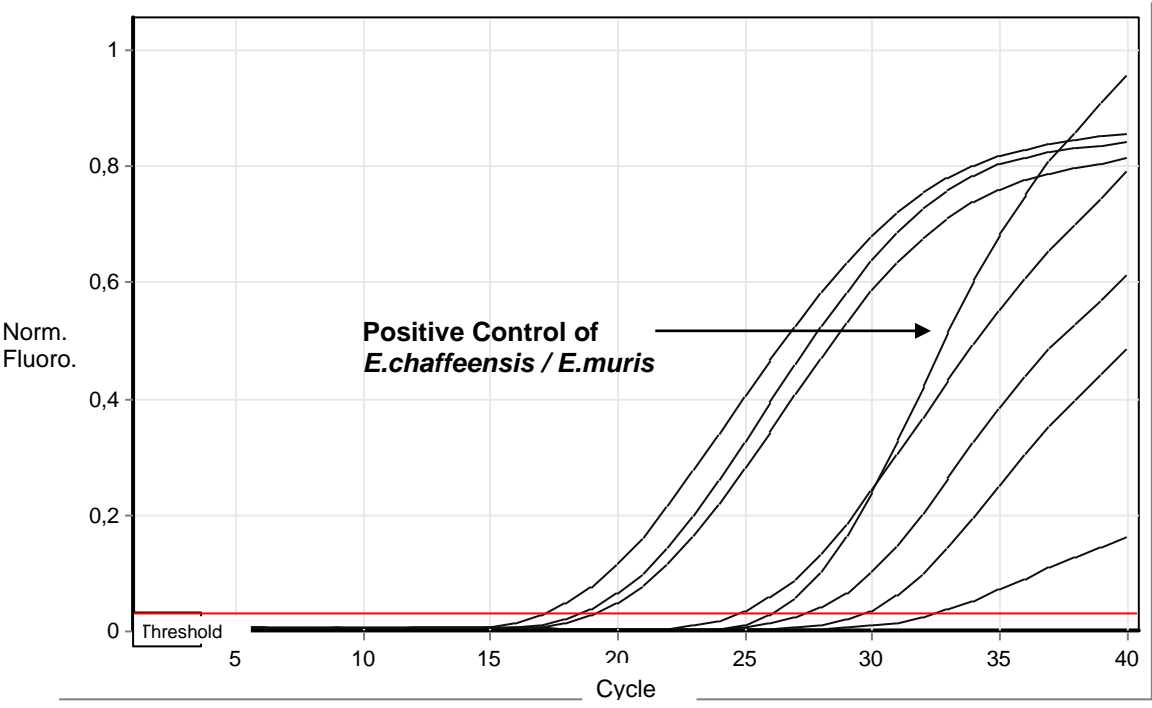
FAM/Green channel (amplification of *TBEV* cDNA for PCR-mix-1-FRT *TBEV*, *A.ph.*, *E.ch.* / *E.m.* and amplification of IC cDNA for PCR-mix-1-FRT *B.b. sl* / IC)



JOE channel (amplification of *A.phagocytophilum* DNA for PCR-mix-1-FRT *TBEV*, *A.ph.*, *E.ch.* / *E.m.* and amplification of *B.burgdorferi sl* cDNA for PCR-mix-1-FRT *B.b. sl* / IC)



**ROX channel (amplification of *E.chaffeensis* / *E.muris* cDNA for PCR-mix-1-FRT
TBEV, A.ph., E.ch. / E.m.)**



AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ5 and iCycler iQ (Bio-Rad, USA) INSTRUMENTS

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 *iCycler iQ/iQ5* program.

3. Define the plate setup (the order of the tubes in the reaction chamber and the detection of fluorescent signal in all tubes in FAM, JOE/HEX and ROX channels if **PCR-mix-1-FRT TBEV, A.ph., E.ch. / E. m.** is used or in FAM and JOE/HEX channels if **PCR-mix-1-FRT B.b. sl / IC** is used).

— For **iCycler iQ 5** instrument. Click the **Create New** or **Edit** button in the **Selected Plate Setup** window of the **Workshop** module. 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**. Save the set plate setup by clicking the **Save &Exit Plate Editing** button.

— For **iCycler iQ** instrument. Edit the plate setup in the **Edit Plate Setup** window of the **Workshop** module. To do this, define the samples' position in the reaction module in the **Samples: Whole Plate Loading** option and name each sample in the **Sample Identifier** window. In the **Select and Load Fluorophores** option, assign the detection of fluorescence signal in all tubes in FAM, JOE, and ROX channels if **PCR-mix-1-FRT TBEV, A.ph., E.ch. / E. m.** is used or in FAM and JOE channels if **PCR-mix-1-FRT B.b. sl / IC** is used.

— 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 and select the needed 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 4).

Amplification program for iCycler iQ and iQ5

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
1	95	15 min	—	1
2	95	10 s	—	5
	60	35 s	—	
	72	15 s	—	
3	95	10 s	—	40
	56	35 s	FAM, JOE/HEX, ROX	
	72	15 s	—	

- For **iCycler iQ5** instrument. In the **Selected Protocol** window of the **Workshop** module click the **Create New** or **Edit** buttons. Set the amplification parameters and save the protocol by activating the **Save&Exit Protocol Editing** button. Later, for further runs one may select the file containing this program in the **Protocol** box (the protocol files are saved in the **Users** folder on default).
 - For **iCycler iQ** instrument. To set the amplification program, select the **Edit Protocol** option of the **Workshop** module. Set the number of cycles, time, and temperature in the bottom window. Specify readout step for the fluorescence signal in the window at the right as follows: **Cycle 3 – Step**. To save the protocol, name the file in the **Protocol Filename** window (use the .tmo file suffix) and click the **Save this protocol** button (in the upper part of the screen). Later, for further runs one may select the file containing this program from the **View Protocol** tab of the **Library** module. Press the **Run with selected plate setup** button to save and activate the created program.
5. Insert the prepared tubes into the reaction chamber according to the specified tube order.

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.

- For **iCycler iQ5** instrument. Before a run 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. Make sure that amplification and calibration are performed with the same type of plastic consumables. Click the **Begin Run** button, name the experiment (result data will be automatically saved to this file) and click **OK**. Select the caps type (**Seal Type**) as **Domed cap**, and the tubes type (**Vessel Type**) as **Tubes**.

- For **iCycler iQ** instrument. Before a run it is obligatory to check in the **Run Prep** window if the selected protocol and the plate scheme are correct For the well factors measurement the select the **Persistent Plate** option in the **Select well factor source** menu. Set the reaction volume (**Sample Volume**) as **25 µl**. Click the **Begin Run** button, name the experiment (the results of this experiment will be automatically saved in this file) and click **OK**.

Analyze results after the amplification program is completed.

Data analysis:

The results are interpreted by the presence (or absence) of the *Ct* (threshold cycle) value in the corresponding column of the results table Moreover, the fluorescence curve of the sample should have the area of typical exponential growth (S-shape) of fluorescence and once cross the threshold line.

- For **iCycler iQ** instrument. Activate the **View Post-Run** window in the **Library** module. Select the needed file with the analysis data in the **Data Files** window and click the **Analyze Data** button. Select the desired channel in the **PCR Quantification** option of the **Select a Reporter** menu. Make sure that **PCR Base Line Subtracted Curve Fit** mode is activated (selected by default). In the **Threshold Cycle Calculation** menu indicate that the threshold is to be set manually and the base line is to be calculated automatically. To do this, select **Auto Calculated** in the **Baseline Cycles** submenu and select **User Define** in the **Threshold Position** submenu. To set the threshold line it should be move with the cursor while the left mouse button is pushed. When data obtained with **PCR-mix-1-FRT TBEV, A.ph., E.ch. / E. m.** are processed, disable the wells that were analyzed with **PCR-mix-1-FRT B.b. sl / IC** in the **Display wells** menu. When data obtained with **PCR-mix-1-FRT B.b. sl / IC** are processed, disable the wells that were analyzed with **PCR-mix-1-FRT TBEV, A.ph., E.ch. / E. m.** in the **Display wells** menu. Click the **Recalculate Threshold Cycles** button. *Ct* values will appear in the results grid.
- For **iQ5** instrument. Select the needed file with the analysis data (in the **Data File** window of the **Workshop** module) and click the **Analyze** button. Select data obtained in the required channel in the window of the module. Make sure that the **PCR Base Line Subtracted Curve Fit** mode is activated (selected by default).

Amplification data analysis in case of using PCR-mix-1-FRT TBEV, A.ph., E.ch. /

E. m:

Data analysis of TBEV cDNA amplification

1. Click the **FAM** button in the **Data Analysis** menu.
2. Select the **Baseline Threshold** option using the right mouse button on the plot of the fluorescence curves.
3. Set following parameters: select **User Defined, Select all,** and **Edit Range** in the **Base Line Cycles** menu and set **Start Cycle=2, Ending Cycle=25**; select **User Defined** in the **Crossing Threshold** menu and set **Threshold Position=200**. Click **OK**.
4. In the results grid (the **Results** window) one will be able to see the *Ct* values.

Data analysis of A.phagocytophilum DNA amplification

1. Click the **JOE** button in the **Data Analysis** menu.
2. Select the **Baseline Threshold** option using the right mouse button on the plot of the fluorescence curves.
3. Set following parameters: select **User Defined, Select all,** and **Edit Range** in the **Base Line Cycles** menu and set **Start Cycle=2, Ending Cycle=25**; select **User Defined** in the **Crossing Threshold** menu and set **Threshold Position=100**. Click **OK**.
4. In the results grid (the **Results** window) one will be able to see the *Ct* values.

Data analysis of E.muris, E.chaffensis cDNA amplification

1. Click the **ROX** button in the **Data Analysis** menu.
2. Select the **Baseline Threshold** option using the right mouse button on the plot of the fluorescence curves.
3. Set following parameters: select **User Defined, Select all,** and **Edit Range** in the **Base Line Cycles** menu and set **Start Cycle=2, Ending Cycle=25**; select **User Defined** in the **Crossing Threshold** menu and set **Threshold Position=100**. Click **OK**.
4. In the results grid (the **Results** window) one will be able to see the *Ct* values.

Amplification data analysis in case of using PCR-mix-1-FRT B.b. sl/IC:

Data analysis of IC amplification

1. Click the **FAM** button in the **Data Analysis** menu.
2. Select the **Baseline Threshold** option using the right mouse button on the plot of the fluorescence curves.
3. Set following parameters: select **User Defined, Select all,** and **Edit Range** in the **Base Line Cycles** menu and set **Start Cycle=2, Ending Cycle=25**; select **User Defined** in the **Crossing Threshold** menu and set **Threshold Position=50**. Click **OK**.

4. In the results grid (the **Results** window) one will be able to see the *Ct* values.

Data analysis of *B.burgdorferi* *sl* DNA amplification

1. Click the **JOE** button in the **Data Analysis** menu.
2. Select the **Baseline Threshold** option using the right mouse button on the plot of the fluorescence curves.
3. Set following parameters: select **User Defined**, **Select all**, and **Edit Range** in the **Base Line Cycles** menu and set **Start Cycle=2**, **Ending Cycle=25**; select **User Defined** in the **Crossing Threshold** menu and set **Threshold Position=100**. Click **OK**.
4. In the results grid (the **Results** window) one will be able to see the *Ct* values.

Result interpretation

The result of the PCR analysis is considered reliable only if the results of Positive and Negative Controls of amplification as well as Negative Control of extraction are correct (Table 5). The results of Positive and Negative controls should not exceed *Ct* values specified in Table 5.

Table 5

Results for controls

Control	Stage for control	Ct value		
		FAM	HEX	ROX
PCR-mix-1-FRT <i>TBEV</i>, <i>A.ph.</i>, <i>E.ch.</i> / <i>E.m.</i>				
		Detection of <i>TBEV</i>	Detection of <i>A.phagocytophilum</i>	Detection of <i>E.chaffeensis</i> / <i>E.muris</i>
C-	RNA/DNA extraction	Absent	Absent	Absent
NCA	PCR	Absent	Absent	Absent
C+ <i>TBEV</i> , <i>B.b. sl</i> , <i>A.ph.</i> , <i>E.ch.</i> / <i>E.m.</i> / STI	PCR	<30	<31	<30
PCR-mix-1-FRT <i>B.b. sl</i> / IC				
		Detection of IC	Detection of <i>B.burgdorferi sl</i>	—
C-	RNA/DNA extraction	<33	Absent	—
NCA	PCR	Absent	Absent	—
C+ <i>TBEV</i> , <i>B.b. sl</i> , <i>A.ph.</i> , <i>E.ch.</i> / <i>E.m.</i> / STI	PCR	<30	<30	—

Principle of interpretation is the following:

- *TBEV* cDNA is **detected** if the *Ct* value determined in the FAM channel (with the use of **PCR-mix-1-FRT *TBEV*, *A.ph.*, *E.ch.* / *E.m.***) is less than the boundary *Ct* value specified in the Table 6.
- *A.phagocytophilum* DNA is **detected** if the *Ct* value determined in the JOE/HEX

channel (with the use of **PCR-mix-1-FRT TBEV, A.ph., E.ch. / E.m.**) is less than the boundary *Ct* value specified in the Table 6.

- *E.chaffeensis / E.muris* cDNA is **detected** if the *Ct* value determined in the ROX channel (with the use of **PCR-mix-1-FRT TBEV, A.ph., E.ch. / E.m.**) is less than the boundary *Ct* value specified in the Table 6.
- *Borrelia burgdorferi sl.* cDNA is **detected** in a sample if the *Ct* value determined in the JOE/HEX channel (with the use of **PCR-mix-1-FRT B.b. sl / IC**) is less than the boundary *Ct* value specified in the Table 6.

Moreover, the fluorescence curve of the sample should cross the threshold line in the area of typical exponential growth of fluorescence

- cDNA/DNA of the above-mentioned microorganisms **are not detected** if the *Ct* value determined in the FAM channel (with the use of **PCR-mix-1-FRT B.b. sl / IC**) is less than the boundary *Ct* value specified in Table 6, whereas the *Ct* value is not determined (absent) in the channel assigned for detection of the specific pathogen.
- The result is **invalid** if the *Ct* value is not determined (absent) in the channels for detection of specific signal, whereas the *Ct* value in the FAM channel (with the use of **PCR-mix-1-FRT B.b. sl / IC**) is also not determined (absent) or greater than the specified boundary *Ct* value. In such cases, the PCR analysis should be repeated for such samples.

Table 6

Results for test samples obtained with iCycler iQ and iQ5

PCR-mix-1-FRT	Signal in channel (<i>Ct</i>)		
	FAM	HEX	ROX
<i>TBEV, A.ph., E.ch. / E.m.</i>	Detection of <i>TBEV</i>	Detection of <i>A.phagocytophilum</i>	Detection of <i>E.chaffeensis / E.muris</i>
	<39	<39	<39
<i>B.b. sl / IC</i>	Detection of IC	Detection of <i>B.burgdorferi sl</i>	—
	<38	<39	—

Troubleshooting

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

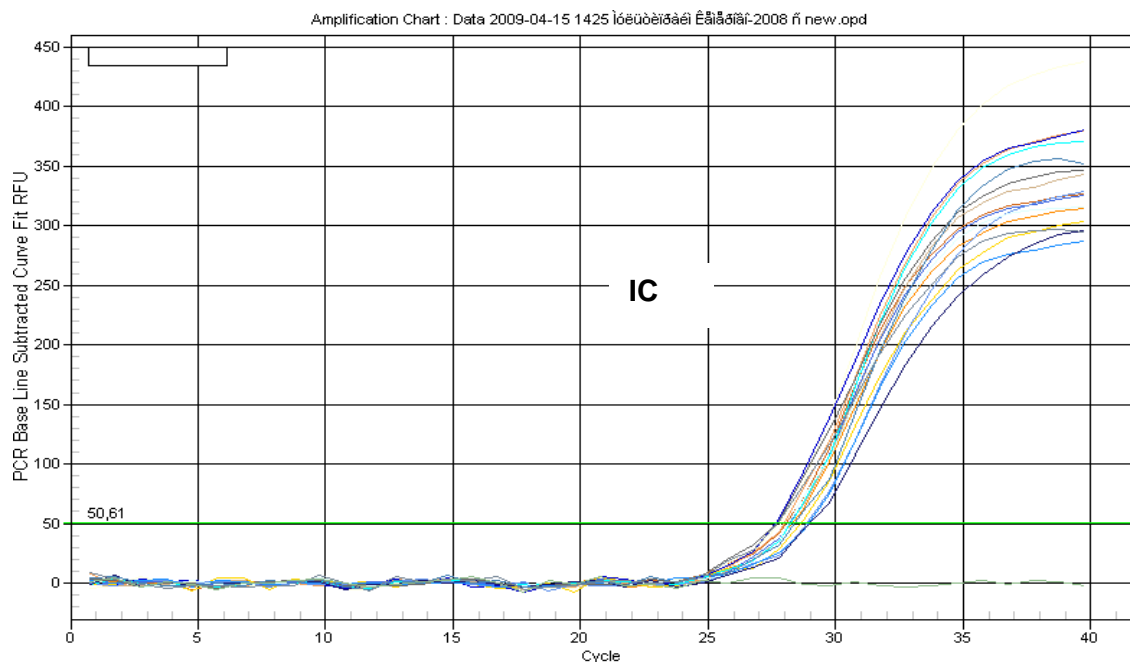
- If the negative result in all channels was obtained for the samples (except for NCA), the amplification and detection should be repeated for such samples. If the same result is obtained once again, repeat the analysis of the sample starting from the extraction stage. The negative result obtained in all channels is accepted as normal for NCA.
- If the *Ct* value determined for the Positive Control of amplification (C+*TBEV, B.b. sl, A.ph., E.ch. / E.m. / STI*) is absent or greater than the specified boundary *Ct* value in the FAM, JOE/HEX,

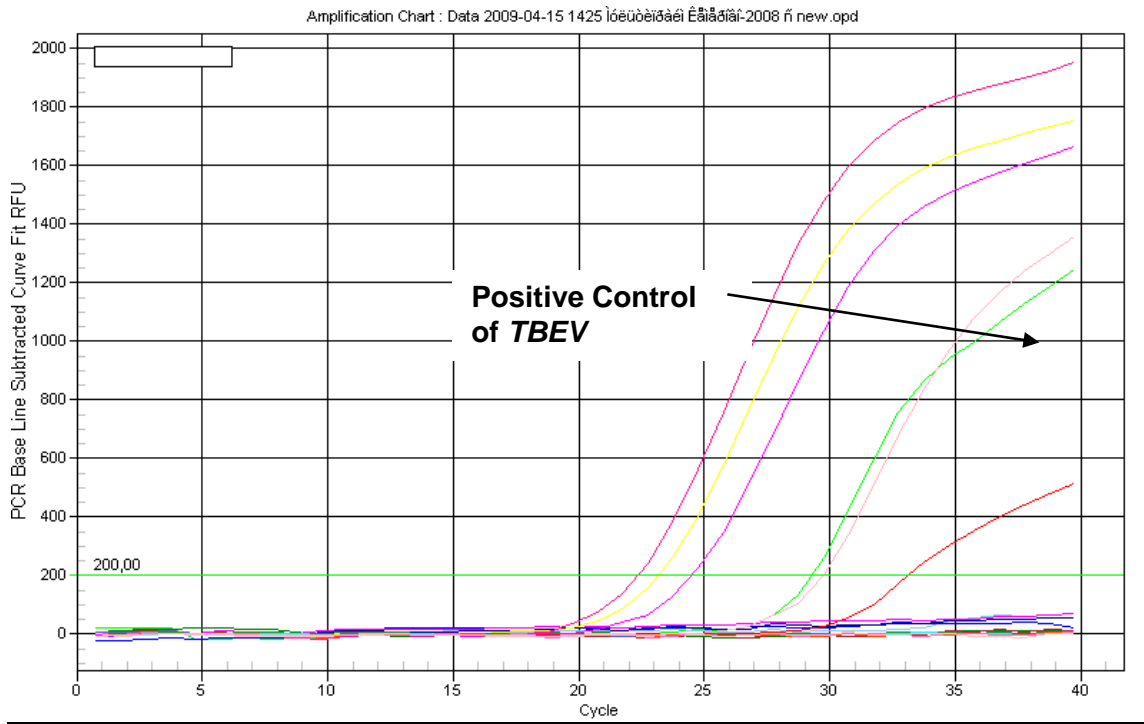
ROX channels (with the use of **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.***) or in the FAM and JOE/HEX channels (with the use of **PCR-mix-1-FRT *B.b. sl / IC***), the amplification should be repeated for all samples in which specific cDNA/DNA was not found in the appropriate channel.

- If the *Ct* value for the Negative Control of extraction (C-) in the FAM/Green, JOE/Yellow, ROX/Orange channels (with the use of **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.***) and in the JOE/Yellow channel (with the use of **PCR-mix-1-FRT *B.b. sl / IC***) and/or Negative Control of amplification (NCA) (in any channel) is detected in the results grid, PCR analysis should be repeated for all samples in which specific cDNA/DNA was detected in the appropriate channel.

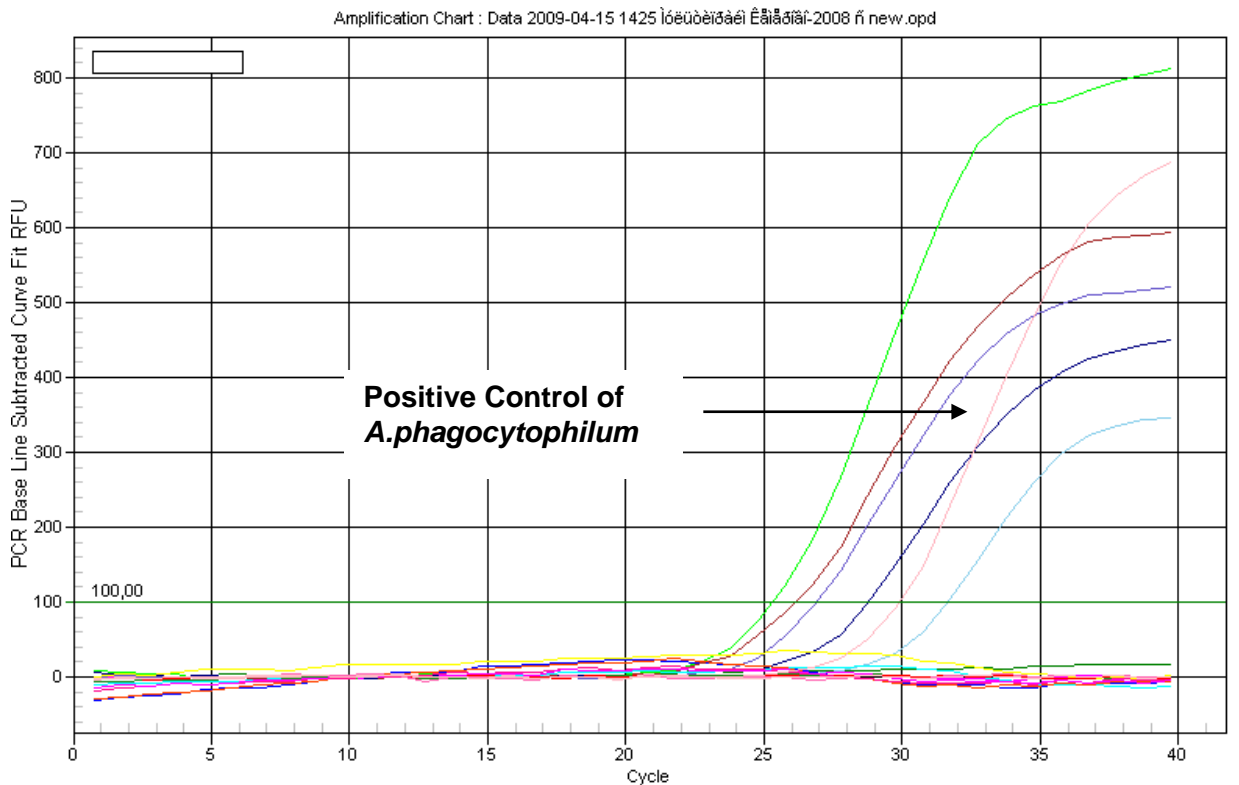
Examples of obtained results

FAM channel (amplification of *TBEV* cDNA for PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.* and amplification of IC cDNA for PCR-mix-1-FRT *B.b. sl / IC*)





JOE channel (amplification of *A.phagocytophilum* DNA for PCR-mix-1-FRT TBEV, *A.ph.*, *E.ch.* / *E.m.* and amplification of *B.burgdorferi* *sl* cDNA for PCR-mix-1-FRT *B.b. sl* / IC)



AMPLIFICATION AND DATA ANALYSIS USING Mx3000P (Stratagene, USA)

INSTRUMENT

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

Program the instrument according to the *Instruction Manual* provided by the manufacturer.

1. Switch on the instrument. Start the **Stratagene Mx3000P** program.
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. In the **Dye Assignment** tab set **FAM** parameter next to the **FAM filter set** item, **JOE** parameter next to the **HEX/JOE filter set** item, and **ROX** parameter next to the **ROX filter set** item.
5. Set the fluorescence detection parameters in the **Plate Setup** menu. To do this, select all the cells with **PCR-mix-1-FRT TBEV, A.ph., E.ch. / E. m.** test tubes and mark them as **Unknown** in the **Well type** window. Select **FAM**, **JOE**, and **ROX** fluorophores in the **Collect fluorescence data** option. Then, select all the cells with **PCR-mix-1-FRT B.b. sl / IC** test tubes and mark them as **Unknown** in the **Well type** window. Select **FAM** and **JOE** fluorophores in the **Collect fluorescence data** option.
6. Enter the names of the test samples in the **Well Information** window.
7. In the **Plate Setup** menu, select all cells with the tubes and select **Unknown** in the **Well type** drop-down window. Activate **FAM**, **JOE**, and **ROX** fluorophores in the **Collect fluorescence data** field.
8. Set the amplification program in **Thermal Profile Setup** (see Table 7).

Table 7

Amplification program for Mx3000P instrument

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
1	95	15 min	—	1
2	95	10 s	—	5
	60	35 s	—	
	72	15 s	—	
3	95	10 s	—	40
	56	35 s	FAM, JOE/HEX, ROX	
	72	15 s	—	

9. Select **Run** in the menu. Ensure that amplification program is correct. Click the **Start** button. Tick the **Turn lamp off at end of run** box. Save the experiment.

Data analysis:

1. Open the saved data file and shift to **Analysis** mode.
2. Activate the **Results** window in the menu.
3. Select the **Amplification plots** line in the **Area to analyze** unit.
4. In the **Threshold fluorescence** unit set the threshold line at a level where fluorescence curves are linear. It is recommended to set the threshold level as **500** for all channels. Normally, the threshold line should cross only sigmoid curves of positive samples and controls and should not cross the base line. Otherwise, raise the threshold.
5. Select the **Text report** line in the **Area to analyze** unit.

Result interpretation

The result of the PCR analysis is considered reliable only if the results of Positive and Negative Controls of amplification as well as Negative Control of extraction are correct (Table 8). The results of Positive and Negative controls should not exceed *Ct* values specified in Table 8.

Table 8

Results for controls

Control	Stage for control	Ct value		
		FAM	JOE	ROX
PCR-mix-1-FRT TBEV, A.ph., E.ch. / E.m.				
		Detection of TBEV	Detection of A.phagocytophilum	Detection of E.chaffeensis / E.muris
C-	RNA/DNA extraction	Absent	Absent	Absent
NCA	PCR	Absent	Absent	Absent
C+ <i>TBEV, B.b. sl, A.ph., E.ch. / E.m. / STI</i>	PCR	<30	<31	<30
PCR-mix-1-FRT B.b. sl / IC				
		Detection of IC	Detection of B.burgdorferi sl	-
C-	RNA/DNA extraction	<34	Absent	-
NCA	PCR	Absent	Absent	-
C+ <i>TBEV, B.b. sl, A.ph., E.ch. / E.m. / STI</i>	PCR	<30	<30	-

Principle of interpretation is the following:

- *TBEV* cDNA is **detected** if the *Ct* value determined in the FAM channel (with the use of **PCR-mix-1-FRT TBEV, A.ph., E.ch. / E.m.**) is less than the boundary *Ct* value specified in the Table 9.
- *A.phagocytophilum* DNA is **detected** if the *Ct* value determined in the JOE/HEX channel

(with the use of **PCR-mix-1-FRT TBEV, A.ph., E.ch. / E.m.**) is less than the boundary *Ct* value specified in the Table 9.

- *E.chaffeensis / E.muris* cDNA is **detected** if the *Ct* value determined in the ROX channel (with the use of **PCR-mix-1-FRT TBEV, A.ph., E.ch. / E.m.**) is less than the boundary *Ct* value specified in the Table 9.
- *Borrelia burgdorferi sl.* cDNA is **detected** in a sample if the *Ct* value determined in the JOE/HEX channel (with the use of **PCR-mix-1-FRT B.b. sl / IC**) is less than the boundary *Ct* value specified in the Table 9.

Moreover, the fluorescence curve of the sample should cross the threshold line in the area of typical exponential growth of fluorescence

- cDNA/DNA of the above-mentioned microorganisms **are not detected** if the *Ct* value determined in the FAM channel (with the use of **PCR-mix-1-FRT B.b. sl / IC**) is less than the boundary *Ct* value specified in Table 9, whereas the *Ct* value is not determined (absent) in the channel assigned for detection of the specific pathogen.
- The result is **invalid** if the *Ct* value is not determined (absent) in the channels for detection of specific signal, whereas the *Ct* value in the FAM channel (with the use of **PCR-mix-1-FRT B.b. sl / IC**) is also not determined (absent) or greater than the specified boundary *Ct* value. In such cases, the PCR analysis should be repeated for such samples.

Table 9

Results for test samples obtained with Mx3000P

PCR-mix-1-FRT	Signal in channel (<i>Ct</i>)		
	FAM	HEX	ROX
<i>TBEV, A.ph., E.ch. / E.m.</i>	Detection of <i>TBEV</i>	Detection of <i>A.phagocytophilum</i>	Detection of <i>E.chaffeensis / E.muris</i>
	<39	<39	<39
<i>B.b. sl / IC</i>	Detection of IC	Detection of <i>B.burgdorferi sl</i>	–
	<38	<39	–

Troubleshooting

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

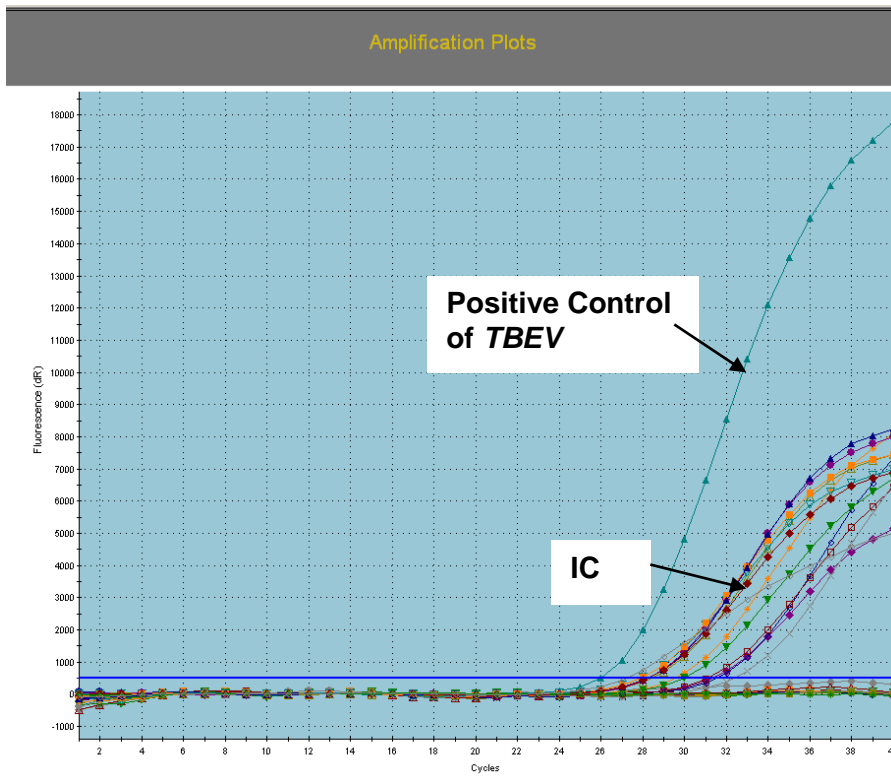
- If the negative result in all channels was obtained for the samples (except for NCA), the amplification and detection should be repeated for such samples. If the same result is obtained once again, repeat the analysis of the sample starting from the extraction stage. The negative result obtained in all channels is accepted as normal for NCA.
- If the *Ct* value determined for the Positive Control of amplification (**C+*TBEV, B.b. sl, A.ph., E.ch.***)

/ *E.m.* / ST1) is absent or greater than the specified boundary *Ct* value in the FAM, JOE/HEX, ROX channels (with the use of **PCR-mix-1-FRT TBEV, A.ph., E.ch. / E.m.**) or in the FAM and JOE/HEX channels (with the use of **PCR-mix-1-FRT B.b. sl / IC**), the amplification should be repeated for all samples in which specific cDNA/DNA was not found in the appropriate channel.

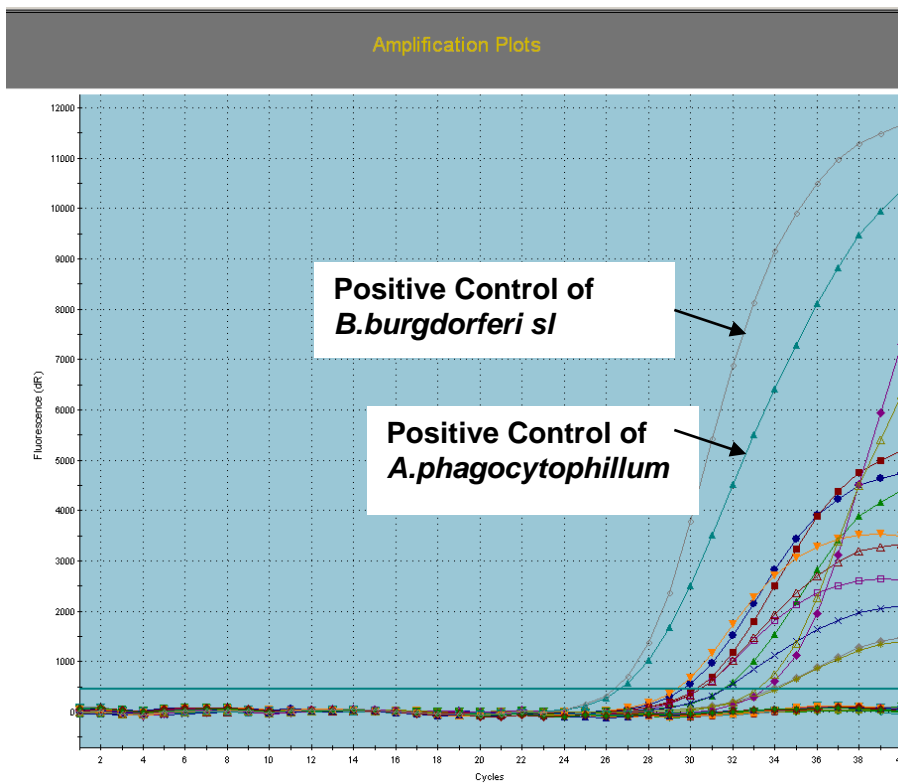
- If the *Ct* value for the Negative Control of extraction (C-) in the FAM, JOE/HEX, ROX channels (with the use of **PCR-mix-1-FRT TBEV, A.ph., E.ch. / E.m.**) and in the JOE/HEX channel (with the use of **PCR-mix-1-FRT B.b. sl / IC**) and/or Negative Control of amplification (NCA) (in any channel) is detected in the results grid, PCR analysis should be repeated for all samples in which specific cDNA/DNA was detected in the appropriate channel.

Examples of obtained results

FAM channel (amplification of *TBEV* cDNA for PCR-mix-1-FRT *TBEV*, *A.ph.*, *E.ch.* / *E.m.* and amplification of IC cDNA for PCR-mix-1-FRT *B.b. sl*/IC)



JOE channel (amplification of *A.phagocytophillum* DNA for PCR-mix-1-FRT *TBEV*, *A.ph.*, *E.ch.* / *E.m.* and amplification of *B.burgdorferi sl* cDNA for PCR-mix-1-FRT *B.b. sl*/IC)



ROX channel (amplification of *E.chaffeensis* / *E.muris* cDNA for PCR-mix-1-FRT TBEV, A.ph., *E.ch.* / *E.m.*)

