

## GUIDELINES

### to AmpliSens<sup>®</sup> *HHV6*-screen-titre-FRT PCR kit

for qualitative and quantitative detection of *human herpes virus* type 6 (*HHV6*) DNA in biological material 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® HHV6-screen-titre-FRT** PCR kit for qualitative and quantitative detection of *human herpes virus* type 6 (*HHV6*) DNA in biological material (whole blood, white blood cells, viscera biopsy material, saliva, oropharyngeal swabs 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);
- iCycler iQ, iQ5 (Bio-Rad, USA);
- Mx3000P/Mx3005P (Stratagene, USA);
- CFX96 (Bio-Rad, USA).

### Correspondence of names of fluorophores and detection channels

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

<sup>1</sup> 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 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.
2. Insert the tubes in the rotor so that the first tube is in No. 1 well, insert the rotor in the reaction module, and secure the lid (the rotor cells are numbered, the numbers are used for the further programming of the samples' position in the thermocycler).

**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 studied 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 program main menu.
2. In the newly opened window select **Advanced** and click **Dual Labeled Probe/Hydrolysis probes**. Activate the **New** button.
3. In the newly opened window select rotor for 36 wells, **36-Well Rotor** (or rotor for 72 wells, **72-Well Rotor**), and outline that tubes with domed lids are not being used (**No Domed Tubes** (Rotor-Gene 3000)) and that the locking ring is adjusted (**Locking Ring Attached** (Rotor-Gene 6000)). Press the **Next** button.
4. In the newly opened window set the name of the operator and choose the reaction mix volume: **Reaction volume – 25 µl**. Tick the box next to **15 µl oil layer volume**. Press 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	
	72	15 s	–	

6. Click the **OK** button.
7. Select the **Calibrate/Gain Optimisation** button in the **New Run Wizard** window.
  - perform the calibration in FAM/Green and JOE/Yellow channels (activate the **Calibrate Acquiring/Optimise Acquiring** button);
  - perform the calibration before the first detection (select **Perform Calibration Before 1<sup>st</sup> Acquisition/Perform Optimisation Before 1<sup>st</sup> Acquisition**);
  - to set channels calibration, indicate **5** in the **Min Reading** box and **10** in the **Max Reading** box. Tick **Perform Calibration Before 1<sup>st</sup> Acquisition/Perform Optimization Before 1<sup>st</sup> Acquisition**. Activate 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. Enter the data in the grid of the samples (it opens automatically after the amplification has been started).

Indicate names/numbers of the test samples in the box **Name**. Designate the Negative Control of amplification as **NCA**; the Positive Control of amplification, as **C+**. Set the **Unknown** type for all the test samples, the **Positive control** type for the Positive Control of amplification, and **NTC** type for the Negative Control of amplification. Set the type **Standard** in case of carrying out a qualitative analysis for calibrators and indicate their concentration in the box **Given** (the data concerning the concentration of the calibrators is given in the *Bulletin* for the PCR kit).

Set the type **None** for the cells matching with the corresponding empty tubes.

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

**Data analysis for the HHV6 DNA amplification (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, the concentration values (**Calc Conc (copies/reaction)**) will appear for the 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 the Positive Control of Extraction (**Positive Control DNA 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 concentrations should fall within the value range specified in the *Important Product Information Bulletin*.
11. For Positive Control of Amplification - **KSG2** – the *Ct* value should be less than the value specified in the *Important Product Information Bulletin* (for qualitative analysis).
12. For DNA-calibrators – **KSG1** and **KSG2** – the *Ct* values and concentration values (**Calc Conc (copies/reaction)**) (for quantitative analysis) should appear.

#### **Data analysis for 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. 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 for **IC Glob DNA** will appear for each test sample, the concentration values (**Calc Conc (copies/reaction)**) will appear for the quantitative analysis. Herewith the **Ct** values should not exceed the value specified in the *Important Product Information Bulletin*.
8. For the Negative Control of Extraction – **Negative Control (C-)** – **Ct** values should be absent.
9. For the Negative Control of Amplification (**NCA**) – **RNA-buffer** - **Ct** values should be absent.
10. For the Positive Control of Extraction (PCE) - **Positive Control DNA HHV6 and human DNA** - the **Ct** values should be less than the value specified in the *Important Product Information Bulletin*; for quantitative analysis, the concentration values should be determined.
11. For the Positive Control (C+) - **KSG2** – the **Ct** value should be less than the value specified in the *Important Product Information Bulletin* (for qualitative analysis).
12. For DNA-calibrators - **KSG1** and **KSG2** – the **Ct** values and the concentration values (**Calc Conc (copies/reaction)**) (for quantitative analysis) will appear.

### **Results interpretation**

The result of the PCR analysis is considered reliable only if the results for the controls of the amplification and the extraction are correct in accordance with the table of assessment of results for controls (see the Instruction manual) and boundary values specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

The interpretation of the test samples is to be carried out in accordance with the Instruction Manual and the *Important Product Information Bulletin* enclosed to the PCR kit.

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

Carry out the sample pretreatment and reaction mixture preparation stages according to the PCR kit *Instruction Manual*. When carrying out the amplification it is recommended to use thin-walled PCR tubes (0.2 ml) with domed or optically transparent flat caps (e.g. Axygen, USA) (detection through the cap of the tube).

1. Switch on the instrument. Start the 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	
	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 chamber).
5. Mark all test samples as **Unknown**, positive controls as “+”, negative controls as “–” in the opened window. Set the fluorescence detection in **FAM** and **HEX** channels for all samples. Select **Standard** for DNA calibrators and enter the values specified in the *Important Product Information Bulletin* enclosed in the PCR kit. Make sure that the **Whole Plate Loading** button is disabled when calibrators are set.
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 test.

### **Data analysis**

1. Start the software and open the saved file: select **Data file** in the **Workshop** module and select the data file. Proceed to the **Data Analysis** mode.
2. The data for each channel are 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, click the **Log View** button and set the threshold (with the left mouse button) at a level where fluorescence curves are linear and do not cross the curves of negative samples. Ct values detected in the analyzed channel will appear in the results grid.
4. Activate the **Results** button (for iQ5) (situated under the buttons with the fluorophores' names) or the **PCR Quant** button (for iCycler iQ) for result analysis.

### **Data analysis for HHV6 DNA (JOE/HEX channel)**

Check the sample table for the presence of the calibrators and their concentration values in case of quantitative analysis.

1. In the results grid the Ct values for **DNA HHV6** will appear, the concentration values (**copies/reaction**) will appear for the quantitative analysis.
2. For Negative Control of Extraction (C-) – **Negative Control (C-)** – Ct values should be absent.
3. For Negative Control of Amplification (NCA) – **RNA-buffer** – Ct values should be absent.
4. For Positive Control of Extraction (PCE) – **Positive Control DNA HHV6 and human DNA** – Ct value should be less than the values specified in the *Important Product Information Bulletin*; for quantitative test, the calculated concentrations should be within the range of values specified in the *Important Product Information Bulletin*.
5. For Positive Control of PCR (C+) – **KSG2** – the Ct values should be less than the boundary Ct values specified in *Important Product Information Bulletin* (for qualitative analysis).
6. For DNA calibrators – **KSG1** and **KSG2** – the Ct values and concentration values will appear (for quantitative analysis).

### **Data analysis for the IC Glob (FAM channel)**

Check the sample table for presence of the calibrators and their concentration values in case of quantitative analysis.

1. In the results grid the *Ct* values for **IC Glob** will appear in each tested sample, and for quantitative analysis, the concentration values (**copies/reaction**) will appear. Herewith the *Ct* value should be less than values specified in *Important Product Information Bulletin*.
2. For Negative Control of Extraction (C–) – **Negative Control (C–)** – *Ct* values should be absent.
3. For Negative Control of Amplification (NCA) – **RNA-buffer** – *Ct* values should be absent.
4. For Positive Control of Extraction (PCE) – **Positive Control DNA HHV6 and human DNA** – the *Ct* values should be less than the boundary values specified in the *Important Product Information Bulletin*; for quantitative analysis, the concentration values should be defined.
5. For Positive Control of PCR (C+) – **KSG2** – the *Ct* values should be less than the boundary *Ct* values specified in *Important Product Information Bulletin* (for qualitative analysis).
6. For DNA calibrators – **KSG1** and **KSG2** – the *Ct* values and concentration values (**Calc Conc (copies/reaction)**) (for quantitative analysis) will appear.

### **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/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 domed or optically transparent flat caps (e.g. Axygen, USA) (detection through the cap of the tube).

### Programming the thermocycler

1. Switch on the instrument.

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

2. Start the program Mx3000P/Mx3005P.
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. In the **Dye Assignment** window set **FAM** parameter next to the **FAM filter set** item, set **JOE** parameter next to the **HEX/JOE filter set**.
5. Select **Quantitative PCR (Multiple Standards)** in the **New Experiment Options** window and check the **Turn lamp on for warm-up** box.
6. Set fluorescence detection parameters in the **Plate Setup** menu. To do this,
  - Select all wells with tested tubes or strips (select the required range using the mouse and holding the **Ctrl** button);
  - Mark all selected wells as **Unknown** in the **Well type** window. Tick **FAM** and **JOE** in the **Collect fluorescence data** option. Double click each cell and enter the name of the sample (the **Well Information** window). Sample names can be specified during or after the end of an amplification run in the **Plate Setup** menu.

Set calibrators in the **FAM** and **JOE/HEX** channels as **Standard** and enter their concentrations specified in the *Important Product Information Bulletin*.

Proceed to the **Thermal Profile Setup** tab and 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	
	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. Check the sample table for presence of the calibrators and their concentrations (for quantitative analysis).
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. Select the **Results** tab.
5. Make sure that both fluorescence channels are activated (**JOE** and **FAM** buttons are pushed in the **Dyes Shown** field at the bottom of the program window).
6. Make sure that **JOE** and **FAM** channels are ticked in the **Threshold fluorescence** field. Ensure that the threshold automatically set 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.

By default, all fluorescence curves are displayed as linear. To shift from the linear to the logarithmic mode, double click one axis (X or Y) with the left mouse button and tick the **Log** option in the **Scale** field of the **Graph properties** window.

7. In the results grid one will be able to see the *Ct* values for **HHV6 DNA** (JOE/HEX

channel), **IC Glob DNA** (FAM channel); for quantitative analysis, the concentration values (**copies/reaction**) will appear. In the FAM channel the *Ct* value 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 and JOE/HEX channels.
9. For Negative Control of Amplification (NCA) – **RNA-buffer** – *Ct* values should be absent in the FAM and JOE/HEX channels.
10. For Positive Control of Extraction (PCE) – **Positive Control DNA HHV6 and human DNA** – the *Ct* values should be less than the boundary values for all the channels; for quantitative analysis, the concentration values should fall within range of the values specified in *Important Product Information Bulletin*.
11. For Positive Control of PCR (C+) – **KSG2** – the *Ct* values should be less than the boundary *Ct* values specified in *Important Product Information Bulletin* for all channels (for qualitative analysis).
12. For DNA-calibrators – **KSG1** and **KSG2** – the *Ct* values and concentration values (**copies/reaction**) (for quantitative analysis) will appear for all channels.

### **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 optically transparent flat caps (for example, 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	
	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 sample except 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 the *HHV6* 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 enter names of the samples and calibrators in the opened window, if required.
4. Click **Tools** on the toolbar, then **Reports...**, and then save the generated report (select **File**, then **Save As** and name the file).
5. In the results grid one will be able to see the *Ct* values for **HHV6 DNA** (JOE/HEX channel), **IC Glob DNA** (FAM channel); for quantitative analysis, the concentration values (**copies/reaction**) will appear. In the FAM channel the *Ct* value should be less than the value specified in the *Important Product Information Bulletin*.
6. For Negative Control of Extraction (C-) – **Negative Control (C-)** – *Ct* values should be absent in the FAM and HEX channels.
7. For Negative Control of Amplification (NCA) – **RNA-buffer** – *Ct* values should be absent in the FAM and HEX channels.
8. For Positive Control of Extraction (PCE) – **Positive Control DNA HHV6 and human DNA** – the *Ct* values should be less than the boundary values for all the channels; for quantitative analysis, the concentration values should fall within range of the values specified in *Important Product Information Bulletin*.
9. For Positive Control of PCR (C+) – **KSG2** – the *Ct* values should be less than the boundary *Ct* values specified in *Important Product Information Bulletin* for all channels (for qualitative analysis).
10. For DNA-calibrators – **KSG1** and **KSG2** – the *Ct* values and concentration values (**copies/reaction**) (for quantitative analysis) will appear for all channels.

### **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 the *Ct* value is determined in the FAM/Green and JOE/Yellow/HEX channels for Negative Control of Amplification (NCA) and Negative Control of Extraction (C–). The results testify the presence of contamination of reagents or samples. In that case the PCR analysis (beginning with the extraction stage) should be repeated for all samples, in which DNA was found.
2. In qualitative analysis, if the *Ct* value for the Positive Control of Amplification (C+) - **KGS2** in the JOE/Yellow/HEX (*HHV6*) or FAM/Green channel is absent or greater than the threshold value in the results grid, repeat amplification of all samples in which *HHV6* DNA was not detected.
3. If the *Ct* value for the Positive Control of Extraction (PCE) - **Positive Control DNA *HHV6* and human DNA** - in JOE/Yellow/HEX (*HHV6*) or FAM/Green channel is absent or greater than the threshold value, the results of analysis for all samples are considered invalid. Repeat analysis of all samples starting from the DNA amplification stage.
4. If the *Ct* value for a sample in the JOE/Yellow/HEX channel is absent or exceeds the boundary *Ct* value and the *Ct* value for the same sample in the FAM/Green channel exceeds the boundary *Ct* value specified for Internal Control, repeat analysis of the sample starting from the DNA extraction stage. This may be caused by errors in preparation of biological material, which entailed the loss of DNA, or by the presence of inhibitors.
5. If the *Ct* value for a test sample in the JOE/Yellow/HEX channel (*HHV6* DNA) exceeds the boundary *Ct* value, the result of analysis is considered **equivocal**. Repeat analysis of such samples in duplicate. If a positive result is obtained in both replicates, the result of analysis is considered as **positive**. If the result is not reproduced in two replicates, the result of analysis is considered **equivocal**.
6. If the number of copies per reaction in DNA calibrators in quantitative tests exceeds the specified *Ct* value by more than 30%, check the order of the tubes in the rotor (DNA calibrators should be inserted into the cells named **Standard** in the table of samples, the concentration of samples should correspond to the concentration specified in the *Important Product Information Bulletin*, and cell no. 1 in rotor-type instruments should not be empty (fill it with any test tube).
7. If the correlation coefficient **R** in the **Standard Curve** window in quantitative tests is less than 0.9, this indicates error in calibration. Check whether the settings for DNA calibrators are correct and change them, if necessary. If this does not help, run PCR for

all samples and calibrators once again.

### DNA HHV6 CONCENTRATION CALCULATION

The concentration of HHV6 DNA per ml of a sample (CS HHV6 DNA) is calculated by the formula below for DNA samples extracted from oropharyngeal swabs and cerebrospinal fluid:

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

where K HHV6 DNA is a number of HHV6 DNA copies in a DNA sample.

The logarithm of concentration of HHV6 DNA copies per the standard cell quantity ( $10^5$ ) in control and test samples is calculated by the formula below for DNA samples extracted from whole human blood, white blood cells, or viscera biopsy material:

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

Use the following recalculation coefficient to convert relative concentration of HHV6 DNA per standard number of cells (for example  $10^5$ ):

$$10^5 \text{ cells} = 2 \cdot 10^5 \text{ human genomes}$$

Each PCR kit should include an *Important Product Information Bulletin* with specified concentrations of DNA calibrators which must be used for calculation of concentration of analyzed samples and estimation of result reliability.

### Example of calculation of the logarithm of HHV6 DNA copies per $10^5$ cells using an Excel spreadsheet

1. Copy data from the **Name** column (names of samples) from the opened **Text report** window and enter in the first column of the Excel sheet (column A).
2. Copy the concentrations of the HHV6 DNA detected in the Yellow (HEX/JOE) channel (**Calc Conc (copies/reaction)**) and enter them in the second column of the Excel sheet (column B).
3. Copy the concentrations of the Glob DNA detected in the Green (FAM) channel (**Calc Conc (copies/reaction)**) and enter them in the third column of the Excel sheet (column C).
4. Enter the formula (**E=LOG(B/C\*200,000)**) in the fourth column of the Excel sheet (column D) as shown in the table below. Values of lg of HHV6/ $10^5$  will appear.


5. Enter the formula ( **$F=B/C*200,000$** ) in the fifth column of the Excel sheet (column E) as shown in the table below. Values of *HHV6* copies/ $10^5$  will appear.

Table 1

**Example of calculation of Ig *HHV6* DNA copies per  $10^5$  cells  
if DNA is extracted from cell suspension (whole blood,  
white blood cells, or biopsy material of visceral organs)**

Name	Calc Conc (copies/reaction) Yellow (HEX/JOE)	Calc Conc (copies/reaction) Green (FAM)	Ig <i>HHV6</i> / $10^5$	<i>HHV6</i> copies / $10^5$
A	B	C	$E=LOG(B/C*200,000)$	$F=B/C*200,000$
1	8742	125640	4.1	13916
2	253	87787	2.8	576
3		43564		
4	648	16354	3.9	7925
5		88736		
KSG1	9962	9793		
KSG1	10038	10211		
KSG2	94	102		
KSG2	107	98		
K-				

### List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
19.06.11 RT	Cover page	The name of Institution was changed to Federal Budget Institute of Science “Central Research Institute for Epidemiology”
28.06.13 FN	Footer	Catalogue number <b>REF</b> R-V10-T(RG,iQ,Mx)-CE-B was deleted
23.12.14 PM	Intended use	The CFX96 (Bio-Rad, USA) instrument was added
	Through the text	Corrections according to the template
	AMPLIFICATION AND DATA ANALYSIS USING Mx3000P/Mx3005P INSTRUMENTS (Stratagene, USA)	The chapter “Programming the thermocycler” was rewritten
	AMPLIFICATION AND DATA ANALYSIS USING CFX96 (Bio-Rad, USA)	The chapter was added
04.06.21 MM	Through the text	The symbol  was changed to NOTE:
	Cover page	The phrase “For research use only. Not for diagnostic procedures” was added
	Through the text	Corrections according to the template, amplification programs and results interpretation were added to the each section with instruments
	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) instruments	Data analysis for C– and NCA in the FAM channel was corrected
	Amplification and data analysis with the use of iCycler iQ5 (Bio-Rad, USA) instrument	
	Amplification and data analysis using CFX96 (Bio-Rad, USA)	
Amplification and data analysis using Mx3000P and Mx3005P instruments (Stratagene, USA)		
21.08.23 EM	Footer	<b>REF</b> R-V10-T(RG,iQ,Mx)-CE was added