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For Professional Use Only

GUIDELINES

to AmpliSens® *CMV*-screen/monitor-FRT PCR kit

for qualitative detection and quantification of human cytomegalovirus (*CMV*) DNA in the biological material by using real-time hybridization-fluorescence detection

AmpliSens®

Federal Budget Institute of
Science “Central Research
Institute for Epidemiology”
3A Novogireevskaya Street
Moscow 111123 Russia

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INTENDED USE

The guidelines describe the procedure of using **AmpliSens® *CMV*-screen/monitor-FRT** PCR kit for qualitative detection and quantification of *human cytomegalovirus* (*CMV*) DNA in the biological material (peripheral blood plasma, amniotic fluid, cerebrospinal fluid (liquor), 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);
- iCycler iQ5 (Bio-Rad, USA);
- CFX96 (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 AUTOMATED NUCLEIC ACID EXTRACTION SYSTEM

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

This method of extraction allows reducing the consumption of NucliSens lysis buffer. It is preferred for working with test samples that contain clots.

1. Switch on the NucliSENS easyMAG instrument and prepare it for the RNA/DNA extraction according to the instruction manual.
2. In the window for input of test samples, enter the following parameters for each sample:
   - Sample name;
   - ***Matrix*** for DNA extraction (select ***Plasma***);
   - ***Volume*** – ***0.1 ml***;
   - ***Eluate*** – ***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 the sample table into the created protocol.
5. Take the required number of special disposable tubes intended for DNA extraction in the NucliSENS easyMAG instrument (including negative and positive control of DNA extraction). Add **10 µl** of **Internal Control STI-87 (IC)** into each tube, on the internal walls. Add **550 µl** of **NucliSens lysis buffer.**

NOTE: When working with the material which contains clots, lysis should be carried out in 1.5-ml tubes. After finishing the incubation, tubes should be centrifuged at 10,000 rpm for 1 min. Then transfer the supernatant into special tubes intended for DNA extraction in the NucliSENS easyMAG instrument.

6. Add **100 µl** of the prepared samples into the tubes with **NucliSens lysis buffer** and **Internal Control STI-87 (IC)** using disposable tips with aerosol barriers and carefully mix by pipetting. Avoid adding mucus clots and large particles into the tube.
7. Add **100 µl** of **Negative Control (C–)** into the tube with Negative Control of Extraction (C–). Add **90 µl** of **Negative Control (C–)** and **10 µl** of **Positive Control DNA *CMV* and human DNA** into the tube with Positive Control of Extraction (PCE).
8. Incubate the tubes for 10 min at room temperature.
9. Resuspend the tube with **magnetic silica NucliSens** by intensive vortexing. Add **10 µl** of **magnetic silica** using disposable tips with aerosol barriers and carefully mix by pipetting. Magnetic silica should be distributed evenly over the whole tube volume.
10. Place the tubes with the samples into the instrument and start the DNA extraction

program with lysis of samples by selecting ***off board*** mode.

11. After finishing DNA extraction, take the tubes out of the instrument.

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

1. Switch on the NucliSENS easyMAG instrument and prepare it for 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 ml***;
   - ***Eluate*** – ***55 µl***;
   - ***Type*** – ***Primary***;
   - ***Priority*** – ***Normal***.
3. Create a new protocol of DNA extraction and save it. Select ***On-board Lysis Buffer Dispensing – yes***, ***On-board Lysis Incubation – yes***.
4. Relocate the programmed sample into the created protocol.
5. Add **100 µl** of prepared samples into the tubes intended for DNA extraction in NucliSENS easyMAG instrument by using of disposable tips with aerosol barriers.
6. Add **100 µl** of **Negative Control (C–)** into the tube with Negative Control of Extraction (C–) intended for DNA extraction in the NucliSens easyMAG instrument. Add **90 µl** of **Negative Control (C–)** and **10 µl** of **Positive Control DNA *CMV* and human DNA** into the tube with Positive Control of Extraction (PCE).
7. Mix **magnetic silica NucliSens** and **Internal Control STI-87 (IC)** in an individual sterile 2-ml tube using sterile disposable tips with aerosol barriers at the following ratio:

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

8. Mix the contents of the tube. The mixture of **magnetic silica NucliSens** and **Internal Control STI-87 (IC)** can be stored for at most 30 min.
9. Place tubes with samples into the instrument and start up the DNA extraction program with lysis of samples by selecting the ***on board*** mode.
10. Wait until the NucliSENS easyMAG instrument stops working at the ***Instrument State – Idle*** position (~ 15 min).
11. Carefully mix the tube with the prepared mixture of **magnetic silica NucliSens** and **Internal Control STI-87 (IC)** by vortexing to a homogeneous state.

12. Open the lid of the instrument and add **20 µl** of the **mixture** to each tube. Mix carefully the contents of each tube by pipetting using multichannel pipettes. Use 200-µl individual tips with aerosol filters.
13. Continue the DNA extraction program.
14. After the extraction is completed, take the tubes out of the instrument.

POSITIVE RESULT *Ct* DETERMINATION

Before starting the work with a new reagents lot, *Ct* values need to be determined for positive samples in the JOE/HEX/Yellow channel for each instrument. Dilute **Positive Control DNA *CMV* 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 *CMV* and human DNA**). Run the PCR amplification of the 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*** or ***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*. The obtained value is the cycle threshold value for the positive result.

An example is given in Table 1.

Table 1

Example of calculation of the threshold value of a positive result

	***Ct* (JOE/HEX/ Yellow)**	**Average value**	**Threshold value for positive samples**
Positive Control DNA *CMV* 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 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, ROX/Orange	
	72	15 s	–	

6. After setting up the temperature profile click the ***OK*** button.
7. Select the ***Calibrate/Gain Optimisation*** button in the ***New Run Wizard*** window:
   - perform the calibration in FAM/Green, JOE/Yellow, ROX/Orange channels (activate the ***Calibrate Acquiring/Optimise Acquiring*** button);
   - perform the calibration before the first detection (select ***Perform Calibration Before 1st Acquisition/Perform Optimisation Before 1st Acquisition)***;
   - to set channels calibration, indicate **5** in the ***Min Reading*** box and **10** in the ***Max Reading*** box. Tick ***Perform Calibration Before 1st Acquisition/Perform Optimization Before 1st 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 experiment will be automatically saved in this file).
10. Enter the data into 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,** the Positive Control of amplification as **C+**. Set the type ***Unknown*** opposite all the test samples, the type ***Positive control*** – for the Positive control of amplification, the type ***NTC*** – for the Negative control of amplification. Set the type ***Standard*** for DNA-calibrators C1 and C2 and enter the values from the *Important product information bulletin* enclosed to the PCR kit into the ***Given conc.*** column. Set the type ***None*** for the cells matching with the corresponding empty tubes.

NOTE: Samples indicated as ***None*** won't be analysed.

Data analysis for the *CMV* 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 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 (PCE) – **Positive Control DNA *CMV* and human DNA** – the *Ct* value should be less than the boundary value specified in *Important Product Information Bulletin* and for quantitative analysis the calculated concentration value should fall within the range of values specified in the *Important Product Information Bulletin*.
11. For Positive Control of Amplification (C+) – **KSG2** – the *Ct* value should be less than the value specified in the *Important Product Information Bulletin* (for qualitative analysis).
12. For DNA calibrations – **KSG1** and **KSG2** – the *Ct* values and concentration values (***Calc Conc (copies/reaction)***) (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* value should not exceed the value specified in the *Important Product Information Bulletin.*
8. For the Negative Control of Extraction (C–) – **Negative Control (C–)** – the *Ct* value should be absent.
9. For the Negative Control of Amplification (NCA) – **RNA-buffer** – the *Ct* value should be absent.
10. For the Positive Control of Extraction (PCE) – **Positive Control DNA *CMV* 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 value 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 concentrations values (***Calc Conc (copies/reaction)*** (for quantitative analysis) should appear.

Data analysis of the Internal Control STI-87 (IC) (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, 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 the 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 the Negative Control of Amplification (NCA) – **RNA-buffer** – *Ct* value should be absent.
10. For the Positive Control of Extraction (PCE) – **Positive Control DNA *CMV* and human DNA** – the *Ct* value should be less than the value specified in the *Important Product Information Bulletin*, for quantitative analysis, the concentration value 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 concentration values (***Calc Conc (copies/reaction)***) (for quantitative analysis) should 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) INSTRUMENT

Carry out the sample pretreatment and reaction mixture preparation stages according to the PCR kit *Instruction Manual*. When carrying out the amplification it is recommended to use thin-walled PCR tubes (0.2 ml) with 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	
	72	15 s	–	

3. Name the new protocol and save it.
4. Set the ***Plate Setup*** (set the order of the tubes in the reaction chamber).
5. In the opened window, mark all the test samples as ***Unknown*****,** positive controls as **"+",** negative controls as **"–".** Set the calibrators in **JOE/HEX, FAM and ROX** channels as ***Standard*** and indicate concentration from the *Important Product Information Bulletin*. When setting the calibrators, the ***Whole Plate Loading*** button should be deactivated. Set fluorescence detection in **JOE/HEX, FAM, and ROX** channels for all the samples and calibrators.
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 (***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 the *CMV* 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 ***CMV*** **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 *CMV* and human DNA** – *Ct* value should be less than values specified in the *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* value should be less than the value 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 be less than values specified in *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 *CMV* 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 value should be defined.
5. For Positive Control of PCR (C+) – **KSG2** – the *Ct* value should be less than the value specified in the *Important Product Information Bulletin* (qualitative analysis).
6. For DNA calibrations – **KSG1** and **KSG2** – *Ct* values and concentration values (***Calc Conc (copies/reaction)***) will appear (quantitative analysis).

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

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

1. In the results grid (the ***Quant. Results*** window) one will be able to see the *Ct* values for **Internal Control STI-87 (IC) DNA** for each test sample and 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* value should be less than the value specified in the *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 *CMV* 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 value should be defined.
5. For DNA calibrations – **KSG1** and **KSG2** – *Ct* values and concentrations (***Calc Conc (copies/reaction)***) (for quantitative analysis) should 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 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.

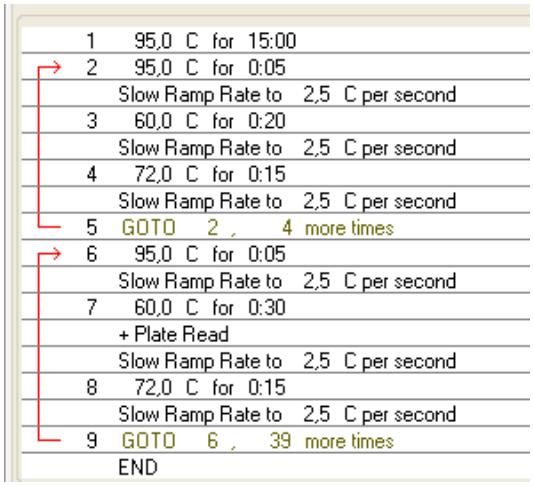
Creating the template for the run

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***.



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 the *CMV* DNA concentration are performed automatically in accordance with the *Ct* values.

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.

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.***

4. In the results grid one will be able to see the *Ct* values for ***CMV* DNA** (HEX channel), **IC Glob DNA** (FAM channel), **Internal Control STI-87 (IC) DNA** (ROX channel); 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 *Important Product Information Bulletin.*
5. For Negative Control of Extraction (C–) – **Negative Control (C–)** – *Ct* values should be absent in the FAM and HEX channels; the *Ct* value should be less than the value specified in *Important Product Information Bulletin* in the ROX channel.
6. For Negative Control of Amplification (NCA) – **RNA-buffer** – *Ct* values should be absent in the FAM, HEX and ROX channels.
7. For Positive Control of Extraction (PCE) – **Positive Control DNA *CMV* and human DNA** – the *Ct* value should be less than the value specified in *Important Product Information Bulletin* in all the channels; for quantitative analysis the concentration values should be within range of the values specified in *Important Product Information Bulletin*.
8. For DNA calibrators – **KSG1** and **KSG2** – the *Ct* values and concentration values (***Calc Conc (copies/reaction)***) (for quantitative analysis) should 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 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 and close the lid.

NOTE: Do not rotate the strips when loading the instrument.

4. Select ***Optics Configuration*** in the ***Options*** menu, set **JOE/HEX** parameters opposite the ***HEX/JOE filter set*** item, **FAM** parameters opposite the ***FAM filter set*** item, and **ROX** parameters opposite the ***ROX filter set*** item.
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 the selected wells in the ***Well type*** window as ***Unknown***. Set **FAM**, **JOE,** and **ROX** in 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 concentrations 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:

<u>Using of the template file</u> 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. Check the sample table for presence of the calibrators and their concentrations in case of quantitative analysis.
2. Proceed to the ***Analysis*** mode (select the corresponding button on the toolbar).
3. Make sure that all the samples in the ***Analysis Selection/Setup*** tab are active (cells corresponding to samples should have another color). Otherwise select all tested samples and, holding down the ***Ctrl*** button, mark the necessary region with the mouse.
4. Proceed to the ***Results*** tab.
5. Make sure that **JOE/HEX**, **FAM** and **ROX** channels are active (**JOE**, **FAM** and **ROX** buttons are pressed in the ***Dyes Shown*** field at the bottom of the window).
6. Make sure that **JOE/HEX**, **FAM** and **ROX** buttons are activated in the ***Threshold fluorescence*** field. Make sure that the automatic selection of the threshold level is correct. Normally, the threshold line should intercept only the sigmoid curves of signal accumulation for positive samples and for controls, yet it should not intercept the base line. Otherwise, the threshold level should be raised. Curves of signal accumulation are displayed linear by default. To change curve from linear to logarithmic, click with the left mouse button on one of axis area (X or Y) and indicate ***Scale*** next to the ***Log*** item in ***Graph properties*** window (Y axis).
7. In the results grid one will be able to see the *Ct* values for ***CMV*** **DNA** (JOE/HEX channel), **IC Glob DNA** (FAM channel), **Internal Control STI-87 (IC) DNA** (ROX

channel); and for quantitative analysis the concentration values (***CaIc Conc (copies/reaction)***) will appear. In the FAM and ROX channels the *Ct* value should be less than the value specified in *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 *CMV* and human DNA** – the *Ct* value should be less than the value specified in *Important Product Information Bulletin* in all the channels; for quantitative analysis the concentration values should be within range of the values specified in *Important Product Information Bulletin*.
11. For DNA calibrators – **KSG1** and **KSG2** – the *Ct* values and concentration values (***CaIc Conc (copies/reaction)***) (for quantitative analysis) should 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 ROX/Orange channel for Negative Control of Amplification (NCA); 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. If *Ct* value is absent or greater than the threshold in the results grid for the Positive Control of Amplification (C+) – **KSG2** – in the JOE/Yellow/HEX (*CMV*), FAM/Green or ROX/Orange channels, the amplification must be repeated for all samples where ***CMV* 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 *CMV* and human DNA –** in the JOE/Yellow/HEX (*CMV*), FAM/Green or ROX/Orange channels, 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 JOE/Yellow/HEX channel and the *Ct* value in the FAM/Green or ROX/Orange channels 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 JOE/Yellow/HEX channels and the *Ct* value in the FAM/Green or ROX/Orange channels 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 quantitative 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.

CALCULATION OF *CMV* DNA CONCENTRATION

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

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

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

10^5 of cells = $2\cdot10^5$ human genome equivalents.

The concentration of *CMV* DNA per ml of sample when DNA is extracted from whole human blood, peripheral blood plasma, amniotic fluid, cerebrospinal fluid (liquor), bronchoalveolar lavage together with the internal control sample is calculated by the following formula:

CS *CMV* DNA = (К *CMV* DNA / К STI-87) x IC coefficient, (*CMV* DNA copies/ml)

where **К *CMV* DNA is the number of copies of *CMV* DNA in DNA sample;**

$К_{STI-87}$ 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, ml. It is indicated in the *Important Product Information Bulletin* and is **specific for each lot.**

NOTE: **AmpliSens® *CMV*-screen/monitor-FRT** reagents kit is validated is validated in compliance with the international WHO standard - 1st WHO International Standard for Human Cytomegalovirus (HCMV) for nucleic acid amplification techniques NIBSC code 09/162, version 3.0, 30/11/2010 (Great Britain). The calculation coefficient of *CMV* DNA copies/ml in IU/ml for the **AmpliSens® *CMV*-screen/monitor-FRT** reagent kit is equal to 0.6:

1 IU *CMV* DNA/ml = 1.67 *CMV* DNA copies/ml

The concentration of *CMV* DNA in the samples of peripheral blood plasma, whole blood, amniotic fluid, cerebrospinal fluid (liquor), bronchoalveolar lavage in IU *CMV* DNA/ml is calculated by the following formula:

$$\frac{К_{\textit{CMV}\text{ DNA}}}{К_{\text{STI-87}}}\ \textbf{IC coefficient (copies/ml) 0.6 = IU } \textit{CMV}\textbf{ DNA/ml}$$

where **К *CMV* DNA is the number of copies of *CMV* DNA in DNA sample;**

K_{STI-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, ml. It is indicated 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
31.07.12 BM	Cover page	Cover page was added
	Text	Table of contents was added
		Names of sections were corrected
20.01.13 LA	Page footer	Reference number R-V7-100-S(RG,iQ,Mx)-CE-B was deleted
05.09.13 GA	Work with NucliSENS easyMAG automated nucleic acid extraction platform (bioMérieux, France). Variant 2. DNA extraction on-board sample lysis (on-board mode)	The paragraph "This method of extraction allows reducing the consumption of NucliSens lysis buffer. It is preferred for working with clinical samples which contain clots" was deleted
	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia). Programming the Rotor-Gene 3000/6000 instrument	In item 7 channel Cy5/Red was deleted
	Through the text	Misprints were corrected
	Calculation of *CMV* DNA concentration	The composition of the clinical samples was changed
		The procedure of calculation of *CMV* DNA concentration in the samples was changed
05.02.15 PM	Cover page	Address of European representative was added
	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) AND Rotor-Gene Q (QIAGEN, Germany) instruments, Amplification and data analysis with the use of iCycler iQ5 (Bio-Rad, USA) instrument, Amplification and data analysis using Mx3000P and Mx3005P instruments (Stratagene, USA)	Data analysis of test and control samples was corrected for each channel
	Troubleshooting	The chapter was added
	Calculation of *CMV* DNA concentration	Calculation coefficient of *CMV* DNA copies/ml in IU/ml was added
	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
28.12.15 ME	Text	The clinical material saliva, oropharyngeal swabs, urine samples was deleted
31.05.21 KK	Through the text	The symbol  was changed to NOTE:
	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)	
	Footer	The phrase “For research use only. Not for diagnostic procedures” was added
22.06.23 EM	Footer	**REF** R-V7-100-S(RG,iQ,Mx)-CE was added