GUIDELINES

to AmpliSens® *EBV / CMV / HHV*6-screen-FRT PCR kit

for qualitative and quantitative detection of *Epstein-Barr virus* (*EBV*) DNA, *Human Herpes virus* type 6 (*HHV*6) DNA and *human cytomegalovirus* (*CMV*) DNA in the clinical material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

AmpliSens®

Not for use in the Russian Federation

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INTENDED USE

The guidelines describe the procedure of using **AmpliSens® *EBV / CMV / HHV6*-screen-FRT** PCR kit for qualitative and quantitative detection of *Epstein-Barr virus* (*EBV*) DNA, *Human Herpes virus* type 6 (*HHV*6) DNA and *human cytomegalovirus* (*CMV*) DNA in clinical material (whole blood, white blood cells, viscera biopsy material and cerebrospinal fluid) 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);
- CFX96 (Bio-Rad, USA);
- iCycler iQ, iQ5 (Bio-Rad, USA).
- Mx3000P, Mx3005P (Stratagene, 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
ROX	ROX/Orange/TxR
Cy5	Cy5/Red

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[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 the 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) (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: Balance the rotor of the instrument if it is not loaded entirely. Fill the spare wells with empty tubes (don't use the tubes left after previous experiments). Well 1 must be filled with any test tube except for an empty one.

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.

2. In the opened window select the **Advanced** menu and outline the **Dual Labeled Probe** or **Hydrolysis probes**. 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, select the reaction volume – **25 µl**. Tick the **15 µl oil layer volume** option. Click the **Next** button.

5. In the newly opened window select the **Edit profile** button to set the amplification program:

"AmpliSens-1" amplification program for rotor-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
Hold	95	15 min	–	1
Cycling 1	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling 2	95	5 s	–	40
	60	20 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:

   – perform the calibration in FAM/Green, JOE/Yellow, ROX/Orange and Cy5/Red channels (activate the **Calibrate Acquiring/Optimise Acquiring** button);

   – perform the calibration in the selected channels before the first detection (tick the **Perform Calibration Before 1st Acquisition/ Perform Optimisation Before 1st Acquisition** option);

   – to set channels calibration, indicate **5** in the **Min Reading** box and **10** in the **Max Reading** box (tick the **Perform Calibration Before 1st Acquisition/ Perform Optimisation Before 1st Acquisition** option). Click the **Close** button.

8. Click **Next** button, select the **Start run** button for amplification program to start.

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

10. Enter the data in 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**, and the Positive control of Amplification as **C+**. Tick the box next to **Unknown** type for all tested clinical samples, tick the box next to **Positive control** type for Positive control of Amplification and **NTC** type for Negative control of Amplification. For **calibrators** tick the box next to **Standard** type and set their concentrations in the **Given Conc.** column. Concentration values for calibrators are indicated in *the Important Product Information Bulletin*. Set the type **None** for the cells matching with the corresponding empty tubes.

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

Amplification data analysis of the IC Glob (FAM/Green channel)

1. Make sure that DNA calibrators are specified and their concentrations (for quantitative analysis) are entered in the sample table.

2. Activate the **Analysis** button then select the mode of the analysis **Quantitation** and activate the buttons **Cycling A. FAM**, **Cycling A. Green**, **Show**.

3. Cancel the automatic choice of the threshold line level by activating the **Threshold** button.

4. Activate the **Dynamic tube** and **Slope Correct** buttons in the main window menu (**Quantitation analysis**)

5. Choose the parameter **More settings/Outlier Removal** (in the right part of the window) and set **10 %** for the value of negative samples threshold (**NTC Threshold**).

6. In the **Calculation** menu indicate the threshold line level **0.03** in the **Threshold** box.

7. In the results grid (the **Quant. Results** window) the *Ct* values for **IC Glob DNA** will appear for each test sample, and the concentration values (**Calc Conc (copies/reaction)**) will appear for the quantitative analysis. Herewith the *Ct* value should not exceed the value specified in the *Important Product Information Bulletin*.

8. For Negative Control of Extraction (C–) – **Negative Control (C–)** – the *Ct* value should be absent.

9. For Negative Control of Amplification (NCA) – **RNA-buffer** – the *Ct* value should be absent.

10. For Positive Control of Extraction (PCE) – **Positive Control DNA *EBV / CMV / HHV*6 and human DNA** – the *Ct* value should be less than the value specified in the *Important Product Information Bulletin*, and for quantitative analysis, the concentration values should be defined.

11. For Positive Control of Amplification (C+) – **KSG2** – the *Ct* value should be less than the value specified in the *Important Product Information Bulletin* (qualitative analysis).

12. For DNA calibrations – **KSG1** and **KSG2** – the *Ct* values and the concentration values (**Calc Conc (copies/reaction)** (for quantitative analysis) should appear.

Amplification data analysis of the *EBV* DNA (JOE/Yellow channel)

1. Make sure that DNA calibrators are specified and their concentrations (for quantitative analysis) are entered in the sample table.

2. Activate the **Analysis** button then select the mode of the analysis **Quantitation** and activate the buttons **Cycling A. JOE/Cycling A. Yellow, Show**.

3. Cancel the automatic choice of the threshold line level by activating the **Threshold** button.

4. Activate the **Dynamic tube** and **Slope Correct** buttons in the main window menu (**Quantitation analysis**).

5. In the **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** window), the *Ct* values will appear (or the concentration values (**Calc Conc (copies/reaction)**) will appear for quantitative analysis).

8. For Negative Control of Extraction (C-) – **Negative Control (C–)** – *Ct* values should be absent.

9. For Negative Control of Amplification (NCA) – **RNA-buffer** – *Ct* values should be absent.

10. For Positive Control of Extraction (PCE) – **Positive Control DNA *EBV / CMV / HHV*6 and human DNA** – *Ct* value should be less than the value specified in the *Important Product Information Bulletin,* for quantitative analysis the calculated concentration values should fall within the range of values specified in the *Important Product Information Bulletin.*

11. For Positive Control of Amplification (C+) – **KSG2** – the *Ct* values should be less than the value specified in the *Important Product Information Bulletin* (qualitative analysis).

12. For DNA calibrators – **KSG1** and **KSG2** – the *Ct* values and the concentrations values (**Calc Conc (copies/reaction)** (for quantitative analysis) should appear.

Amplification data analysis of the *CMV* DNA (ROX/Orange channel)

1. Make sure that DNA calibrators are specified and their concentrations (for quantitative analysis) are entered in the sample table.

2. Activate the **Analysis** button then select the mode of the analysis **Quantitation** and activate the buttons **Cycling A. ROX**, **Cycling A. Orange**, **Show**.

3. Cancel the automatic choice of the threshold line level by activating the **Threshold** button.

4. Activate the **Dynamic tube** and **Slope Correct** buttons in the main window menu (**Quantitation analysis**).

5. In the **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** window), the *Ct* values will appear (or the

concentration values (**Calc Conc (copies/reaction)**) will appear for quantitative analysis).

8. For Negative Control of Extraction (C-) – **Negative Control (C–)** – *Ct* values should be absent.

9. For Negative Control of Amplification (NCA) – **RNA-buffer** – *Ct* values should be absent.

10. For Positive Control of Extraction (PCE) – **Positive Control DNA *EBV / CMV / HHV*6 and human DNA** – *Ct* value should be less than the value specified in the *Important Product Information Bulletin,* for quantitative analysis the calculated concentration values should fall within the range of values specified in *Important Product Information Bulletin.*

11. For Positive Control of Amplification (C+) – **KSG2** – the *Ct* values should be less than the value specified in *Important Product Information Bulletin* (qualitative analysis).

12. For DNA calibrators – **KSG1** and **KSG2** – the *Ct* values and the concentrations values (**Calc Conc (copies/reaction)**) (for quantitative analysis) should appear.

Amplification data analysis of the *HHV*6 DNA (Cy5/Red channel).

1. Make sure that DNA calibrators are specified and their concentrations (for quantitative analysis) are entered in the sample table.

2. Activate the **Analysis** button then select the mode of the analysis **Quantitation** and activate the buttons **Cycling A. Cy5/Cycling A. Red**, **Show**.

3. Cancel the automatic choice of the threshold line level by activating the **Threshold** button.

4. Select the **Dynamic tube** and the **Slope Correct** buttons in the main window menu (**Quantitation analysis**).

5. In the **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** window), the *Ct* values will appear (or the concentration values (**Calc Conc (copies/reaction)**) will appear for quantitative analysis).

8. For Negative Control of Extraction (C–) – **Negative Control (C–)** – *Ct* values should be absent.

9. For Negative Control of Amplification (NCA) – **RNA-buffer** – *Ct* values should be

absent.

10. For Positive Control of Extraction (PCE) – **Positive Control DNA** *EBV / CMV / HHV***6 and human DNA** – *Ct* value should be less than the value specified in the *Important Product Information Bulletin,* for quantitative analysis the calculated concentration values should fall within the range of values specified in the *Important Product Information Bulletin*.

11. For Positive Control of Amplification (C+) – **KSG2** – the *Ct* values should be less than the value specified in the *Important Product Information Bulletin* (qualitative analysis).

12. For DNA calibrators – **KSG1** and **KSG2** – the *Ct* values and concentration values (***Calc Conc (copies/reaction)***) will appear (quantitative analysis).

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 (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 (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**…).
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.**

"AmpliSens-1" amplification program for plate-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
1	95	15 min	–	1
2	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
3	95	5 s	–	40
	60	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**.


```
   1    95,0  C  for  15:00
→  2    95,0  C  for  0:05
        Slow Ramp Rate to   2,5  C per second
   3    60,0  C  for  0:20
        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  0:05
        Slow Ramp Rate to   2,5  C per second
   7    60,0  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**. This protocol can be used for further runs by clicking the **Select Existing...** button in the **Protocol** tab. When the required program is entered or edited, click **OK** at the bottom of the window.

4. Set the plate scheme. In the **Plate** tab click the **Create new...** button. Set the tube order in the opened **Plate Editor – New** window. Click the **Select Fluorophore** button**,** then indicate with a checkmark **Selected** required fluorophores, and click **OK.** In the **Sample type** menu select **Unknown** for all samples except for DNA-calibrators. Then indicate with a checkmark **Load** (in the right part of window) measuring the fluorescence signal of all samples in the required channels. Define sample names in the **Sample name** window, with a **Load** must be indicate with a checkmark.

For DNA-calibrators **K1** and **K2** set for all the channels **Sample type** – **Standard** and indicate their concentrations in the **Concentration** field according to the *Important Product Information Bulletin*. The parameter **Load** should be checked with a mark.

5. In the **Plate Editor New** window select **File**, then **Save As**, and name the plate. When the required plate is entered or edited, click **OK** at the bottom of the window.

6. Select **Start Run** tab. Click **Open Lid** button to open the instrument lid. Insert the test tubes into the thermocycler's wells according to the preprogrammed plate scheme. Click **Close Lid** button to close the instrument lid.

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 **Start Run** button to start the selected program with the specified scheme tablet, select the directory to save the fail staging, names file, click **Save.**

8. Proceed to the analysis of results after the end of the run.

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 (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. The calibration curve plotting and calculation of *EBV*, *CMV*, *HHV*6 DNA concentration are performed automatically in accordance with the *Ct* values of the calibrators.

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.

For each channel indicate *Log Scale* with a checkmark. 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 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.

3. Click the *View/Edit Plate…* button on the toolbar and set the samples names and calibrators concentrations in the opened window.

4. 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.

AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ5 and iCycler iQ (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 optically transparent domed or flat caps, or tubes (0.1 ml) with transparent caps from the eight-pieces-strips (e.g. Axygen, USA) (detection through the cap of the tube).

1. Switch on the instrument and start the iCycler iQ5 program.

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

2. Insert the tubes or strips into the reaction module of the thermocycler and program the instrument.

NOTE: Monitor the tubes. There must not be drops left on the walls of the tubes as falling drops during the amplification process may lead to the signal failure and complicate the results analysis. Don't turn the strips upside down while inserting them into the instrument.

Program the thermocycler only according to the *Instruction Manual* given by the manufacturer of the instrument:

1. Select the **Create new** option in the **Workshop** module to create a new amplification protocol.

2. In the newly opened window set the amplification program.

"AmpliSens-1" amplification program for plate-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
1	95	15 min	–	1
2	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
3	95	5 s	–	40
	60	30 s	FAM, JOE/HEX, ROX, Cy5	
	72	15 s	–	

3. Name the new protocol and save it.

4. Create the **Plate setup**. Set the order of the tubes in the reaction module.

5. In the opened window, set all clinical samples as **Unknown,** positive controls as "+", and negative controls as "–". Set calibrators in **FAM, JOE/HEX, ROX** and **Cy5** channels as **Standard** and indicate concentration from the *Important product information bulletin*. If calibrators are used, **Whole Plate Loading** should be non-activated. Set fluorescence detection in FAM, JOE/HEX, ROX and Cy5 channels.

6. Name the plate setup and save it.

7. Activate the **Run** button. In the opened window, select **Use Persistent Well Factors** and click the **Begin Run** button. Save the experiment.

Data analysis

1. Start the software and open the saved file: select **Data file** in the **Workshop** module and select data file. Proceed to the **Data Analysis** mode.
2. The data for each channel is to be browsed separately.
3. Make sure that automatic selection of the threshold level is correct. Normally, the threshold line should cross only sigmoid curves of signal accumulation of positive samples and controls and should not cross the baseline; otherwise, the threshold level should be raised. To do this, select **Log View** and set the threshold with the left mouse button at a level where the fluorescence curves are linear and do not cross the curves of negative samples. In the results grid (the **Quant. Results** window) the *Ct* values for analyzed channel will appear.
4. For results analysis, press the **Results** button (situated under the buttons for the fluorophore names).

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 Mx3000P and Mx3005P instruments (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 optically transparent domed or flat caps (e.g. Axygen, USA) (detection through the cap of the tube).

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 **JOE/HEX filter set**.

5. Lock the fixing arm and the door of the instrument.

6. Select **Quantitative PCR (Multiple Standards)** and **Turn lamp on for warm-up** in the **New Experiment Options** window.

7. Set fluorescence detection parameters in the **Plate Setup** menu. To do this:

    – Select all wells with test tubes or stripes (holding **Ctrl** and selecting needed range).

    – Set all selected wells as **Unknown** in the **Well type** window. Set **FAM, JOE/HEX, ROX, Cy5** in the **Collect fluorescence data** option. Name each sample by double clicking on each cell (**Well Information** window). *The names of the samples may also be added during or after amplification by returning to **Plate Setup** menu.*

    – Set calibrators in **JOE/HEX**, **FAM**, **ROX** and **Cy5** channels as **Standard**. Indicate concentration from the *Important Product Information Bulletin*.

8. In the **Thermal Profile Setup** tab, set the amplification program. To do this, use one of the following methods:

Use the template file for setting the amplification program (recommended).

Press the **Import…** button right to the thermocycling profile picture. Proceed to the folder containing previous experimental file and open it. In the **Thermal Profile** window, the required thermocycling profile will appear.

Manual programming

1. After setting all necessary values and parameters, select all wells with the tested tubes once again. Proceed to the **Thermal Profile Setup** menu and set the corresponding amplification program.

"AmpliSens-1" amplification program for plate-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
1	95	15 min	–	1
2	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
3	95	5 s	–	40
	60	30 s	FAM, JOE/HEX, ROX, Cy5	
	72	15 s	–	

2. To set detection parameter of fluorescent signal at desired temperature, select the **All points** option for **Data collection by marker dragging** parameter and move it with your mouse from right side to the box with desired temperature.

3. Select **Run** and **Start** to run amplification and name the experiment file.

Data analysis

1. Make sure that DNA calibrators are specified and their concentrations (for quantitative analysis) are entered in the sample table.

2. Select **Analysis** by clicking the corresponding button of the tool bar.

3. 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). Otherwise select all the test samples by holding down the **Ctrl** button and selecting the needed range with the mouse.

4. Proceed to the **Results** tab.

5. Make sure that **JOE/HEX, FAM, ROX** and **Cy5** channels are active (the **JOE/HEX, FAM, ROX** and **Cy5** buttons are pressed in the **Dyes Shown** field at the bottom of the window).

6. Select the **Threshold fluorescense** field and make sure that tick marks are put against three fluorescence channels: **JOE/HEX, FAM, ROX** and **Cy5**. Check the correctness of the automatically chosen threshold line. 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. The curves of the signal accumulation are shown by the instrument in the linear form by default. In order to change the form of the curves and

make them logarithmical double click the left mouse button near one of the coordinate axes (X or Y) in the **Graph properties** window for Y axis (**Y axis**) and put the tick mark against the **Log** point in the **Scale** field.

7. In the results grid the *Ct* values for **EBV DNA** (JOE/HEX channel), **CMV DNA** (ROX channel), **HHV6 DNA** (Cy5 channel), **IC Glob DNA** (FAM channel) will appear; for quantitative analysis, the concentration values (**Calc Conc (copies/reaction)**) will appear. The *Ct* value for test samples should be less than the value specified in the *Important Product Information Bulletin.*

8. For Negative Control of Extraction (C–) – **Negative Control (C–)** – *Ct* values should be absent in the FAM, JOE/HEX, ROX and Cy5 channels.

9. For Negative Control of Amplification (NCA) – **RNA-buffer** – *Ct* values should be absent in the FAM, JOE/HEX, ROX and Cy5 channels.

10. For Positive Control of Extraction (PCE) – **Positive Control DNA *EBV / CMV / HHV6* and human DNA** – the *Ct* value should be less than the value specified in the *Important Product Information Bulletin,* for quantitative analysis the calculated concentration values should fall within the range of values specified in the *Important Product Information Bulletin.*

11. For Positive Control of Amplification (C+) – **KSG2** – the *Ct* values should be less than the values specified in the *Important Product Information Bulletin* in all channels (qualitative analysis).

12. For DNA calibrators – **KSG1** and **KSG2** – the *Ct* values and concentration values (**Calc Conc (copies/reaction)**) will appear in all the channels (quantitative analysis).

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.

TROUBLESHOOTING

1. If any *Ct* value appears in the channels for the FAM, JOE, ROX and Cy5 fluorophores for the Negative Control of Amplification (NCA) and Negative Control of Extraction (C–) these results testify the presence of contamination of reagents or samples. In that case the PCR-analysis should be repeated (beginning with the extraction stage) for all samples, in which DNA was detected.

2. If *Ct* value is absent or greater than the threshold in the results grid for the Positive Control of Amplification (C+) – **KSG2** – for the qualitative analysis in the channels for JOE, FAM, ROX or Cy5 fluorophores, the amplification must be repeated for all samples where pathogen agent DNA was not detected.

3. If the *Ct* value is absent or greater than the threshold for the Positive Control of Extraction (PCE) – **Positive Control DNA *EBV / CMV / HHV*6 and human DNA –** in the channels for JOE, FAM, ROX or Cy5 fluorophores, the results of analysis must be considered as **invalid** for all samples. PCR should be repeated for all samples.

4. If the *Ct* value for given sample was not defined or the *Ct* value exceeds the threshold value in the channel for JOE, ROX or Cy5 fluorophores, and *Ct* value defined in the channel for **FAM** fluorophore exceeds the maximal value specified for IC, the experiment needs to be repeated, starting with the extraction stage. Possible reason is an error in the clinical material pretreatment procedure that leads to the DNA loss or the presence of PCR inhibitors.

5. If the *Ct* value for the clinical samples exceeds the maximal threshold value in the channel for JOE, ROX or Cy5 fluorophore, the results of analysis must be considered as **equivocal**. In that case, it is necessary to conduct additional analysis for that DNA sample with two repeats. If the repeated positive *Ct* value is obtained, the result is considered positive. If the positive *Ct* value can't be reproduced in two repeats, the result is considered **equivocal**.

6. If in quantitative analysis the copies/reaction values in calibrators differ by more than 30 % from the set values, it is necessary to check the tube order in the rotor (calibrators should be placed in the wells indicated as ***Standard*** in sample table, concentration should correspond to concentration specified in the *Important Product Information Bulletin*, well no.1 must be filled with some test tube (not empty)).

7. If the correlation coefficient R in ***Standard Curve*** window is less than 0.9 (in case of quantitative analysis), it means that calibration failed. Check the settings of calibrators and correct inaccuracies, if no effect, repeat PCR for all samples and calibrators.

CALCULATION OF DNA CONCENTRATION

***EBV* DNA concentration per ml of sample when DNA is extracted from cerebrospinal fluid (liquor)** is calculated by the following formula:

$$\text{CS}_{\,EBV\,\text{DNA}} = \text{C}_{\,EBV\,\text{DNA}} \times 100 \text{ (copies/ml)},$$

where $\text{C}_{\,EBV\,\text{DNA}}$ is the number of *EBV* DNA copies in DNA sample (JOE/Yellow/HEX channel).

***CMV* DNA concentration per ml of sample if DNA is extracted from cerebrospinal fluid (liquor)** is calculated by the following formula:

$$\text{CS}_{\,CMV\,\text{DNA}} = \text{C}_{\,CMV\,\text{DNA}} \times 100 \text{ (copies/ml)},$$

where $\text{C}_{\,CMV\,\text{DNA}}$ is the number of *CMV* DNA copies in DNA sample (ROX/Orange channel).

***HHV*6 DNA concentration per ml of sample if DNA is extracted from cerebrospinal fluid (liquor)** is calculated by the following formula:

$$\text{CS}_{\,\text{DNA}\,HHV6} = \text{C}_{\,\text{DNA}\,HHV6} \times 100 \text{ (copies/ml)},$$

where $\text{C}_{\,\text{DNA}\,HHV6}$ is the number of *HHV6* DNA copies in DNA sample (Cy5/Red channel).

The logarithmic concentration of *EBV* DNA (in log of *EBV* DNA copies per 10^5 cells) if DNA is extracted from whole blood, white blood cells and viscera biopsy material is calculated by the following formula:

$$\lg \left\{ \frac{\text{number of } EBV \text{ DNA copies in PCR sample}}{\text{number of Glob DNA copies in PCR sample}} \times 2{\cdot}10^5 \right\} = \lg \{ EBV \text{ DNA copies}/10^5 \text{ cells}\}$$

The logarithmic concentration of *CMV* DNA (in log of *CMV* DNA copies per 10^5 cells) if DNA is extracted from whole blood, white blood cells and viscera biopsy material is calculated by the following formula:

$$\lg \left\{ \frac{\text{number of } CMV \text{ DNA copies in PCR sample}}{\text{number of Glob DNA copies in PCR sample}} \times 2{\cdot}10^5 \right\} = \lg \{ CMV \text{ DNA copies}/10^5 \text{ cells}\}$$

The logarithmic concentration of *HHV*6 DNA *(*in log of *HHV*6 DNA copies per 10^5 cells) if DNA is extracted from whole blood, white blood cells and viscera biopsy material is calculated by the following formula:

$$\lg \left\{ \frac{\text{number of } HHV6 \text{ DNA copies in PCR sample}}{\text{number of Glob DNA copies in PCR sample}} \times 2{\cdot}10^5 \right\} = \lg \{ HHV6 \text{ DNA copies}/10^5 \text{ cells}\}$$

To express relative DNA concentration ratio in copies per the standard cell number (for example, 10^5), the following conversion equation is used:

$$10^5 \text{ of cells} = 2*10^5 \text{ of human genome equivalents.}$$

DNA calibrators concentrations, needed for calculation of the test sample concentrations and evaluation of the obtained results validity, are specified in the *Important Product Information Bulletin* enclosed to each PCR kit.

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
31.07.12 BM	Cover page	Cover page was added
	Text	Table of contents was added
		Names of sections were corrected
12.03.13 PE	Footer	Reference number R-V48(RG,iQ,Mx)-CE-B was deleted
01.04.14 SA	Cover page	Address of European representative was added
17.06.14 ME	Main text	General document formatting and grammar correction in accordance with the template.
	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) instruments, Amplification and data analysis using iCycler iQ5 (Bio-Rad, USA) instruments, Amplification and data analysis using Mx3000P and Mx3005P instruments (Stratagene, USA)	Results interpretation for control and tested samples was corrected. The results interpretation for NCA and C– in the FAM/Green channel was changed.
	Troubleshooting	The chapter was added
26.03.15 PM	Intended use	The CFX96 (Bio-Rad, USA) instrument was added
	Through the text	Corrections according to the template
	Amplification and data analysis using CFX96 (Bio-Rad, USA)	The chapter was added
	Amplification and data analysis using iCycler iQ5 (Bio-Rad, USA) instruments	Results interpretation were added
28.12.15 ME	Text	The clinical material saliva and oropharyngeal swabs was deleted
27.02.20 PM	Front page	The phrase "Not for use in the Russian Federation" was added
23.09.20 EM	Through the text	Corrections according to the template, amplification programs were added to the each section with instruments
	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) instruments	The information about Rotor-Gene Q instrument was added; data analysis of the IC Glob (FAM/Green channel) for C– and NCA was corrected
	Amplification and data analysis using Mx3000P and Mx3005P instruments (Stratagene, USA)	Data analysis for C– and NCA in the FAM channel was corrected
15.01.21 EM	Through the text	The symbol ⚠ was changed to NOTE:
01.03.21 MM	Front page	The name, address and contact information for Authorized representative in the European Community was changed