RUO

For Professional Use Only

GUIDELINES

to AmpliSens® *HIV*-Monitor-FRT PCR kit

for quantitative detection of *human immunodeficiency virus* type 1 (*HIV*-1) RNA in the biological material by polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

AmpliSens®

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

For research use only. Not for diagnostic procedures

TABLE OF CONTENTS

INTENDED USE

The guidelines describe the procedure of using **AmpliSens® *HIV*-Monitor-FRT** PCR kit for quantitative detection of *human immunodeficiency virus* type 1 (*HIV*-1) RNA in biological material (blood plasma) by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using the following instruments:

- Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia),
- Rotor-Gene Q (QIAGEN, Germany),
- iCycler iQ, iCycler iQ5 (Bio-Rad, USA),
- CFX96 (Bio-Rad, USA),
- Mx3000P, Mx3005P (Stratagene, USA),

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

The guidelines describe also the procedure of calculation of *HIV* RNA concentration.

CONTROL SAMPLES PREPARATION WHEN EXTRACTING FROM 1,000 µL

When working with forms 2 and 3 and extracting nucleic acids from 1,000 µl, the control samples can be prepared with extra volume.

For **the positive controls of extraction (PCE-1, PCE-2)** carry out the dilution in two steps:

1. Dilute the **Positive Control-1-*HIV*** and **Positive Control-2-*HIV* 10 times** with the use of **Negative Control (C–)** (from the content of forms 2 and 3). For example, **135 µl** of **Negative Control (C–)** and **15 µl of Positive Control-1-*HIV*** or **Positive Control-2-*HIV*.**
2. Dilute the obtained samples **10 times** with sterile H_2O (is not a component of forms 2 and 3) to the required volume. For example, **130 µl** of **Positive Control-1-*HIV*** or **Positive Control-2-*HIV*** diluted in Negative Control (C–) and **1170 µl** of **H_2O**.

For **the negative control of extraction (C–)** dilute **Negative Control (C–) 10 times** with sterile H_2O (is not a component of forms 2 and 3) to the required volume. For example, **130 µl** of **Negative Control (C–)** and **1170 µl** of **H_2O**.

For **Calibratior *HIV*-Q** carry out the dilution in two steps with the use of **Solvent Q only:**

1. Sediment the content of the tube with **Calibratior *HIV*-Q** on vortex. Open the cap carefully. Add **400 µl** of **Solvent Q** avoiding spraying the contents. Tightly cap the tube and incubate it at room temperature for 20 min vortexing occasionally. After complete dissolution sediment the drops from the cap of the tube with **Calibratior *HIV*-Q** on vortex for 3-5 sec.

2. Dilute the obtained sample of **Calibratior *HIV*-Q 10 times** with **Solvent Q** to the required volume. For example, **125 µl** of dissolved **Calibratior *HIV*-Q** and **1125 µl** of **Solvent Q**.

WORK with the NucliSENS easyMAG AUTOMATED NUCLEIC ACID EXTRACTION SYSTEM

Variant 1

RNA extraction from 100 µl - sample with lysis of sample outside of the instrument (off-board mode)

1. Switch on the NucliSENS easyMAG instrument and prepare it to the RNA extraction according to the instruction manual.
2. In the window for input of test samples enter the following parameters:
   - Sample name
   - ***Matrix*** for RNA/DNA extraction (select *Plasma*)
   - ***Volume*** – 0.1 ml
   - ***Eluate*** – 55 µl
   - ***Type*** – Lysed
   - ***Priority*** – Normal.
3. Create a new protocol of RNA 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. Add **450 µl** of **NucliSens lysis buffer** to the wells of the reagent cartridge intended for RNA extraction in the NucliSENS easyMAG instrument.
6. Add **100 µl** of test plasma into each well of the reagent cartridge by using disposable tips with filters and carefully mix by pipetting.
7. For each panel it is necessary to carry out the control reactions as follows:

PCE-1 – Add **90 µl of Negative Control (C–)** and **10 µl of Positive Control-1-*HIV*** to the tube with lysis solution labelled PCE-1 (Positive control of Extraction);

PCE-2 – Add **90 µl of Negative Control (C–)** and **10 µl of Positive Control-2-*HIV*** to the tube with lysis solution labelled PCE-2 (Positive control of Extraction);

C– – Add **100 µl of Negative Control (C–)** to the tube with lysis solution labelled C- (Negative control of Extraction).

Mix by pipetting.

8. Incubate the reagent cartridge for 10 min at room temperature to ensure lysis.
9. Mix in a new sterile 1.5-ml tube NucliSens magnetic silica and Internal Control by using disposable tips with filters (see table 1).

Table 1

Quantity of samples for RNA/DNA extraction	Quantity of magnetic silica NucliSens, µl	Quantity of Internal Control, µl
1	10	10
8	90	90
16	170	170
24 (complete load instrument)	250 (extra for 25 samples)	250

10. Add **20 µl of prepared mixture of NucliSens magnetic silica and Internal Control** to each well of the reagent cartridge. Carefully mix the contents of each well by using a pipette with 1,000 µl disposable tips with filters.
11. Place the reagent cartridge with samples into the instrument, insert tips and start the RNA extraction program with lysis of samples by selecting the ***off board*** mode.
12. After the extraction procedure is completed, remove the reagent cartridge from the instrument and carry out the RT-PCR not later than 30 min after the completion of RNA extraction.

For long-time storage transfer the supernatant without disturbing the sorbent into a sterile tube and store at the temperature from minus 24 to minus 16 °C for 1 month or at the temperature below minus 68 °C for 1 year.

Variant 2

RNA/DNA extraction from 0.1 – 1 ml sample with automated lysis of sample in the instrument (on-board mode)

1. Sample lysis is carried out in the NucliSENS easyMAG instrument in automatic mode. Add 100 µl or 1 ml of test plasma by using disposable tips with filters to each wells of the reagent cartridge intended for RNA extraction in the NucliSENS easyMAG instrument.
2. For each panel it is necessary to carry out the control reactions as follows:

PCE-1 – Add **90 µl of Negative Control (C–)** and **10 µl of Positive Control-1-*HIV*** to the tube with lysis solution labelled PCE-1 (Positive control of Extraction);
PCE-2 – Add **90 µl of Negative Control (C–)** and **10 µl of Positive Control-2-*HIV*** to the tube with lysis solution labelled PCE-2 (Positive control of Extraction);
C– – Add **100 µl of Negative Control (C–)** to the tube with lysis solution labelled C- (Negative control of Extraction).

Mix by pipetting.

3. Switch on the NucliSENS easyMAG instrument and prepare it for the RNA extraction according to the instruction manual.
4. In the window for input of test samples enter the following parameters:
   - Sample name
   - ***Matrix*** for RNA extraction (select ***Plasma***)

- ***Volume*** – from 100 µl to 1 ml
- ***Eluate*** – 55 µl
- ***Type*** – Primary
- ***Priority*** – Normal.

5. Create a new protocol of RNA extraction and save it. In the protocol, select ***On-board Lysis Buffer Dispensing - yes***, ***On-board Lysis Incubation - yes*****.**
6. Relocate programmed sample to created protocol.
7. Place the reagent cartridge with samples into the instrument, insert aspiration tip sets, and run the RNA extraction with sample lysis in the instrument (***on board*** mode).
8. Wait for the NucliSENS easyMAG instrument stop working in ***Instrument State-Idle*** position (near 15 min).
9. Mix in a new sterile 1.5-ml tube NucliSens magnetic silica and Internal Control by using disposable tips with filters (see table 1).
10. Open the lid of the instrument and add **20 µl of prepared mixture of NucliSens magnetic silica and Internal Control** to each well of the reagent cartridge. Thoroughly mix the contents of each well by using a pipette with 1000 µl disposable tips with filter.
11. Continue the RNA extraction program.
12. After the RNA extraction procedure is completed, remove the reagent cartridge from the instrument and carry out the RT-PCR not later than 30 min after completion of RNA extraction.

For long-time storage transfer the supernatant without disturbing the sorbent into a sterile tube and store at the temperature from minus 24 to minus 16 °C for 1 month or at the temperature below minus 68 °C for 1 year.

AMPLIFICATION AND DATA ANALYSIS USING Rotor-Gene 3000/6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany)

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. It is recommended to use 0.2-ml tubes with flat caps (detection through the bottom of the tube) or 0.1-ml tubes.

Place tubes into the rotor ensuring that the first tube appears in well no. 1, place the rotor into the instrument, and close the lid (cells are numbered, these numbers are used for subsequent tube order programming).

NOTE: Well No. 1 should be loaded with a test tube from the current experiment

Programming the instrument

1. Press the ***New*** button in the main menu of the program.
2. In the opened window select ***Advanced*** menu and ***Dual Labeled Probe/Hydrolysis probes***. Activate the ***New*** button.
3. Select ***36-Well Rotor*** (or ***72-Well Rotor***) and ***No Domed 0.2 ml Tubes***/***Locking ring attached***. Click the ***Next*** button.
4. Set an operator and specify the ***Reaction volume*** as **50 µl**. Click the ***Next*** button.
5. Select the ***Edit profile*** button and set the amplification program (see table 2)

Table 2

***HIV*-Monitor-FRT program for rotor-type instruments**

Step	Temperature, °C	Time	Fluorescence detection	Cycles
Hold 1	50	30 min	–	1
Hold 2	95	15 min	–	1
Cycling 1	95	20 s	–	5
	52	30 s	–	
	72	30 s	–	
Cycling 2	95	20 s	–	40
	55	30 s	FAM/Green, JOE/Yellow	
	72	30 s	–	

6. Click ***OK***.
7. In the ***New Run Wizard*** window select the ***Calibrate***/**Gain** ***Optimisation***.
   - For calibration in FAM/Green and JOE/Yellow channels select ***Calibrate Acquiring/Optimise Acquiring***.
   - Check the ***Perform Calibration Before 1st Acquisition/Perform Optimisation Before 1st Acquisition***.

- For all channels set calibration from **5Fl** to **10Fl (*Edit…*** button, ***Auto gain calibration channel settings*** window). Press the ***Close*** button.

8. Click the ***Next*** button. Select the ***Start run*** button.
9. Name the experiment and save it to the disk (results of the run will be automatically saved in this file).
10. Set the data in the table of samples (open automatically after thermocycling process starts). Indicate the names/numbers of test samples in the ***Name*** column. For empty wells indicate ***None***.

NOTE: For DNA calibrators PIC1 *HIV* and PIC2 *HIV*, indicate ***Standard*** type and in the ***Given conc.*** column enter values specified in the *Important Product Information Bulletin.*

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

Data analysis

The Internal Control cDNA amplified product is detected in the FAM/Green channel. The *HIV* cDNA amplified product is detected in the JOE/Yellow channel. Results are interpreted by the presence (or absence) of the intercept between the fluorescence curve and the threshold line set at a certain level (in the middle of linear fragment of the positive control fluorescence growth in log scale), which determines presence (or absence) of *Ct* (cycle threshold) value of a sample in the corresponding cell of the result grid.

Data analysis *HIV* cDNA amplification (JOE/Yellow)

1. Activate the ***Analysis*** button then select ***Quantitation*** button and activate the ***Cycling A. JOE/Cycling A. Yellow*** button. Click ***Show***.
2. Cancel the ***Threshold*** automatic choice.
3. Activate the ***Dynamic tube*** and ***Slope Correct*** buttons (in the ***Quantitation analysis*** window) for each channel.
4. In the ***CT Calculation*** menu, set ***Threshold* = 0.03.**
5. Select the ***More Settings/Outlier Removal*** parameter and set ***NTC threshold*** value **5%.**
6. In the results grid (***Quant. Results*** window) the *Ct* (cycle threshold) values will appear.

Data analysis of the Internal Control cDNA amplification (FAM/Green channel)

1. Activate the ***Analysis*** button, then select the ***Quantitation*** button and activate the ***Cycling A. FAM/Cycling A. Green*** button. Click ***Show***.
2. Cancel the ***Threshold*** automatic choice.
3. Activate the ***Dynamic tube*** and ***Slope Correct*** buttons (in the ***Quantitation analysis*** window).

4. In ***CT Calculation*** menu, set ***Threshold* = 0.03.**
5. Select the ***More Settings*/*Outlier Removal*** parameter and set ***NTC threshold*** value **10 %.**
6. In the results grid (***Quant. Results*** window), the *Ct* (cycle threshold) values will appear.

Calculation of concentration in biological and control samples

Based on the *Ct* values (the intercept of the fluorescence curve and the threshold line set at a certain level) and on the specified values for the calibrators, PIC1 *HIV* and PIC2 *HIV*, the calibration line will automatically plot and produce the values for the number of *HIV* cDNA copies (JOE/Yellow channel) and for the number of Internal Control cDNA copies (FAM/Green channel) in a PCR sample. The retrieved values are used for the *HIV* RNA concentration calculation in tested and control samples, using the formulae:

$$\frac{\textit{HIV}\text{ cDNA copies per PCR-sample}}{\text{IC cDNA copies per PCR-sample}} \times \text{coefficient A} \times \text{coefficient B} = \textit{HIV}\text{ RNA copies/ml of plasma}$$

$$\text{Coefficient A} = \frac{100}{\text{extraction volume, µl}}$$

NOTE: Coefficient A = 1 when calculating PCE-1 and PCE- 2 concentrations

Coefficient B (number of copies of IC per ml of plasma) is specified in the *Important Product Information Bulletin* provided with the PCR kit and is specific for each lot. It cannot be used with PCR kits of different lots. If form 2 and 3 are used then coefficient B is calculated as the result of calibration during the first PCR run of PCR kit of a specific lot (procedure is described in Instruction Manual, Section 8.1.4).

NOTE:
If the result is greater than 10,000,000 copies/ml then it is interpreted as the **greater than 10,000,000 copies of *HIV* RNA/ml result.** If the obtained value is greater than the linear range, then the sample may be re-tested after 10x dilution; the produced result is multiplied by 10.
If the result is less than 500 copies/ml (extraction from 100 µl), or less than 250 copies/ml (extraction from 200 µl), or less than 50 copies/ml (extraction from 1 ml), than it is interpreted as the **less than 500,** or **less than 250,** or **less than 50 copies of *HIV* RNA/ml result**, respectively.

Note – To convert results from copies/ml into International Units (IU/ml), multiply results obtained in copies/ml by 1.75 (1 copy= 1.75 IU, 1 IU=0.57 copy)

Interpretation of results for control samples

The result of the analysis is considered reliable only if the results obtained for controls are correct (see table 3).

Table 3

Results for controls

Control	Stage for control	Result of amplification in the channel	
		FAM/Green	**JOE/Yellow**
C–	RNA extraction, PCR	**Positive** (IC concentration is greater than the boundary value)	Negative (*Ct* value is absent)
PCE-1	RNA extraction, PCR	**Positive** (IC concentration is greater than the boundary value)	**Positive** (concentration calculated with IC copies/ml should be within range)
PCE-2	RNA extraction, PCR	**Positive** (IC concentration is greater than the boundary value)	**Positive** (concentration calculated with IC copies/ml should be within range)
$C+_1$	PCR	Positive	Positive
$C+_2$	PCR	Positive	Positive
NCA	PCR	Negative *(Ct* value is absent)	Negative (*Ct* value is absent)

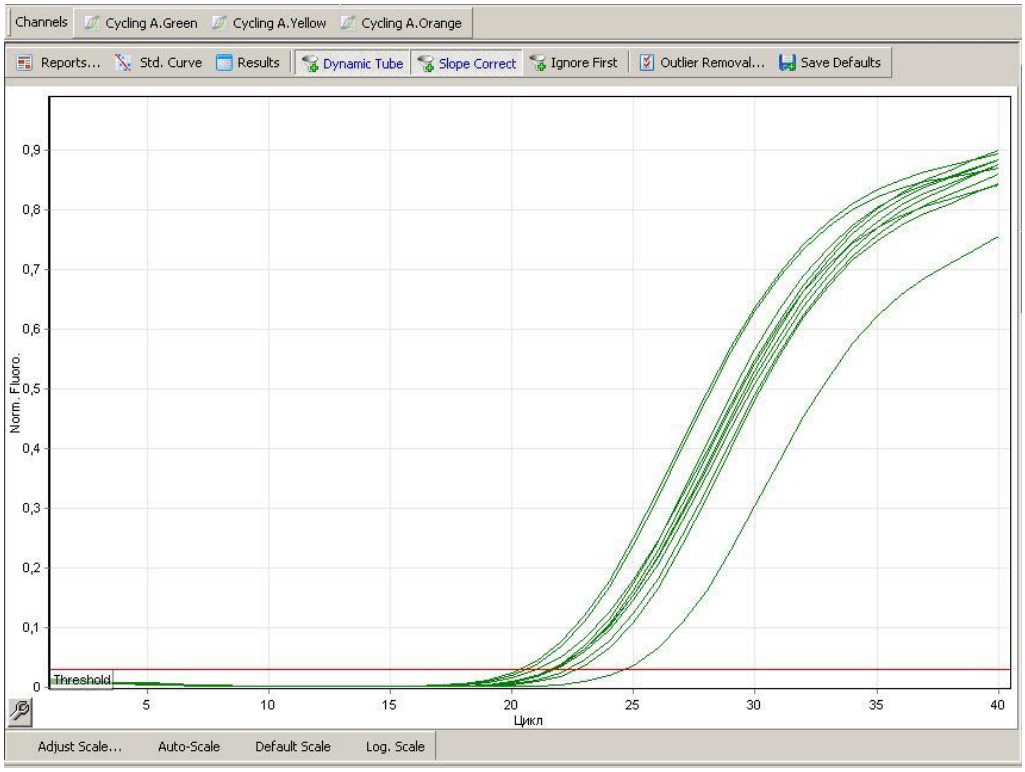
NOTE: The boundary (minimum allowable) concentration values for IC and the range of values for **PCE-1 (Positive Control-1-*HIV)*** and **PCE-2 (Positive Control-2-*HIV)*** calculated with IC copies/ml are specified in the *Important Product Information Bulletin* enclosed to the PCR kit of specific lot.

Results of analysis are not taking into account in the following cases

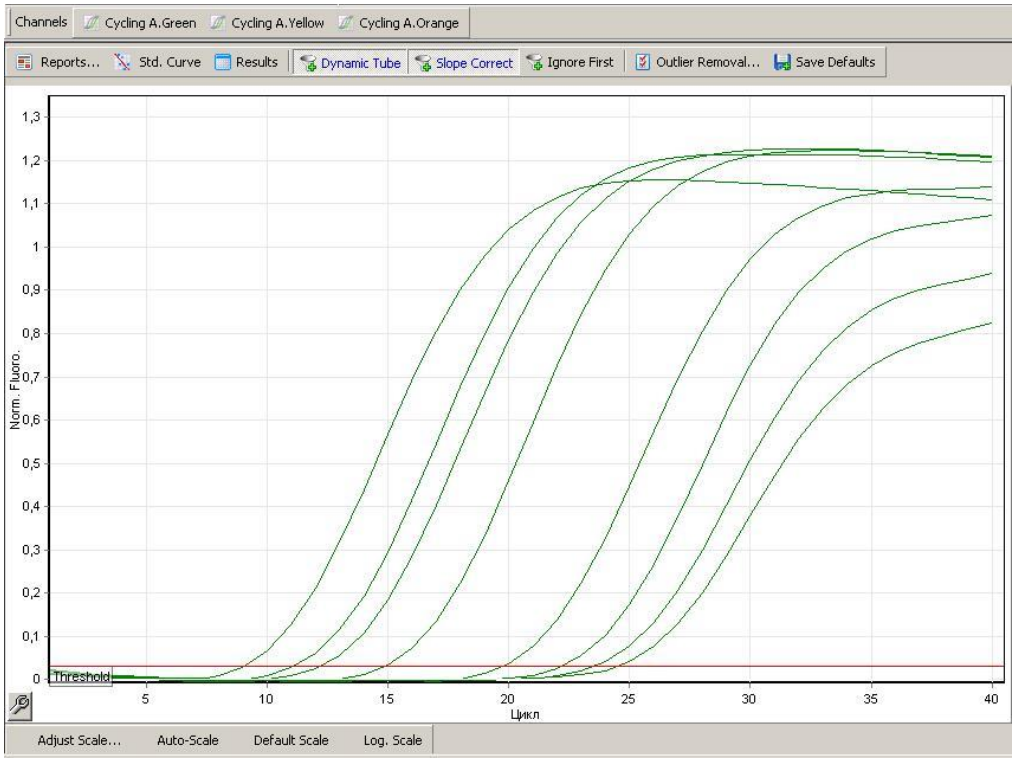
1. If *Ct* value is obtained for the Negative Control of extraction (C–) in the JOE/Yellow channel and/or Negative Control of amplification (NCA) in the FAM/Green and JOE/Yellow channels, analysis (beginning with RNA extraction) should be repeated for all samples in which *HIV* RNA was detected.
2. If concentration value of IC in the results grid is less than the boundary value specified in the *Important Product Information Bulletin*, repeat analysis of the sample starting from the first stage of the analysis.
3. If the correlation coefficient R^2 is less than 0.98, PCR should be repeated for all samples
4. If the calculated concentration values of Positive Control-1-*HIV* and Positive Control-2-*HIV* do not fit in the range specified in the *Important Product Information Bulletin*, analysis (starting from RNA extraction) should be repeated for all samples.

Example of results

FAM/Green channel data – Internal Control



JOE/Yellow channel data – samples with the specific target



AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ, iQ5 (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 0.2-ml PCR tubes with domed caps (e.g. Axygen, USA).

Programming the thermocycler

1. Turn on the instrument and the power supply unit of the optical block of the instrument.

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

2. Start the program iCycler iQ/iQ5.
3. Insert the tubes or strips into the reaction module of the thermocycler and program the instrument according to the *Instruction Manual* given by the manufacturer of the instrument.

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

Creating the template for the run

1. Select ***Create new*** in the ***Selected Protocol*** of the ***Workshop*** module.
2. Set the amplification program (see table 4).

Table 4

***HIV*-Monitor-FRT program for plate-type instruments**

Step	Temperature, °C	Time	Fluorescence detection	Cycle repeats
1	50	30 min	–	1
2	95	15 min	–	1
3	95	20 s	–	5
	52	30 s	–	
	72	30 s	–	
4	95	20 s	–	42
	55	40 s	FAM, JOE/HEX	
	72	30 s	–	

3. Name the new protocol and save it.
4. Set the tube order (***Plate Setup***) in the reaction module.
5. In the opened window mark all biological samples as ***Unknown***. Set fluorescence acquiring in FAM and JOE/HEX channels for all samples.

NOTE: For DNA calibrators PIC1 *HIV* and PIC2 *HIV*, indicate ***Std*** type and in the ***Quantity*** column enter values specified in the *Important Product Information Bulletin* enclosed in the PCR kit of the required lot.

6. Set the ***Sample Volume*** as **50 µl**, ***Seal Type*** as ***Domed Cap***, and ***Vessel Type*** as ***Tubes***. Save the plate setup. Make sure that plastic consumables are the same as those used for instrument calibration.

7. Start the amplification run:
   - iCycler iQ. Click the ***Run with Selected Plate*** button. In the opened window set reaction volume as ***50 µl***, select ***PCR Quantification Melt Curve*** and ***Experimental Plate***. Click ***Begin Run*** and save the experiment.
   - iCycler iQ5. Click ***Run***. In the open window mark ***Use Persistent Well Factors***, select ***Begin Run*** and save the experiment.

Data analysis

The Internal Control cDNA amplified product is detected in the FAM channel. The *HIV* cDNA amplified product is detected in the JOE/HEX channel. Results are interpreted by the presence (or absence) of the intercept between the fluorescence curve and the threshold line set at a certain level (in the middle of linear fragment of the positive control fluorescence growth in log scale), which determines presence (or absence) of *Ct* (cycle threshold) value of a sample in the corresponding cell of the result grid.

Data processing

1. Start the software and open the saved file: select ***Data file*** in the ***Workshop*** module. Proceed to ***Data Analysis*** mode.
2. View data on each channel one at a time.
3. Make sure that the threshold line is set correctly for each channel. Normally, the threshold line should cross s-shaped curves of positive samples and should not cross the baseline. Otherwise, the threshold level should be raised. To do this, click the ***Log View*** button. Set the threshold (using the left mouse button) at the level, where fluorescence curves are linear and do not cross the curves of negative samples.
4. For result analysis select the ***Results*** button.

Calculation of concentration in test and control samples

Based on the *Ct* values (the intercept of the fluorescence curve and the threshold line set at a certain level) and on the specified values for the calibrators, PIC1 *HIV* and PIC2 *HIV*, the calibration line will automatically plot and produce the values for the number of *HIV* cDNA copies (JOE/HEX channel) and for the number of Internal Control cDNA copies (FAM channel) in a PCR sample. The retrieved values are used for the *HIV* RNA concentration calculation in tested and control samples, using the formula:

$$\frac{\textit{HIV}\text{ cDNA copies per PCR-sample}}{\text{IC cDNA copies per PCR-sample}} \times \text{coefficient A} \times \text{coefficient B} = \textit{HIV}\text{ RNA copies/ml of plasma}$$

$$\text{Coefficient A} = \frac{100}{\text{extraction volume, µl}}$$

NOTE: Coefficient A = 1 when calculating PCE-1 and PCE- 2 concentrations

Coefficient B (number of copies of IC per ml of plasma) is specified in the *Important Product Information Bulletin* provided with the PCR kit and is specific for each lot. It cannot be used with PCR kits of different lots. If form 2 and 3 are used then coefficient B is calculated as the result of calibration during the first PCR run of PCR kit of a specific lot (procedure is described in Instruction Manual, Section 8.1.4).

NOTE: If the result is greater than 10,000,000 copies/ml then it is interpreted as the **greater than 10,000,000 copies of *HIV* RNA/ml result.** If the obtained value is greater than the linear range, then the sample may be re-tested after 10x dilution; the produced result is multiplied by 10.
If the result is less than 500 copies/ml (extraction from 100 µl), or less than 250 copies/ml (extraction from 200 µl), or less than 50 copies/ml (extraction from 1 ml), than it is interpreted as the **less than 500,** or **less than 250,** or **less than 50 copies of *HIV* RNA/ml result**, respectively.

Note – To convert the results from copies/ml into the International Units (IU/ml), multiply the results obtained in copies/ml by 1.75 (1 copy= 1.75 IU, 1 IU=0.57 copy)

Interpretation of results for control samples

The result of the analysis is considered reliable only if the results obtained for controls are correct (see table 5).

Table 5

Results for controls

Control	Stage for control	Result of amplification in the channel	
		FAM	JOE/HEX
C–	RNA extraction, PCR	**Positive** (IC concentration is greater than the boundary value)	Negative (*Ct* value is absent)
PCE-1	RNA extraction, PCR	**Positive** (IC concentration is greater than the boundary value)	**Positive** (concentration calculated with IC copies/ml should be within range)
PCE-2	RNA extraction, PCR	**Positive** (IC concentration is greater than the boundary value)	**Positive** (concentration calculated with IC copies/ml should be within range)
$C+_1$	PCR	Positive	Positive
$C+_2$	PCR	Positive	Positive
NCA	PCR	Negative (*Ct* value is absent)	Negative (*Ct* value is absent)

NOTE: The boundary (minimum allowable) concentration values for IC and the range of values for **PCE-1 (Positive Control-1-*HIV)*** and **PCE-2 (Positive Control-2-*HIV)*** calculated with IC copies/ml are specified in the *Important Product Information Bulletin* enclosed to the PCR kit of specific lot.

Results of analysis are not taking into account in the following cases

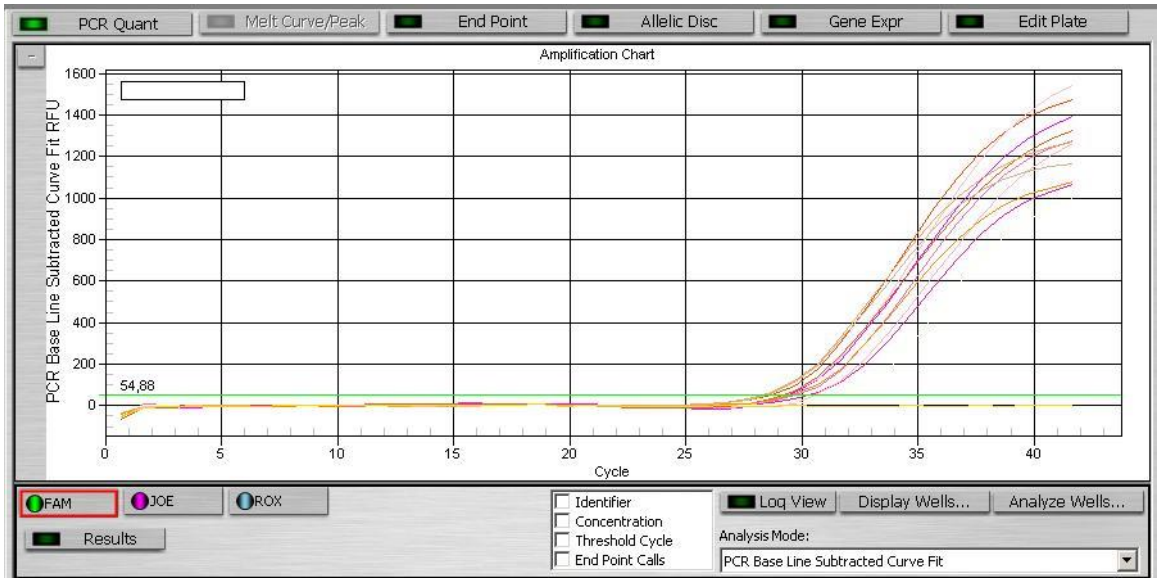
1. If *Ct* value is obtained for the Negative Control of extraction (C–) in the JOE/HEX channel and/or Negative Control of amplification (NCA) in the FAM and JOE/HEX channels, analysis (beginning with RNA extraction) should be repeated for all samples

in which *HIV* RNA was detected.

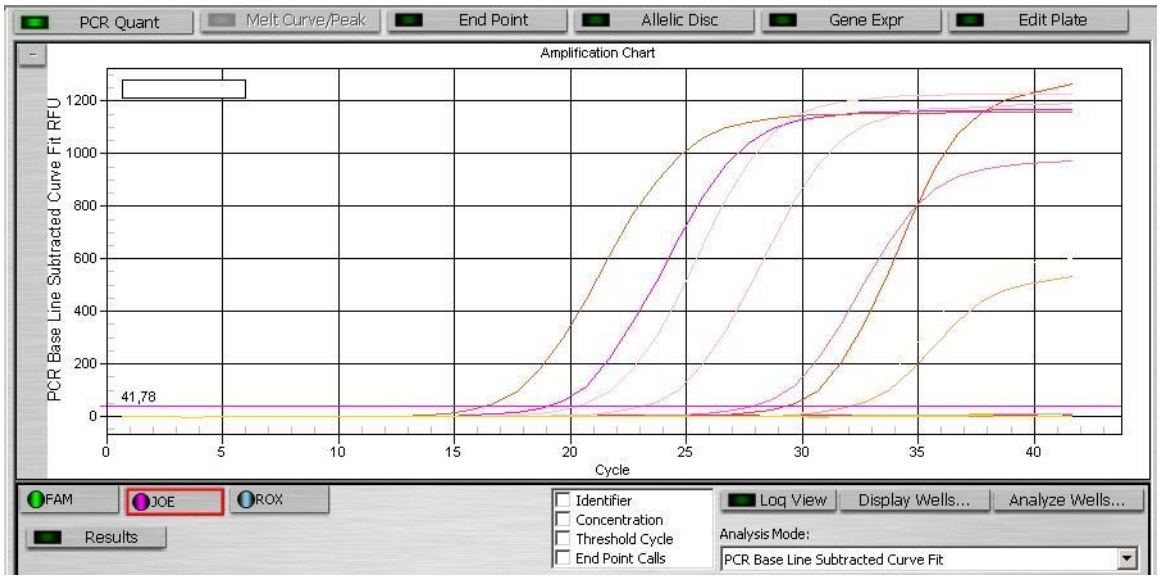
2. If the concentration of IC in the results grid is less than the boundary *Ct* value specified in the *Important Product Information Bulletin*, repeat analysis of the sample starting from the first stage of the analysis.
3. If the correlation coefficient R^2 is less than 0.98, PCR should be repeated for all samples.
4. If the calculated concentrations of Positive Control-1-*HIV* and Positive Control-2-*HIV* do not fit in the range specified in the *Important Product Information Bulletin*, analysis (starting from RNA extraction) should be repeated for all samples.

Example of results

FAM channel data – Internal Control



JOE/HEX channel data – samples with the specific target



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*. It is recommended to use 0.2-ml PCR tubes with domed caps (e.g. Axygen, USA).

Programming the thermocycler

1. Switch on the instrument and run the software.
2. Select ***Quantitative PCR (Multiple Standards)*** and ***Turn lamp on for warm-up*** in ***New Experiment Options*** window.

NOTE: Lamp is to be warmed up during 15 min before the run starts up.

3. Place experimental tubes into the module and secure the lid.
4. Select ***Optics Configuration*** in ***Options*** menu. Set ***JOE*** parameters next to the ***HEX/JOE filter set*** item, ***FAM*** parameters next to the ***FAM filter set*** item.
5. Set fluorescence detection parameters in the ***Plate Setup*** menu. To do this, select all cells with analysis tubes and mark them as ***Unknown*** in the ***Well type*** field. Select **FAM** and **JOE/HEX** fluorophores in the ***Collect fluorescence data*** option.
6. Name each sample in the ***Well Information*** window.
7. In the ***Plate Setup*** menu, set fluorescence detection parameters. To do this, select all analyzed wells and indicate them as ***Unknown*** in the ***Well type*** window.

NOTE: For DNA calibrators PIC1 *HIV* and PIC2 *HIV*, indicate ***Standard*** type and in the ***Standard Quantity*** column enter the values specified in the *Important Product Information Bulletin* enclosed in the PCR kit of the required lot.

8. Select ***FAM*** and ***JOE/HEX*** fluorophores in the ***Collect fluorescence data*** option.
9. Enter sample names in the ***Well Information*** window.
10. In the ***Thermal Profile Setup*** module set the amplification program (see table 6).

Table 6

***HIV*-Monitor-FRT program for plate-type instruments**

Step	Temperature, °C	Time	Fluorescence detection	Cycle repeats
1	50	30 min	–	1
2	95	15 min	–	1
3	95	20 s	–	5
	52	30 s	–	
	72	30 s	–	
4	95	20 s	–	42
	55	40 s	FAM, JOE/HEX	
	72	30 s	–	

11. Click the ***Start*** then ***Run*** button to start the amplification program, name the experiment and save it.

Data analysis

The Internal Control cDNA amplified product is detected in the FAM channel. *HIV* cDNA amplified product is detected in the JOE/HEX channel. Results are interpreted by the presence (or absence) of the intercept between the fluorescence curve and the threshold line set at a certain level (in the middle of linear fragment of the positive control fluorescence growth in log scale), which determines presence (or absence) of *Ct* (cycle threshold) value of a sample in the corresponding cell of the results grid.

Data processing

1. Proceed to ***Analysis*** module by clicking the corresponding button on the tool bar.
2. Ensure that all samples are enabled in the ***Analysis Selection/Setup*** menu (cells corresponding to the tubes are shadowed).
3. Proceed to the ***Results*** menu.
4. Make sure that threshold line is set correctly for each channel. Normally, the threshold line should cross s-shaped curves of positive samples and should not cross the baseline. Otherwise, the threshold level should be raised. To do this, enable displaying of each channel in the ***Dyes shown*** panel (at the bottom), view the level of the threshold line and change if necessary.

Calculation of concentration in biological and control samples

Based on the *Ct* values (the intercept of the fluorescence curve and the threshold line set at a certain level) and on the specified values for the calibrators, PIC1 *HIV* and PIC2 *HIV*, the calibration line will automatically plot and produce the values for the number of *HIV* cDNA copies (JOE/HEX channel) and for the number of Internal Control cDNA copies (FAM channel) in a PCR sample. The retrieved values are used for the *HIV* RNA concentration calculation in tested and control samples, using the formulae:

$$\frac{\textit{HIV}\text{ cDNA copies per PCR-sample}}{\text{IC cDNA copies per PCR-sample}} \times \text{coefficient A} \times \text{coefficient B} = \textit{HIV}\text{ RNA copies/ml of plasma}$$

$$\text{Coefficient A} = \frac{100}{\text{extraction volume, µl}}$$

NOTE: Coefficient A = 1 when calculating PCE-1 and PCE- 2 concentrations

Coefficient B (number of copies of IC per ml of plasma) is specified in the *Important Product Information Bulletin* provided with the PCR kit and is specific for each lot. It cannot be used with PCR kits of different lots. If form 2 and 3 are used then coefficient B is calculated as the result of calibration during the first PCR run of PCR kit of a specific lot (procedure is described in Instruction Manual, Section 8.1.4).

NOTE: If the result is greater than 10,000,000 copies/ml then it is interpreted as the **greater than 10,000,000 copies of *HIV* RNA/ml result.** If the obtained value is greater than the linear range, then the sample may be re-tested after 10x dilution; the produced result is multiplied by 10.
If the result is less than 500 copies/ml (extraction from 100 µl), or less than 250 copies/ml (extraction from 200 µl), or less than 50 copies/ml (extraction from 1 ml), than it is interpreted as the **less than 500,** or **less than 250,** or **less than 50 copies of *HIV* RNA/ml result**, respectively.

Note! To convert the results from copies/ml into the International Units (IU/ml), multiply the results obtained in copies/ml by 1.75 (1 copy= 1.75 IU, 1 IU=0.57 copy)

Interpretation of results for control samples

The results of analysis are accepted as relevant if the results obtained for controls are correct (see table 7).

Table 7

Results for controls

Control	Stage for control	Result of amplification in the channel	
		FAM	JOE/HEX
C–	RNA extraction, PCR	**Positive** (IC concentration is greater than the boundary value)	Negative (*Ct* value is absent)
PCE-1	RNA extraction, PCR	**Positive** (IC concentration is greater than the boundary value)	**Positive** (concentration calculated with IC copies/ml should be within range)
PCE-2	RNA extraction, PCR	**Positive** (IC concentration is greater than the boundary value)	**Positive** (concentration calculated with IC copies/ml should be within range)
$C+_1$	PCR	Positive	Positive
$C+_2$	PCR	Positive	Positive
NCA	PCR	Negative (*Ct* value is absent)	Negative (*Ct* value is absent)

NOTE: Boundary (minimum allowable) concentration values for IC and the range of values for **PCE-1 (Positive Control-1-*HIV)*** and **PCE-2 (Positive Control-2-*HIV)*** calculated with IC copies/ml are specified in the *Important Product Information Bulletin* enclosed to the PCR kit of specific lot.

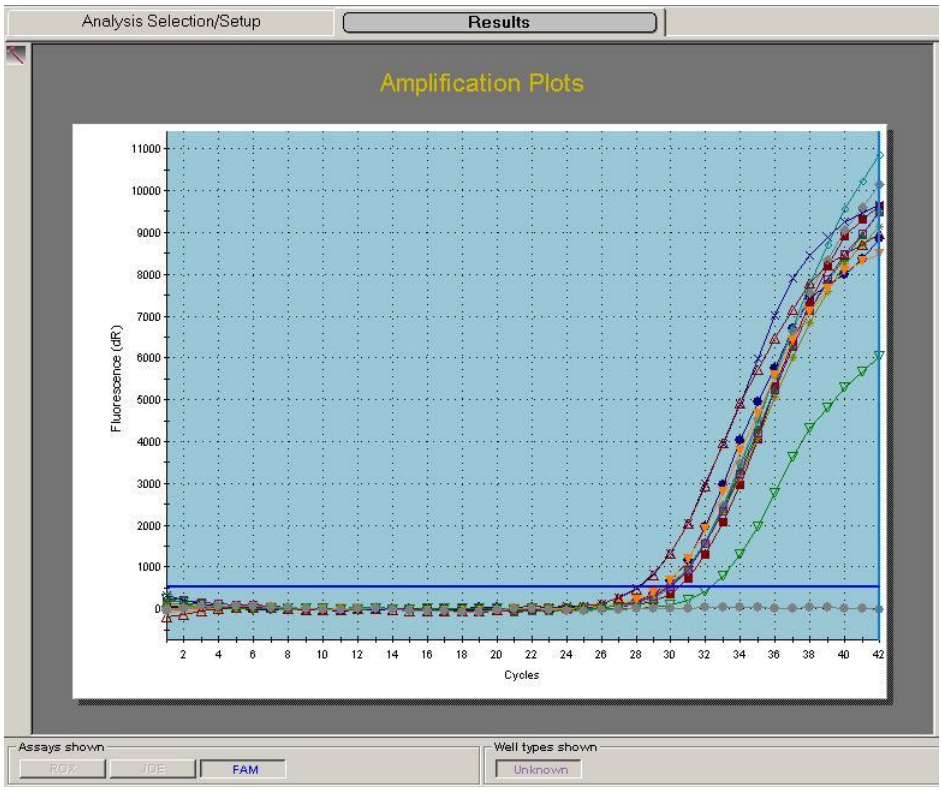
Results of analysis are not taking into account in the following cases

1. If *Ct* value is obtained for the Negative Control of extraction (C–) in the JOE/HEX channel and/or Negative Control of amplification (NCA) in the FAM and JOE/HEX channels, analysis (beginning with RNA extraction) should be repeated for all samples in which *HIV* RNA was detected.
2. If the concentration of IC in the results grid is less than the boundary value specified in the *Important Product Information Bulletin*, repeat analysis of the sample starting from the first stage of the analysis.
3. If the correlation coefficient R^2 is less than 0.98, PCR should be repeated for all samples.

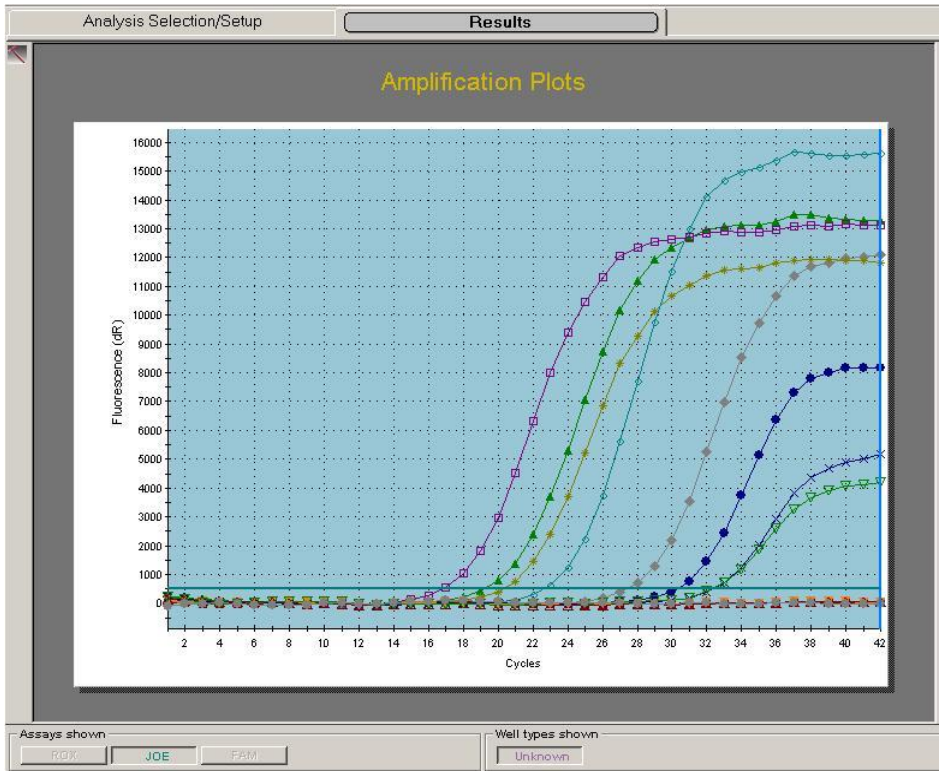
4. If the calculated concentrations of Positive Control-1-*HIV* and Positive Control-2-*HIV* do not fit in the range specified in the *Important Product Information Bulletin*, analysis (starting from RNA extraction) should be repeated for all samples.

Example of results

FAM channel data – Internal Control



JOE/HEX channel data – samples with the specific target



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, or tubes (0.2 ml) with transparent caps from the eight-pieces-strips (e.g. Axygen, USA) (detection through the cap of the tube).

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. Do not turn the tubes (strips) upside down while inserting them into the instrument.

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. Select ***Create a new Run*** (or select ***New*** and then ***Run…*** in the ***File*** menu).
2. In the ***Run Setup*** window, select ***Protocol*** and click the ***Create new…*** button. Set amplification parameters (time, temperature, cycles, and fluorescence acquiring cycle) in the opened ***Protocol Editor – New*** window (see table 8). Set ***Sample Volume – 50 µl.***

Table 8

***HIV*-Monitor-FRT program for plate-type instruments**

Step	Temperature, °C	Time	Fluorescence detection	Cycle repeats
1	50	30 min	–	1
2	95	15 min	–	1
3	95	20 s	–	5
	52	30 s	–	
	72	30 s	–	
4	95	20 s	–	42
	55	40 s	FAM, HEX	
	72	30 s	–	

NOTE: Set ***Ramp Rate* 2,5 °C/s** by clicking the ***Step Options*** button for each step of cycling.

4. In the ***Protocol Editor New*** window select ***File***, then ***Save As***, and name the protocol. This protocol can be used for further runs by clicking the ***Select Existing…*** button in the ***Protocol*** tab. This file can be selected for further runs from the ***Protocol*** tab by clicking the ***Select Existing…*** button. When the required program is entered or edited, click ***OK*** at the bottom of the window.
5. In the ***Plate*** tab click the ***Create new…*** button. Set the tube order in the opened ***Plate Editor – New*** window. In the ***Sample type*** menu select ***Unknown***; click the ***Select***

Fluorophores… button and indicate the required fluorophores with a checkmark; click ***OK***; then indicate with a checkmark the fluorescence signal acquiring for the selected wells in the required channels. Define sample names in the ***Sample name*** window.

6. 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.
7. Place the reaction tubes in the wells of the instrument in accordance with the entered plate setup. In the ***Start Run*** tab click the ***Start Run*** button then save the file of the experiment.
8. Proceed to the analysis of results after the end of the run.

Analysis of results

Obtained data are interpreted by the real-time PCR instrument software by the crossing of a fluorescence curve with the threshold line set at the specific level (that corresponds to the presence of *Ct* value in the results grid).

Curves of accumulation of fluorescence signals are analyzed in two channels:

- the signal of accumulation of the Internal Control cDNA amplification product is detected in the FAM channel;
- the signal of accumulation of the fragment of *HIV* cDNA amplification product is detected in the HEX channel.

Fluorescence curves, plate setup, and the results grid with *Ct* values are displayed in the ***Quantification*** tab. Make sure that the threshold line was set correctly for each channel.

Variant 1

For each channel at a time 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 Controls 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.

Variant 2

For each channel indicate ***Log Scale*** with a checkmark. Set the threshold line at the level where fluorescence curves are linear (use the left mouse button).

Click the ***View/Edit Plate*** button on the toolbar and enter sample names and calibrator concentrations in the opened window, if required.

Click ***Tools*** on the toolbar, then ***Reports…***, and then save the generated report.

Calculation of concentration in biological and control samples

Based on the *Ct* values (the intercept of the fluorescence curve and the threshold line set

at a certain level) and on the specified values for the calibrators, PIC1 *HIV* and PIC2 *HIV*, the calibration line will automatically plot and produce the values for the number of *HIV* cDNA copies (HEX channel) and for the number of Internal Control cDNA copies (FAM channel) in a PCR sample. The retrieved values are used for the *HIV* RNA concentration calculation in tested and control samples, using the formula:

$$\frac{\textit{HIV}\text{ cDNA copies per PCR-sample}}{\text{IC cDNA copies per PCR-sample}} \times \text{coefficient A} \times \text{coefficient B} = \textit{HIV}\text{ RNA copies/ml of plasma}$$

$$\text{Coefficient A} = \frac{100}{\text{extraction volume, µl}}$$

NOTE: Coefficient A = 1 when calculating PCE-1 and PCE- 2 concentrations

Coefficient B (number of copies of IC per ml of plasma) is specified in the *Important Product Information Bulletin* provided with the PCR kit and is specific for each lot. It cannot be used with PCR kits of different lots. If form 2 and 3 are used then coefficient B is calculated as the result of calibration during the first PCR run of PCR kit of a specific lot (procedure is described in Instruction Manual, Section 8.1.4).

NOTE: If the result is greater than 10,000,000 copies/ml then it is interpreted as the **greater than 10,000,000 copies of *HIV* RNA/ml result.** If the obtained value is greater than the linear range, then the sample may be re-tested after 10x dilution; the produced result is multiplied by 10.
If the result is less than 500 copies/ml (extraction from 100 µl), or less than 250 copies/ml (extraction from 200 µl), or less than 50 copies/ml (extraction from 1 ml), than it is interpreted as the **less than 500,** or **less than 250,** or **less than 50 copies of *HIV* RNA/ml result**, respectively.

Note! To convert the results from copies/ml into the International Units (IU/ml), multiply the results obtained in copies/ml by 1.75 (1 copy= 1.75 IU, 1 IU=0.57 copy)

Interpretation of results for control samples

The results of analysis are accepted as relevant if the results obtained for controls are correct (see table 9).

Table 9

Results for controls

Control	Stage for control	Result of amplification in the channel	
		FAM	JOE/HEX
C–	RNA extraction, PCR	**Positive** (IC concentration is greater than the boundary value)	Negative (*Ct* value is absent)
PCE-1	RNA extraction, PCR	**Positive** (IC concentration is greater than the boundary value)	**Positive** (concentration calculated with IC copies/ml should be within range)
PCE-2	RNA extraction, PCR	**Positive** (IC concentration is greater than the boundary value)	**Positive** (concentration calculated with IC copies/ml should be within range)

Control	Stage for control	Result of amplification in the channel	
		FAM	JOE/HEX
$C+_1$	PCR	Positive	Positive
$C+_2$	PCR	Positive	Positive
NCA	PCR	Negative (*Ct* value is absent)	Negative (*Ct* value is absent)

NOTE: Boundary (minimum allowable) concentration values for IC and the range of values for **PCE-1 (Positive Control-1-*HIV)*** and **PCE-2 (Positive Control-2-*HIV)*** calculated with IC copies/ml are specified in the *Important Product Information Bulletin* enclosed to the PCR kit of specific lot.

Results of analysis are not taking into account in the following cases

1. If *Ct* value is obtained for the Negative Control of extraction (C–) in the HEX channel and/or Negative Control of amplification (NCA) in the FAM and HEX channels, analysis (beginning with RNA extraction) should be repeated for all samples in which *HIV* RNA was detected.
2. If the concentration of IC in the results grid is less than the boundary value specified in the *Important Product Information Bulletin*, repeat analysis of the sample starting from the first stage of the analysis.
3. If the correlation coefficient R^2 is less than 0.98, PCR should be repeated for all samples
4. If the calculated concentrations of Positive Control-1-*HIV* and Positive Control-2-*HIV* do not fit in the range specified in the *Important Product Information Bulletin*, analysis (starting from RNA extraction) should be repeated for all samples.

CALCULATION OF CONCENTRATION IN BIOLOGICAL AND CONTROL SAMPLES WITH THE USE OF AmpliSens Soft Monitor FRT SOFTWARE

The procedure of results processing with AmpliSens Soft Monitor FRT software is specified in the "Instruction" worksheet of the software.

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
02.11.12 IvI	Concentration calculation with the AmpliSens Soft Monitor FRT program	AmpliSens Soft Monitor FRT program version was deleted
19.01.13 LA	Text	The names of DNA calibrators were corrected on pages 4, 9, and 14
17.12.14 ME	Carrying out the analysis for qualitative detection of RNA and DNA of *human immunodeficiency virus* type 1 (*HIV*-1) in the clinical material (purified human sperms) by PCR with real-time hybridization-fluorescence detection	The section was added
29.01.15 ME	Footer	**REF** R-V0-MC(RG,iQ,Mx,Dt)-CE-B was added
05.03.15 ME	Text	The text was corrected in accordance with the template
	Intended use	The use of NucliSENS easyMAG and the procedure of concentration calculation were added
	Work with the NucliSENS easyMAG automated nucleic acid extraction system	The section was added
	Carrying out the analysis for qualitative detection of RNA and DNA of *human immunodeficiency virus* type 1 (*HIV*-1) in the clinical material (purified human sperms) by PCR with real-time hybridization-fluorescence detection	The section was deleted
08.05.15 ME	Text	Clinical material was changed to biological
	Calculation of concentration in biological and control samples with the use of AmpliSens Soft Monitor FRT and AmpliSens Soft Monitor FRT-Q software	Additions about using the AmpliSens Soft Monitor FRT-Q software
24.04.17 ME	Control samples preparation when extracting from 1,000 µl	The section was added
07.11.17 PM	Footer	**REF** TR-V0-S-M(RG,iQ,Mx,Dt)-CE and **REF** TR-V0-M-M(RG,iQ,Mx,Dt)-CE were deleted

VER	Location of changes	Essence of changes
29.06.20 EM	Calculation of concentration in biological and control samples with the use of AmpliSens Soft Monitor FRT software	The section was corrected in accordance to the software update
07.10.20 EM	Throughout the text	All the sections were updated according to the template
29.12.20 MA	Through the text	The symbol  was changed to NOTE:
	Cover page	The phrase “Not for use in the Russian Federation” was added
12.02.21 MM	Footer	**REF** R-V0-MC(RG,iQ,Mx,Dt)-CE-B was deleted
02.06.21 KK	Cover page	The phrase “For research use only. Not for diagnostic procedures” was added
04.08.23 BA	Footer	The **REF** R-V0-MC(RG,iQ,Mx,Dt)-CE-B was added