

GUIDELINES

to **AmpliSens[®] EBV-screen/monitor-FRT** PCR kit
for qualitative detection and quantitation of *Epstein-Barr virus (EBV)*
DNA in the biological material by using real-time hybridization-
fluorescence detection

AmpliSens[®]



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INTENDED USE

The guidelines describe the procedure of using **AmpliSens® EBV-screen/monitor-FRT** PCR kit for qualitative detection and quantification of *Epstein-Barr virus (EBV)* DNA in the biological material (peripheral blood plasma, amniotic fluid, cerebrospinal fluid (liquor), saliva, oropharyngeal swabs, bronchoalveolar lavage, whole human blood, white blood cells, and viscera biopsy 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);
- CFX96 (Bio-Rad, USA);
- iCycler iQ5 (Bio-Rad, USA);
- Mx3000P, Mx3005P (Stratagene, USA)

and also in combination with the automatic station for the nucleic acids extraction NucliSENS easyMAG (bioMérieux, France).

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

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

WORK WITH THE NucliSENS easyMAG (bioMérieux,France) AUTOMATED NUCLEIC ACID EXTRACTION SYSTEM

Variant 1. DNA extraction with off-board sample lysis (off-board mode)

This extraction method allows to reduce the consumption of NucliSens lysis buffer. It is preferred for work with test samples which contain clots.

1. Switch on the NucliSENS easyMAG instrument and prepare it to the RNA/DNA extraction according to the *Instruction Manual*.
2. In the test samples input window enter the following parameters:
 - Sample **Name**
 - **Matrix** for DNA extraction (select **Plasma**)
 - **Volume – 0.1 ml**
 - **Euate – 55 µl**
 - **Type – Lysed**
 - **Priority – Normal**.
3. Create a new protocol of DNA extraction and save it. In protocol select **On-board Lysis Buffer Dispensing – no, On-board Lysis Incubation – no**.
4. Relocate sample table into the created protocol.
5. Take the required number of specialized disposable tubes intended for DNA extraction in NucliSENS easyMAG instrument (including negative and positive control of extraction). Add **10 µl** of **Internal Control STI-87** into each tube, on the internal walls. Add **550 µl** of **NucliSens lysis buffer**.

NOTE: When working with material containing clots, it is recommended to carry out lysis in 1.5-ml tubes. After the incubation has finished (step 8) the tubes should be centrifuged at 10,000 rpm for 1 min. Then transfer the supernatant into specialized tubes intended for DNA extraction in NucliSENS easyMAG instrument.

6. Add **100 µl** of prepared samples into tubes with **NucliSens lysis buffer** and **Internal Control STI-87** using disposable tips with filters and thoroughly, but carefully mix by pipetting. Avoid transferring clots and big particles into the tube.
7. Add **100 µl** of **Negative Control** into the tube with Negative Control of Extraction (C-). Add **90 µl** of **Negative Control** and **10 µl** of **Positive Control DNA EBV and human DNA** into the tube with Positive Control of Extraction (PCE).
8. Incubate tubes for 10 min at room temperature.
9. Resuspend the tube with **NucliSens magnetic silica** (bioMérieux) by intensive vortexing. Add **10 µl** of **NucliSens magnetic silica** by using disposable tips with filters and carefully mix by pipetting. The **NucliSens magnetic silica** should be distributed

evenly through the tube volume.

10. Load tubes with samples into the instrument, adjust the lids, and start the DNA extraction program with samples lysis by selecting **off board**.
11. After the DNA extraction is completed, take the tubes out of the instrument. Tubes containing DNA samples should be taken to the PCR amplification zone.

Variant 2. DNA extraction on-board sample lysis (on-board mode)

1. Switch on the NucliSENS easyMAG instrument and prepare it to the DNA extraction according to the *Instruction Manual*.
2. In the window for input of test samples enter the following parameters:
 - Sample **Name**
 - **Matrix** for DNA extraction (select **Plasma**)
 - **Volume – 0.1-1.0 ml**
 - **Eluate – 55 µl**
 - **Type – Primary**
 - **Priority – Normal.**
3. Create a new protocol of DNA extraction and save it. In protocol select **On-board Lysis Buffer Dispensing – yes, On-board Lysis Incubation – yes.**
4. Relocate programmed sample into created protocol.
5. Add **100 µl** of prepared samples into tubes intended for DNA extraction in NucliSENS easyMAG instrument using disposable tips with filters.
6. Add **100 µl** of **Negative Control** into tube with Negative Control of Extraction (C-) intended for DNA extraction in NucliSens easyMAG instrument. Add **90 µl** of **Negative Control** and **10 µl** of **Positive Control DNA EBV and human DNA** into tube with Positive Control of Extraction (PCE).
7. In a separate sterile 2 ml tube mix the **NucliSens magnetic silica** and **Internal Control STI-87** using sterile disposable tips with filters in the following ratio:

Table 1

Quantity of samples for DNA extraction	Quantity of NucliSens magnetic silica (µl)	Quantity of Internal Control STI-87 (µl)
1	10	10
24 (instrument complete load)	250 (for extra 25 samples)	250 (from two tubes)

8. Thoroughly mix the contents of the tube. The mixture of **magnetic silica NucliSens** and **Internal Control STI-87** can be stored for no longer than 30 min.
9. Load the tubes with samples into the instrument and start the DNA extraction program with samples lysis by selecting **on board**.

10. Wait for the NucliSENS easyMAG instrument to stop working in the **Instrument State-Idle** position (approx. 15 min).
11. Carefully mix the tube with prepared mixture of **NucliSens magnetic silica** and **Internal Control STI-87** by vortexing to homogeneous state.
12. Open the lid of instrument and to each sample add **20 µl of mixture** using a new tip for each tube. Carefully, but thoroughly, mix each sample by pipetting with the aid of multichannel pipettes and disposable tips with filters for 200 µl.
13. Set the DNA extraction program for the instrument to continue.
14. After the extraction is completed, take the tubes out of the instrument. Tubes containing DNA samples should be taken to the PCR amplification zone.

POSITIVE RESULT CT DETERMINATION

Before starting work with a new reagents lot, the threshold level of *Ct* values need to be determined for positive samples in JOE/HEX/Yellow channel for the specific instrument in use. Dilute **Positive Control DNA EBV and human DNA** to the ratio 1:100 by the **RNA-buffer** (for example, take **990 µl** of the **RNA-buffer** and **10 µl** of **Positive Control DNA EBV and human DNA**). Run the PCR amplification of diluted sample according to the PCR kit's instruction manual, repeat the amplification five times. For the Rotor-Gene 3000/6000 instrument, choose parameter **More Settings/Outlier Removal** and indicate **NTC threshold = 0%**. Calculate the average *Ct* value for five repetitions in the JOE/HEX/Yellow channel. Add 2 extra cycles to the average *Ct* value. The obtained value is the threshold value for the positive result.

An example is given in Table 2.

Table 2

Example of the threshold value calculation for positive result

	Ct (JOE/HEX/ Yellow)	average value	threshold for positive samples
Positive Control DNA <i>EBV</i> and human DNA, 100-fold diluted	27.15	27.5	29.5
	27.28		
	28.06		
	27.69		
	27.27		

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 software 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, ROX/Orange	
	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 and ROX/Orange 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**, 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 test 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.

Data analysis of 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 *C_t* 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-)** – *C_t* values should be absent.
9. For Negative Control of Amplification (NCA) – **RNA-buffer** – *C_t* values should be absent.
10. For Positive Control of Extraction (PCE) – **Positive Control DNA EBV and human DNA** – the *C_t* value should be less than the boundary values 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 *C_t* values should be less than the boundary *C_t* values specified in the *Important Product Information Bulletin*.
12. For DNA calibrations – **KSG1** and **KSG2** – the *C_t* values and concentration values (**Calc Conc (copies/reaction)**) should appear.

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. 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, 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-)** – **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 and human DNA** – the **Ct** values should be less than the boundary **Ct** value specified in the *Important Product Information Bulletin*, for quantitative analysis, the concentration values should be defined.
11. For Positive Control of Amplification (C+) – **KSG2** – the **Ct** values should be less than the boundary **Ct** values specified in the *Important Product Information Bulletin* (qualitative analysis).
12. For DNA calibrations – **KSG1** and **KSG2** – the **Ct** values and the concentrations values (**Calc Conc (copies/reaction)**) (for quantitative analysis) should appear.

Data analysis of the Internal Control STI-87 (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 for **Internal Control STI-87 (IC) 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 less than the value specified in the *Important Product Information Bulletin*.
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 and human DNA** – the **Ct** values should be less than the boundary **Ct** value specified in the *Important Product Information Bulletin*, for quantitative analysis, the concentration values should be defined.
11. For Positive Control of Amplification (C+) – **KSG2** – the **Ct** values should be less than the boundary **Ct** values specified in the *Important Product Information Bulletin* (qualitative analysis).
12. For DNA calibrations – **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 strips (e.g. Axygen, USA) (detection through the cap of the tube).

Programming the thermocycler:

1. Turn on the instrument and start the **Bio-Rad CFX Manager** 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	
	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 *C_t* (threshold cycle) value in the corresponding column of the results table. The calibration curve plotting and calculation of the *EBV* DNA concentration are performed automatically in accordance with the *C_t* 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 *C_t* 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 (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 (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	
	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 newly opened window mark all test samples as **Unknown**, positive controls as “+”, negative controls as “–”. Set calibrators in **JOE/HEX, FAM** and **ROX** channels as **Standard** and set the concentration values from the *Important Product Information Bulletin*. When setting the calibrators, the **Whole Plate Loading** button should be deactivated. For all samples and calibrators set the fluorescence detection in three channels **JOE/HEX, FAM** and **ROX**.
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 the 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).

Data analysis of EBV DNA (JOE/HEX channel):

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

1. In the results grid the *Ct* values for **EBV DNA** will appear, the concentration values (**Calc Conc (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 EBV and human DNA** – *Ct* value should be less than values specified in *Important Product Information Bulletin*, for quantitative test, the calculated concentrations should be within range of values specified in the *Important Product Information Bulletin*.
5. For Positive Control of Amplification (C+) – **KSG2** – the *Ct* values should be less than the boundary *Ct* values specified in the *Important Product Information Bulletin* (qualitative analysis).
6. For DNA calibrators – **KSG1** and **KSG2** – the *Ct* values and concentration values will appear (quantitative analysis)

Data analysis of the IC Glob (FAM channel):

Check the sample grid 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 DNA** will appear in each test sample, and for quantitative analysis the concentration values (**Calc Conc (copies/reaction)**) will appear. Herewith the *Ct* value should not exceed the values specified in the *Important Product Information Bulletin*.
2. For Negative Control of Extraction (C–) – **Negative Control (C–)** – the *Ct* value should be absent.
3. For Negative Control of Amplification (NCA) – **RNA-buffer** – the *Ct* value should be absent.
4. For Positive Control of Extraction (PCE) – **Positive Control DNA EBV and human DNA** – the *Ct* values should be less than the values specified in the *Important Product Information Bulletin*, and for quantitative analysis the concentration values should be defined.
5. For Positive Control of Amplification (C+) – **KSG2** – the *Ct* values should be less than the values specified in the *Important Product Information Bulletin* (qualitative analysis).
6. For DNA calibrators – **KSG1** and **KSG2** – the *Ct* values and concentration values (**Calc Conc (copies/reaction)**) (for quantitative analysis) will appear.

Data analysis of the Internal Control STI-87 (ROX channel):

Check the sample grid for presence of the calibrators and their concentrations in case of quantitative analysis.

1. In the results grid one will be able to see the *Ct* values for **Internal Control STI-87 (IC)** for each test sample and in the Negative Control of Extraction (C–); and for quantitative analysis, the concentration values (**Calc Conc (copies/reaction)**) will appear. Herewith the *Ct* value should not exceed the value specified in the *Important Product Information Bulletin*.
2. For Negative Control of Extraction (C–) – **Negative Control (C–)** – the *Ct* values should be less than the value specified in *Important Product Information Bulletin*.
3. For Negative Control of Amplification (NCA) – **RNA-buffer** – *Ct* values should be absent.
4. For Positive Control of Extraction (PCE) – **Positive Control DNA EBV and human DNA** – *Ct* values should be less than the values specified in the *Important Product Information Bulletin*, for quantitative analysis, the concentration values should be defined.
5. For Positive Control of Amplification (C+) – **KSG2** – the *Ct* values should be less than the values specified in the *Important Product Information Bulletin* (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 (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 and close the lid.

NOTE: Do not rotate the strips when loading 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, set **ROX** parameter next to the **ROX filter set** 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 strips by holding **Ctrl** and selecting the required range.
 - Mark all selected wells as **Unknown** in the **Well type** window. Select **FAM**, **JOE** and **ROX** fluorophores for the **Collect fluorescence data** option. Name each sample by double clicking on each well (**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** and **ROX** channels as **Standard**. Indicate concentration from the *Important Product Information Bulletin*.
8. In **Thermal Profile Setup** set the amplification program. To do this, use one of the following methods:

Using of 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	
	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. Select the **Results** tab.
5. Make sure that three fluorescence channels are active (the **JOE**, **ROX** and **FAM** buttons are pressed in the **Dyes Shown** field at the bottom of the window).
6. Select the **Threshold fluorescence** field and make sure that tick marks are put against three fluorescence channels: **JOE/HEX**, **FAM**, **ROX**. 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), **IC Glob DNA** (FAM channel), **Internal Control STI-87 (IC) DNA** (ROX channel) will appear; and for quantitative analysis the concentration values (**Calc Conc (copies/reaction)**) will appear. In the FAM and ROX channels 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, the *Ct* value should be less than the value specified in *Important Product Information Bulletin* in the ROX channel.
9. For Negative Control of Amplification (NCA) – **RNA-buffer** – *Ct* values should be absent in the FAM, JOE/HEX and ROX channels.
10. For Positive Control of Extraction (PCE) – **Positive Control DNA EBV and human DNA** – the *Ct* values should be less than the values specified in the *Important Product Information Bulletin* for all channels; for quantitative analysis, the concentration values should fall within values range 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)**) (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.

TROUBLESHOOTING

1. If the *Ct* value is determined in the channel for ROX fluorophore for Negative Control of Amplification (NCA); if the *Ct* value is determined in the channels for FAM and JOE fluorophores 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. If *Ct* value is absent or greater than the threshold in the results grid for the Positive Control of Amplification (C+) – **KSG2** – in the channel for JOE (*EBV*), FAM or ROX fluorophores, the amplification should be repeated for all samples where **EBV DNA** was not detected.
3. If the *Ct* value is absent or greater than threshold for the Positive Control of Extraction (PCE) – **Positive Control DNA EBV and human DNA** – in the channel for JOE (*EBV*), FAM or ROX 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 is absent or is greater than the specified boundary value in channel for JOE and the *Ct* value in the channels for FAM or ROX fluorophore is greater than the maximal value for **IC**, the experiment should be repeated starting from DNA extraction stage.
5. If the *Ct* value is greater than the specified boundary value in channel for JOE and the *Ct* value in the channels for FAM or ROX is less than the specified boundary value, 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 quantitation analysis the copies/reaction values in DNA calibrators differ by more than for 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.

DNA EBV CONCENTRATION CALCULATION

Concentration calculation in lg of DNA EBV copies per 10⁵ cells upon DNA extraction from whole human blood, white blood cells, viscera biopsy material uses the following formula:

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

For expression of EBV DNA concentration ratio in copies on standard cell quantity (for example, on 10⁵) the following conversion rate is used

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

DNA EBV concentration per ml of sample in case of DNA extraction from peripheral blood plasma, amniotic fluid, cerebrospinal fluid (liquor), oropharyngeal swabs, bronchoalveolar lavage with internal control sample is calculated using the following formula:

$$\text{CS DNA EBV} = \text{KDNA EBV} / \text{KSTI-87} \times \text{IC coefficient (copies/ml)}$$

KDNA EBV is the number of copies of DNA EBV in DNA-sample;

KSTI-87 is the number of copies of STI-87 DNA in DNA-sample;

IC coefficient is the number of copies of Internal Control STI-87 DNA in 1 ml of the DNA-sample. It is specified in the *Important Product Information Bulletin* and is **specific** for each lot.

AmpliSens® EBV-screen/monitor-FRT PCR kit is validated in compliance with the 1st WHO International Standard for Epstein-Barr virus for nucleic acid amplification techniques, NIBSC code 09/260, version 2.0, 12/01/2012 (Great Britain). The calculation coefficient of EBV DNA copies/ml in IU/ml for the **AmpliSens® EBV-screen/monitor-FRT** reagent kit is equal to 1.7:

$$1 \text{ IU EBV DNA/ml} = 0.59 \text{ EBV DNA copies/ml}$$

The concentration calculation of EBV DNA in peripheral blood samples, whole blood samples, amniotic fluid, cerebrospinal fluid (liquor), saliva, rinsing samples and swabs from pharynx and bronchoalveolar lavage in IU EBV DNA/ml uses the following formulae:


$$\frac{\text{KDNA EBV}}{\text{KSTI-87}} \times \text{IC coefficient} \times 1.7 = 1 \text{ IU EBV DNA/ml}$$

KDNA EBV is the number of copies of DNA EBV in DNA-sample;

KSTI-87 is the number of copies of STI-87 DNA in DNA-sample;

IC coefficient is the number of copies of Internal Control STI-87 DNA in 1 ml of the DNA-sample. It is specified in the *Important Product Information Bulletin* and is **specific for each lot.**

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
11.07.13 FN	Footer	Catalogue number REF R-V9-100-S(RG,iQ,Mx)-CE was deleted
25.07.14	Text	Cover page was created, the contents page was created. Text was corrected in accordance with the template
	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) INSTRUMENTS	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 results interpretation for NCA and C– in the FAM/Green channel was changed
	DNA <i>EBV</i> concentration calculation	“Calculating DNA <i>EBV</i> copies per ml using an Excel table” section was deleted. Calculation from copies/ml to IU/ml was added. Information about validation with respect to International standard WHO 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
27.07.21 KK	Through the text	The symbol  was changed to NOTE:
	Cover page	The phrase “Not for use in the Russian Federation” 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)		
13.07.23 EM	Footer	REF R-V9-100-S(RG,iQ,Mx)-CE was added