RUO

For Professional Use Only

GUIDELINES

to **AmpliSens®** ***HBV*** **/** ***HDV*****-FRT** PCR kit

for simultaneous detection of *hepatitis B virus* (*HBV)* DNA and

hepatitis D virus (*HDV)* RNA in biological materials

by the polymerase chain reaction (PCR)

with real-time hybridization-fluorescence detection

AmpliSens®

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INTENDED USE

Guidelines describe the procedure of the use of **AmpliSens ® *HBV / HDV*-FRT** PCR kit for simultaneous detection of *hepatitis B virus* (*HBV)* DNA and *hepatitis D virus* (*HDV)* RNA in biological materials (blood plasma) by using real-time hybridization-fluorescence detection with

- Rotor-Gene 3000, Rotor-Gene 6000 (five or six channels) (Corbett Research, Australia),
- iCycler iQ5 (Bio-Rad, USA),
- CFX96 (Bio-Rad, USA),
- Mx3000P (Stratagene, USA).

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

WORK WITH THE NucliSENS easyMAG AUTOMATED NUCLEIC ACID EXTRACTION SYSTEM

Variant 1

RNA/DNA 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/DNA 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/DNA extraction and save it. In the 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 quantity of special disposable tubes intended for RNA/DNA extraction in NucliSENS easyMAG instrument. Add **450 µl** of **NucliSens lysis buffer.**
6. Add **100 µl** of test plasma into each tube by using disposable tips with filters and carefully mix by pipetting.
7. For each panel it's necessary to run the **Positive Control of Extraction (PCE).** To

prepare it, add **90 µl** of **Negative Control** and **10 µl Positive Control *HBV* / *HDV*-rec** into the tube with lysis solution and carefully mix by pipetting.

8. For each panel it is necessary to run the **Negative Control of Extraction (C–).** To prepare it, add **100 µl** of **Negative Control** into the tube with NucliSens lysis buffer and carefully mix by pipetting.
9. Incubate the tubes for 10 min at room temperature to ensure lysis.
10. In individual sterile 1.5 - ml tube mix the NucliSens magnetic silica and Internal Control *ICZ*-rec by using disposable tips with aerosol barriers (see table 1).

NOTE: When extracting sample to carry out several analyses (simultaneous extraction of nucleic acids for detection of *HDV* RNA, *HCV* RNA, *HGV* RNA, *HBV* DNA, and *HIV* RNA as well as *HCV*-genotyping can be done), add all required IC preparations (by analogy).

Table 1

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

11. Add **20 µl of the prepared mixture of NucliSens magnetic silica and Internal Control *ICZ*-rec**. Carefully mix each tube by using a pipette with 1000 µl disposable tips with filters.
12. Place the tubes with samples into the instrument and start the RNA/DNA extraction program with lysis of samples by selecting the *off-board* mode.
13. After the extraction is finished, take the tubes out of the instrument and carry out the RT-PCR not later than 30 min after RNA/DNA extraction.

Variant 2

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

1. Place the vial with the NucliSens lysis buffer into the instrument.
2. For method sensitivity increasing test plasma volume can be varied from 0.1 to 1 ml. Sample lysis is carried out in the NucliSENS easyMAG instrument in automatic mode. The volume of the NucliSens lysis buffer is increased to 2 ml. In this case, it is necessary to add 0.1 to 1 ml of test plasma into each tube intended for RNA/DNA extraction in the NucliSENS easyMAG instrument by using disposable tips with aerosol barriers.
3. For each panel it is necessary to run the **Positive Control of Extraction (PCE).** To

prepare it, add **90 µl** of **Negative Control** and **10 µl Positive Control *HBV / HDV*-rec** into the tube with lysis solution and carefully mix by pipetting.

4. For each panel it is necessary to run the **Negative Control of Extraction (C–).** To prepare it, add **100 µl** of **Negative Control** into the tube with NucliSens lysis buffer and carefully mix by pipetting.
5. Switch on the NucliSENS easyMAG instrument and prepare it for the RNA/DNA extraction according to the instruction manual.
6. In the window for input of test samples enter the following parameters:
   - Sample name
   - *Matrix* for RNA/DNA extraction (select *Plasma*)
   - *Volume* – from 0.1 to 1 ml
   - *Eluate* – 55 µl
   - *Type* – Primary
   - *Priority* – Normal.
7. Create a new protocol of RNA/DNA extraction and save it. In the protocol select *On-board Lysis Buffer Dispensing - yes*, *On-board Lysis Incubation - yes*.
8. Relocate the programmed sample into the created protocol.
9. Place the tubes with samples into the instrument and run the RNA/DNA extraction with sample lysis in the instrument (the *on - board* mode).
10. Wait until the NucliSENS easyMAG instrument stops working in the *Instrument State-Idle* position (approximately 15 min).
11. In individual sterile 1.5 - ml tube mix the NucliSens magnetic silica and Internal Control *ICZ*-rec by using disposable tips with filters (see table 1).

NOTE: When extracting sample to carry out several analyses (simultaneous extraction of nucleic acids for detection of *HDV* RNA, *HCV* RNA, *HGV* RNA, *HBV* DNA, and *HIV* RNA as well as *HCV*-genotyping can be done), add all required IC preparations (by analogy).

12. Add **20 µl of prepared mixture of NucliSens magnetic silica, Internal Control *ICZ*-rec**. Carefully mix each tube by using pipette with 1000-µl disposable tips with filters.
13. Continue the RNA/DNA extraction program.
14. After the extraction is finished, take the tubes out of the instrument and carry out the RT-PCR not later than 30 min after RNA/DNA extraction.

When NucliSENS easyMAG instrument is used the reagents kit allows working with sample volumes from 0.1 to 1 ml.

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

When working with Rotor-Gene 3000 one should use the Rotor-Gene Version 6 software and the Rotor-Gene 6000 versions 1.7 (build 67) software or higher for Rotor-Gene 6000 and Rotor-Gene Q instruments.

Hereinafter, all the terms corresponding to different instruments and software are indicated in the following order: for Rotor-Gene 3000 / for Rotor-Gene 6000.

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), or Rotor-Gene PCR tubes (0.1 ml) with caps from the four-pieces-strips (e.g. Corbett Research, Australia; QIAGEN, Germany) (detection through the bottom of the tube).

Programming the thermocycler

1. Switch the instrument on.
2. Insert the tubes into the carousel of the Rotor-Gene 3000/6000/Q instrument (the carousel cells are numbered, the numbers are used for the further programming of the samples' position in the thermocycler). Program the instrument.

NOTE: Well No 1 must be filled with the test tube from the current experiment. If one rotor contains tubes with reagents from different PCR kits, well No 1 should be filled with the tube with the largest quantity of fluorophores. For example, during *HCV* detection and simultaneous *HBV* and *HDV* detection, tubes with reagents for simultaneous *HBV* and *HDV* detection analysis should be placed first into the rotor.

3. In the ***New Run*** window select ***Advanced*** menu and ***Dual Labeled Probe/Hydrolysis probes***. Activate the ***New*** button.
4. Select ***36-Well Rotor*** (or ***72-Well Rotor***) and ***No Domed 0.2 ml Tubes/Locking ring attached***. Click **Next**.
5. Reaction volume is **25 µl**.
6. Activate function ***15 µl oil layer volume***. Click ***Next***.
7. Select the ***Edit profile*** button.
8. Set the amplification program (see table 2). Select ***OK***.

Table 2

AmpliSens-2 RG amplification program for rotor-type instruments

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Cycles***
1 (Hold)	50	15 min	–	1
2 (Hold)	95	15 min	–	1
3 (Cycling)	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
4 (Cycling 2)	95	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red	
	72	15 s	–	

NOTE: Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program (for example, with the tests for *HDV*, *HCV*-genotyping).

NOTE: Channel Cy5 is switched on when necessary (only in MULTIPRIME assays).

9. Select the ***Calibrate/Gain Optimisation*** button in the ***New Run Wizard*** window:
   - perform the fluorescence detection in FAM/Green, JOE/Yellow, ROX/Orange and Cy5/Red channels (activate the ***Calibrate Acquiring/Optimise Acquiring*** button);
   - perform the calibration in FAM/Green, JOE/Yellow, ROX/Orange and Cy5/Red channels before the first detection (activate the ***Perform Calibration Before 1st Acquisition/ Perform Optimisation Before 1st Acquisition*** button);
   - to set channels calibration, indicate **5FI** in the ***Min Reading*** box and **10FI** in the ***Max Reading*** box for all dyes (activate ***Edit…,*** the window ***Auto gain calibration channel settings***). Click the ***Close*** button, then click ***Next.***
10. Select the ***Start run*** button for amplification run.
11. Name the experiment and save it to the disk (results of the run will be automatically saved in this file).
12. Set the data in the table of samples (open automatically after thermocycling process start). Indicate the names/numbers of test clinical samples in column ***Name***. For empty wells indicate ***None***.

NOTE: Samples with name ***None*** will not be analyzed.

Data analysis:

Data analysis of the *HBV* DNA (JOE/Yellow channel).

1. Activate the ***Analysis*** button then select ***Quantitation*** button and activate the button ***Cycling A. JOE***/***Cycling A. Yellow***, and ***Show***.
2. Cancel ***Threshold*** automatic choice.
3. Select the ***Dynamic tube*** and ***Slope Correct*** buttons in the main window menu (set by default).
4. In the main window menu ***More Settings***/***Outlier Removal*** for Rotor-Gene 6000, set the NTC threshold value 15–25 %.
5. In the ***CT Calculation*** menu set ***Threshold* = 0.03.**
6. In the results grid (***Quant. Results*** window) the *Ct* (Threshold cycle) values for each test sample will appear.

Data analysis of the *HDV* cDNA (ROX/Orange channel).

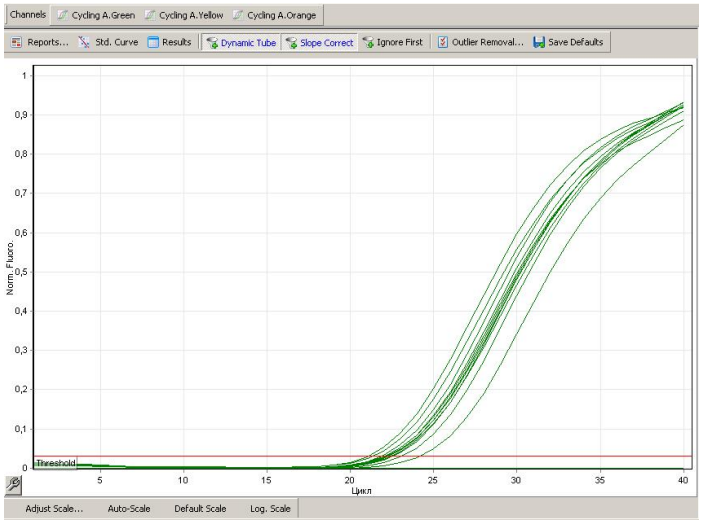
1. Activate the ***Analysis*** button, then select the ***Quantitation*** button and activate the button ***Cycling A. ROX/Cycling A. Orange***, and ***Show***.
2. Cancel the ***Threshold*** automatic choice.
3. Select the ***Dynamic tube*** and ***Slope Correct*** buttons in the main window menu (set by default).
4. In the main window menu ***More Settings/Outlier Removal*** for Rotor-Gene 6000, set the NTC threshold value 10 %.
5. In the ***CT Calculation*** menu set ***Threshold* = 0.03.**
6. In the results grid (***Quant. Results*** window) the *Ct* (Threshold cycle) values in each test sample will appear.

Data analysis of the IC (FAM/Green channel).

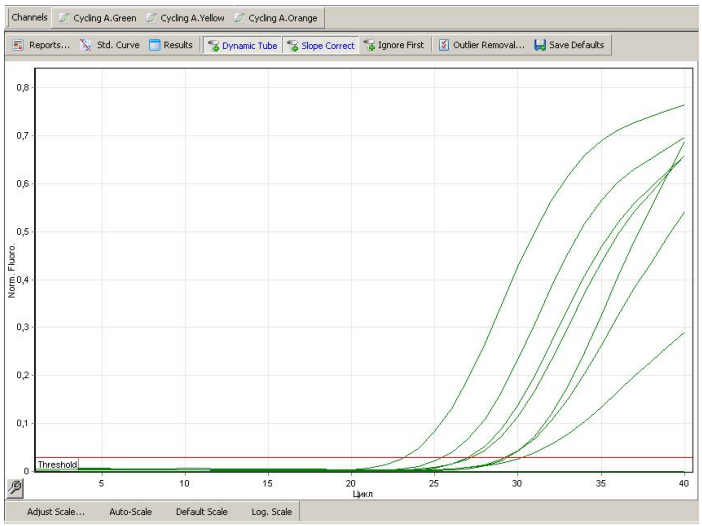
1. Activate the ***Analysis*** button, then select the ***Quantitation*** button and activate the button ***Cycling A. FAM/Cycling A. Green***.
2. Cancel the ***Threshold*** automatic choice.
3. Select the ***Dynamic tube*** and ***Slope Correct*** buttons in the main window menu (set by default).
4. In the main window menu ***More Settings/Outlier Removal*** for Rotor-Gene 6000, set the NTC threshold value 10 %.
5. In the ***CT Calculation*** menu set ***Threshold* = 0.03.**
6. In the results grid (***Quant. Results*** window) the *Ct* (Threshold cycle) values for IC in each studying sample will appear.

Example

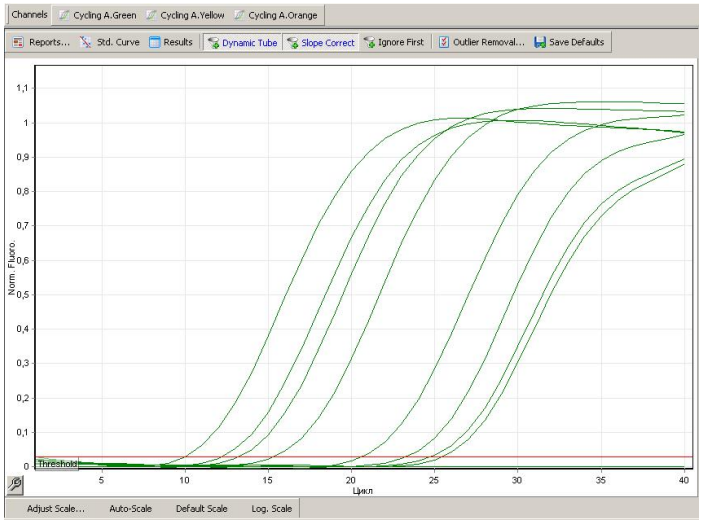
Data in the FAM/Green channel – IC:



Data in the JOE/Yellow channel – sample, which contains *HBV* DNA:



Data in the ROX/Orange channel – sample, which contains *HDV* RNA:



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

Programming the iCycler iQ5 (Bio-Rad, USA)

Carry out the pretreatment and reaction mixture preparation stages according to instruction manual.

1. Switch on the instrument.

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

2. Start the program iCycler iQ5.
3. Insert the tubes or strips into the reaction module of the amplifier (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. Set the plate setup (set the order of the tubes in the reaction chamber and the detection of fluorescent signal). Click the ***Create New*** or ***Edit*** buttons in the ***Selected Plate Setup*** window of the ***Workshop*** module. One can edit the plate setup in the ***Whole Plate loading*** mode. Set the reaction volume (***Sample Volume***) as **25 µl**, the caps type (***Seal Type***), and the tubes type (***Vessel Type***). One should use as plastic type as it was used for the instrument calibration. Select the fluorescent signal detection through the FAM, JOE/HEX and ROX channels. Save the set plate setup by clicking the ***Save&Exit Plate Editing*** button.
2. Set all the clinical samples as ***Unknown.***
3. Set the amplification program (see table 3).

Table 3

AmpliSens-2 iQ program for plate-type instruments

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Cycles*
1	50	15 min	–	1
2	95	15 min	–	1
3	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
4	95	5 s	–	40
	60	30 s	FAM, JOE/HEX, ROX, Cy5	
	72	15 s	–	

NOTE: Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program (for example, with the tests for *HDV*, *HCV*-genotyping).

NOTE: Channel Cy5 is switched on when necessary (only in MULTIPRIME assays).

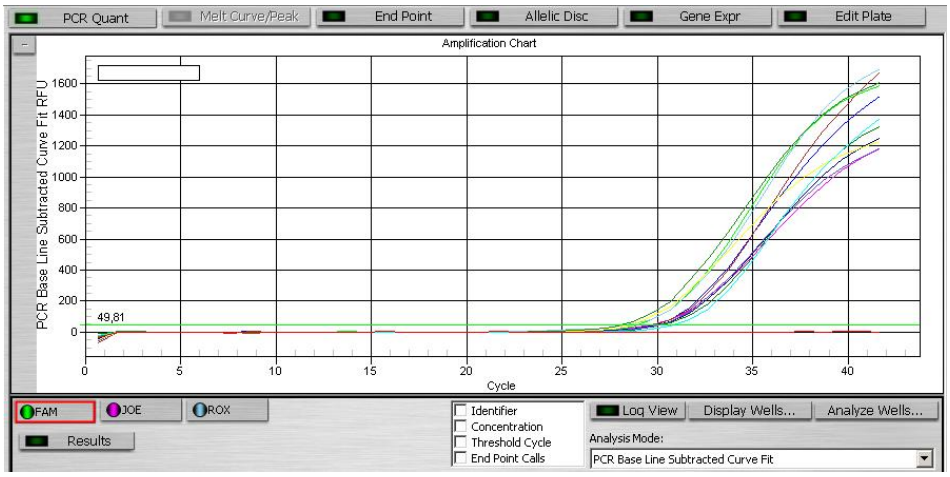
4. Name new protocol and save it.
5. Start the instrument (***Run***), select ***Use Persistant Well Factors,*** click ***Begin Run*** and save the experiment.

Data analysis:

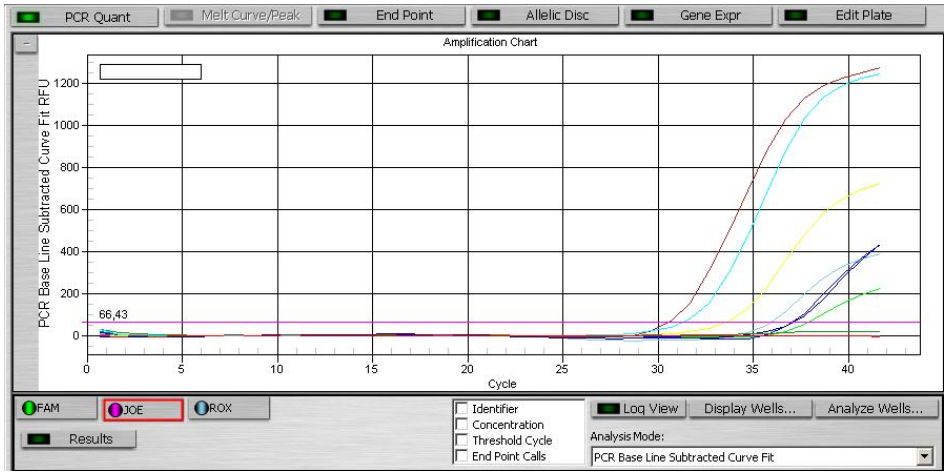
1. Open software and open saved file: select ***Data file*** in ***Workshop*** module and select data file. Pass to ***Data Analysis*** mode.
2. The data for each channel are to be browsed separately.
3. Ensure that automatic selection of threshold level is correct. Normally, the threshold line should cross only sigmoid curves of signal accumulation of positive samples and controls and not to intersect base lane. Otherwise the threshold level should be raised. For this select ***Log View*** and set threshold by left mouse button on level, where fluorescence curves are linear and do not cross curves of negative samples).
4. For result analyses select **Results**.

Example

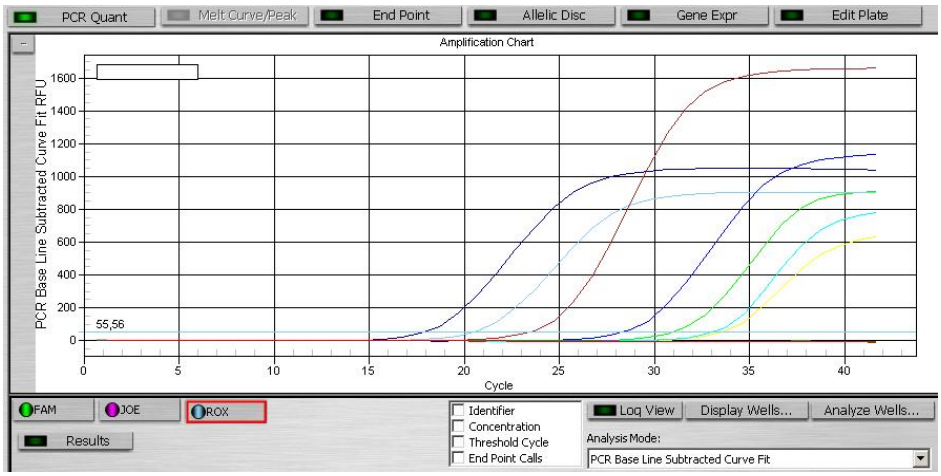
Data in the FAM channel – IC:



Data in the JOE/HEX channel – sample, that contains *HBV* DNA:



Data in the ROX channel – sample, that contains *HDV* RNA:



AMPLIFICATION AND DATA ANALYSIS USING Mx3000P (Stratagene, USA)

1. Switch on the instrument. Run the software Stratagene Mx3000P.
2. Select ***Quantitative PCR (Multiple Standards)*** and ***Turn lamp on for warm-up*** in ***New Experiment Options*** window.

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

3. Insert the tubes into the instrument, lock the fixing arm and the door of the instrument.
4. Select ***Optics Configuration*** in ***Options*** menu and in the ***Dye Assignment*** tab set **JOE** parameters opposite to the ***HEX/JOE filter set*** item, **FAM** parameters opposite to the ***FAM filter set*** item, **ROX** parameters opposite to the ***ROX filter set*** item, **Cy5** parameters opposite to the ***Cy5 filter set*** item.
5. Set the fluorescence detection parameters in the ***Plate Setup*** menu. To do this, select all the cells with the test tubes and mark them as ***Unknown*** in the ***Well type*** field. Select **FAM**, **JOE, ROX, Cy5** fluorophores in the ***Collect fluorescence data*** option.
6. In the ***Thermal Profile Setup*** insert, set the amplification program (see table 4).

Table 3

AmpliSens-2 iQ program for plate-type instruments

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Cycles*
1	50	15 min	–	1
2	95	15 min	–	1
3	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
4	95	5 s	–	40
	60	30 s	FAM, JOE/HEX, ROX, Cy5	
	72	15 s	–	

NOTE: Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program (for example, with the tests for *HDV*, *HCV*-genotyping).

NOTE: Channel Cy5 is switched on when necessary (only in MULTIPRIME assays).

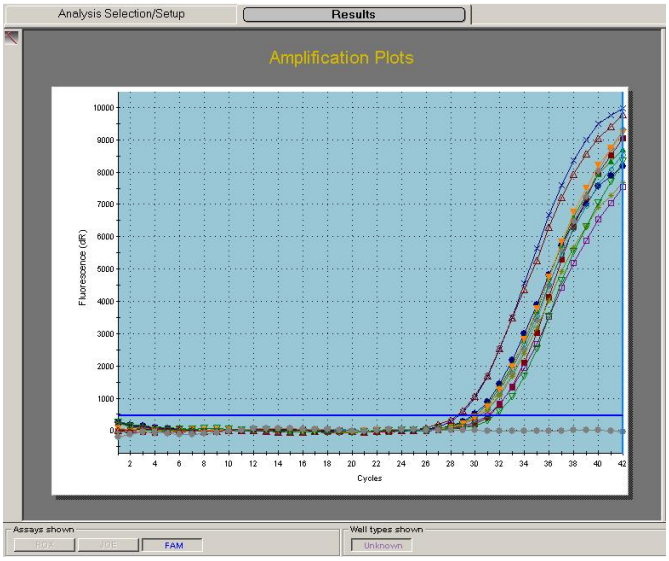
7. Select ***Run*** and ***Start*** and name the experiment file.

Data analysis

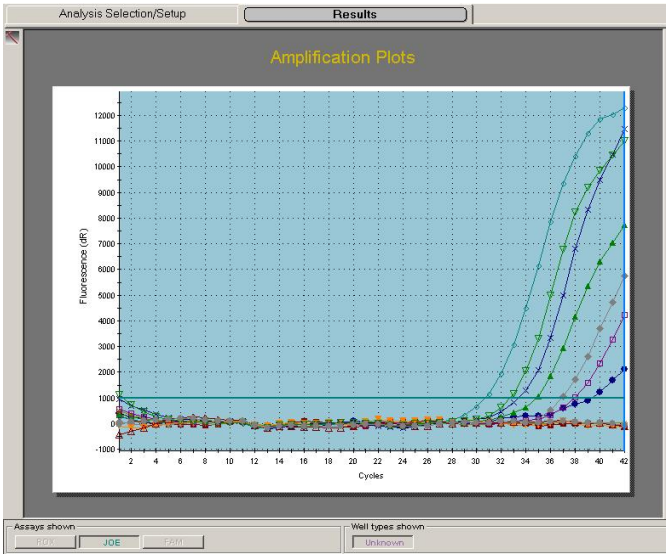
1. Select ***Analysis*** by clicking the corresponding button of the tool bar.
2. The ***Analysis Selection/Setup*** tab will open. Make sure that all the test samples are active (the cells corresponding to the samples should be of a different colour). Otherwise select all the test samples by holding down the ***Ctrl*** button and selecting the needed range with the mouse.
3. Select the ***Results*** tab.
4. Ensure that the automatic selection of the threshold level for each channel is correct. Normally, the threshold line should cross only sigmoid curves of signal accumulation of positive samples and controls and should not intersect the base line. Otherwise, the threshold level should be raised. For this, activate each channel separately in ***Dyes shown*** panel. Look at the threshold line location and change it if necessary.

Example

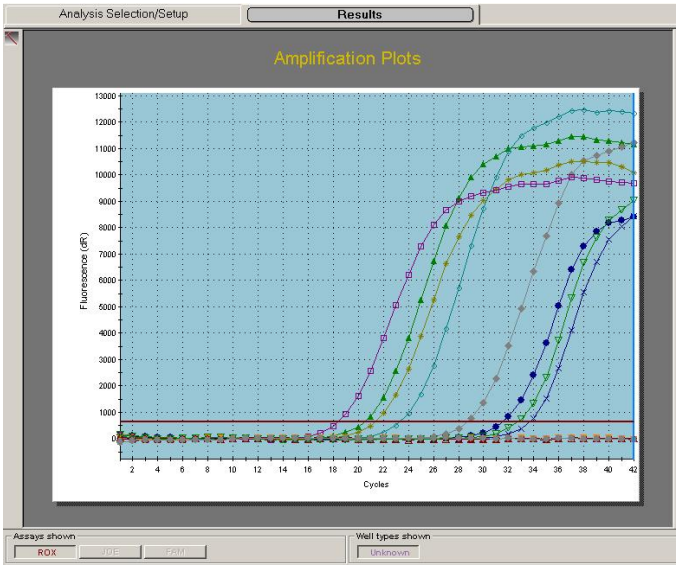
Data in the FAM channel – IC:



Data in the JOE/HEX channel – sample, that contains *HBV* DNA:



Data in the ROX channel – sample, that contains *HDV* RNA:



AMPLIFICATION AND DATA ANALYSIS USING CFX96 (Bio-Rad, USA)

Carry out the pretreatment and reaction mixture preparation stages according to the PCR kit instruction manual. It is recommended that 0.2-ml PCR tubes with optically transparent domed or flat caps are used (detection through the cap of the tube).

NOTE: Make sure that there are no drops left on the walls of the test tubes. Drop fall during amplification run can cause error of signal detection. Do not turn over the tubes before loading them in the instrument.

Program the instrument in accordance with the Operation Manual provided by the manufacturer.

1. Turn on the instrument and start the ***Bio-Rad CFX Manager*** program.
2. Select ***Create a new Run*** (or select ***New*** and then ***Run…*** in the ***File*** menu).
3. 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 5). Set ***Sample Volume – 25 µl.***

Table 5

AmpliSens-2 iQ program for plate-type instruments

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Cycle repeats*
1	50	15 min	–	1
2	95	15 min	–	1
3	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
4	95	5 s	–	40
	60	30 s	FAM, HEX, ROX, Cy5	
	72	15 s	–	

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

NOTE: Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program (for example, with the tests for *HDV*, *HCV*-genotyping).

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. 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 fluorescence signal accumulation are analysed in three channels:

- signal of the amplification product of Internal Control DNA is detected in the FAM channel;
- signal of the amplification product of *HBV* DNA fragment is detected in the HEX channel;
- signal of the amplification product of *HDV* cDNA fragment is detected in the ROX channel.

1. 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 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.

Variant 2

For each channel indicate ***Log Scale*** with a checkmark. Set the threshold line at the level where fluorescence curves are linear

2. Click the ***View/Edit Plate*** button on the toolbar and enter sample names in the opened window if required.
3. Click Tools on the toolbar, then Reports…, and then save the generated report.

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
17.08.11 RT	Programming the Rotor-Gene 3000/6000 (Corbett Research, Australia). Data analysis of the *HBV* DNA (JOE/Yellow channel)	In the item 1.4. the NTC threshold value was changed from 10 % to 15 %
21.06.12 BO	Cover page	Was added
	Table of content	Was added
	Text	"tips with aerosol barriers" was changed to "tips with filters"
		Isolation was changed to extraction
01.10.12 LA	Text	Section "Amplification and data analysis using CFX96 (Bio-Rad, USA)" was added
04.03.14 ME	Work with the NucliSENS easyMAG automated nucleic acid extraction system	Information about using EM-plus was deleted
	Text	The text was complied with the pattern
23.06.14 ChA	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany) Data analysis of the *HBV* DNA (JOE/Yellow channel)	In the item 1.4. the NTC threshold value was changed from 15 % to 15 - 25 %
14.05.15 PM	Text	Clinical material was changed to biological
16.11.20 MM	Footer	**REF** R-V56(RG,iQ,Mx,Dt)-CE-B was deleted
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
02.06.21 KK	Cover page	The phrase "For research use only. Not for diagnostic procedures" was added