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For Professional Use Only

GUIDELINES

to **AmpliSens® *HCV* / *HBV* / *HIV*-FRT** PCR kit

for simultaneous detection of hepatitis C virus RNA (*HCV*), hepatitis B virus DNA (*HBV*) and human immunodeficiency virus RNA (*HIV*) in the biological material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

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INTENDED USE

The guidelines describe the procedure of using **AmpliSens® *HCV* / *HBV* / *HIV*-FRT** PCR kit for simultaneous detection of hepatitis C virus RNA (*HCV*), hepatitis B virus DNA (*HBV*) and human immunodeficiency virus type 1 (*HIV*-1) and type 2 (*HIV*-2) RNA in the biological material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using the following instruments:

- Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia);
- Rotor-Gene Q (QIAGEN, Germany);
- CFX96 (Bio-Rad, USA)

and also in combination with the automatic station for the nucleic acids extraction QIAsymphony SP automated system (QIAGEN, Germany).

WORK WITH QIAsymphony SP AUTOMATED NUCLEIC ACID EXTRACTION SYSTEM (QIAGEN, Germany)

Software Version 3.5

1. Make sure that all branches of the instrument are closed. If the lid is opened while starting the station, an error occurs during system startup.
2. Switch on the power in the lower left corner of the QIAsymphony SP station. Display will show the process of the station start.
3. Log into the system. Click ***Login.*** Select the name from the list. Screen ***Keyboard***. Enter your password to log in and click ***OK.***
4. Nucleic acid extraction reagents are in the cartridges. Each cartridge tray contains a specific reagent: magnetic particles, lysis buffer, washing buffer and buffer for elution. Assembling of the cartridge with the reagents:
   - Put the cartridge holder with the reagents on a hard surface.
   - Remove the cartridge with reagents from the QIAsymphony Virus / Bacteria Midi Kit.
   - Insert the cartridge with reagents in the holder.
   - Remove the foil from the tray with magnetic particles.
   - Place the rack with the enzymes in the hole of the cartridge holder with the reagents.

- Take a piercing lid and remove it from the package. Place the lid on the cartridge with reagents.
- Lightly press down on the piercing lid until it should be closed that indicates tight closing.

NOTE: Do not shake the cartridge with reagents, as it can cause the formation of foam in the buffers, resulting in an error in determining the level of the liquid.

NOTE: Before starting the protocol, make sure that all of the magnetic particles are resuspended. Before use, remove the tray from the magnetic particles from the frame holding the cartridge, shake thoroughly for at least three minutes, and then turn it back into the place.

- If the lid is removed from the tray and the tubes in the rack with enzymes are opened (screw caps should be placed in the slots), assembled cartridge is placed in the ***Reagents and Consumables*** section.

5. Load necessary number of cartridges with reagents and consumables (see Table 1) in ***Reagent and Consumables*** section and do accounting sections scan.

Table 1

Protocol	**Virus Cellfree1000**	
Samples quantity	24	96
Cartridge with reagents	1	2
Cartridges for sample preparation*	18	72
Disposable tips for magnetic rods **	3	12
1500 µl volume tips***	105	402
200 µl volume tips ***	28	104

Note – * Cartridges for 28 samples in a box

** 12 tips for magnetic bars in a box

*** 32 tips / a rack.

6. Make sure that the ***Waste*** section is prepared correctly, and do accounting sections scan, including the discharge and liquid wastes. If necessary, replace the bag for used tips.
7. Load the necessary rack for elution in the ***Elution*** section. Use the slot for elution № 1 with appropriate cooling adapter.
8. Prepare the mix of Internal Control STI-87-rec (IC), RNA carrier and AVE buffer (20 ml vial), based on the calculations in Table 2. Carry out the mix preparation strictly in polystyrene round bottom 14 ml volume, 17 x 100 mm tubes (Becton, Dickinson and Company, USA, reference number 352051).

NOTE: Before first reagent kit use, dilute RNA carrier. Add to a tube, containing 1350 µg of lyophilized RNA carrier, 1350 µl AVE Buffer (2 ml vial) to receive 1 µg/µl concentration solution. Dissolve thoroughly RNA carrier by mixing on vortex, divide into aliquots of appropriate volume and store at ≤ –20 °C. Do not freeze-thaw more than twice.

Table 2

Samples quantity	1 µg/µl RNA carrier quantity, µl	Internal Control STI-87-rec (IC) volume, µl	AVE Buffer volume, µl	Total volume, µl
1	5	10	105	120
12*	90	180	1870	2140
24*	150	300	3130	3580
36*	210	420	4390	5020
48*	270	540	5650	6460
60*	330	660	6910	7900
72*	390	780	8170	9340
84*	450	900	9430	10780
96*	510	1020	10690	12220

Note – * Components volume is calculated with the necessary supply.

9. Place the tube with the mixture “Internal Control STI-87-rec (IC) – RNA carrier – AVE buffer” in the **A** slot of the ***Sample*** section.
10. The status of internal control will change from ***READY TO LOAD*** to ***LOADED***. The system automatically determines the number of loaded tubes and their position in the rack. Loaded tubes are displayed as ***Unknown IC***. Select the tube using touch-screen and then select the ***Virus Cellfree1000*** used protocol of extraction in the ***Optional*** menu for this tube. Color tubes status will change from yellow to blue, then click ***OK***.
11. Place the samples in the QIAsymphony racks and load it in the 1-4 slots of the ***Sample*** section. The rack position status will change from ***READY TO LOAD*** to ***LOADED.***

NOTE: The samples volume must be **no less than 1050 ml**, the samples must be free of clots and foreign particles.

12. If the bar code is present on the tube it is automatically read during rack loading. If the samples are not marked by the bar code, set to them a virtual bar code in the program menu. Select the test samples, and then select used extraction protocol ***Virus Cellfree1000***. Color tubes status will change from yellow to blue, and then click ***OK***.
13. Select used elution plates/tubes types in ***Sample preparation/Elution Slot/Configure Racks***; elution volume - **60 µl** and sample location order in the elution plates/tubes. Click ***OK.***
14. Click the ***Start*** button to start the process. All stages of the process are performed automatically. At the end of the protocol lot status will change from ***Running*** to ***Completed.***
15. Remove the rack with extracted nucleic acids from the ***Eluate*** section of the instrument. The nucleic acids are ready for use or to be stored. For short-term storage (up to 24 hours) it is recommended to store the nucleic acids at temperature from 2 to 8 °C. For long-term storage (more than 24 hours), it is recommended to store the nucleic acids at or below minus 20 °C.

NOTE: If the cartridge with reagents used partly, immediately stop it up by sealing strips after protocol ending and close the tubes with enzymes by screw lids to prevent evaporation.

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

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

Hereinafter, all the terms corresponding to different instruments and software are indicated in the following order: 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), or 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. Turn on the instrument, run the Rotor-Gene software.

2. Insert the tubes or strips into the rotor of the Rotor-Gene 6000/Q instrument beginning from the first well (the rotor wells are numbered, the numbers are used for the further programming of the samples' order in the thermocycler). Insert the rotor into the instrument, close the lid.

NOTE: Well 1 must be filled with any test tube except for an empty one. If the tubes with reagents from different PCR kits or with different PCR-mixes are inserted into the rotor then the tubes' numbers for calibration in each detection channel should be indicated in the Rotor-Gene software. Recommendations about the calibration are described in the information list "Calibration priorities for the AmpliSens kits for Rotor-Gene 3000/6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany) thermocyclers".

3. Program the instrument according to the *Instruction Manual* given by the manufacturer of the instrument.

Creating the template for the run

1. Click the ***New*** button in the software main menu.
2. In the opened window, select ***Advanced*** menu and ***Hydrolysis probes (TaqMan)***. Click the ***New*** button.
3. Select ***36-Well Rotor*** (or ***72-Well Rotor***) and ***Locking ring attached***. Click ***Next***.
4. Select the operator and set the ***Reaction volume*** as **50 µl**. Click ***Next***.
5. In the opened window click the ***Edit profile*** button and set the temperature profile of the experiment as follows (see Table 3):

Table 3

Amplification program for AmpliSens *HBV / HCV / HIV* for rotor-type instruments

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Cycles***
Hold	50	20 min	–	1
Cycling	95	15 min	–	1
Cycling 2	95	20 s	–	4
	46	40 s	–	
Cycling 3	95	5 s	–	42
	60	40 s	–	
	45	30 s	FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red, Cy5.5/Crimson	

6. Click ***OK***.
7. Select the ***Calibrate/Gain Optimisation*** button in the ***New Run Wizard*** window:
   - perform the fluorescence detection in FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red and Cy5.5/Crimson channels (activate the ***Optimise Acquiring*** button);

- perform the calibration in FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red channels before the first detection (activate the ***Perform Optimisation Before 1st Acquisition*** button);
- set channel calibration from **5Fl** to **10Fl** for all dyes (the ***Edit*** button of the ***Auto gain calibration channel settings***). Click ***Close***.

8. Click ***Next***. Start the amplification program by activating 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 (opens automatically after the thermocycling process starts). Indicate the names/numbers of clinical and control samples in the ***Name*** column. Set the ***None*** type for empty wells.

Data analysis

Data analysis for *HCV* cDNA (FAM/Green channel)

1. Activate the button ***Analysis*** in the menu, select the mode of the analysis ***Quantitation***, activate the button ***Cycling A. FAM/ Cycling A. Green, Show***.
2. Cancel the automatic choice of the threshold line level for each of the main open windows (by activating the ***Threshold*** button.
3. Activate the ***Dynamic tube*** and ***Slope Correct*** buttons in the main window menu (***Quantitation analysis***).
4. In ***CT Calculation*** menu (in the right part of the window) indicate the threshold line level **0.05** in the ***Threshold*** box.
5. Choose the parameter ***More settings/Outlier Removal*** and set the value of negative samples threshold (***NTC/Threshold***) specified in the *Important Product Information Bulletin* of reagent kit.
6. In the results grid (the ***Quant. Results*** window) one will be able to see the *Ct* values.

Data analysis for *HIV*-1 cDNA (JOE/Yellow channel)

1. Activate the button ***Analysis*** in the menu, select the mode of the analysis ***Quantitation***, activate the button ***Cycling A. JOE / Cycling A. Yellow, Show***.
2. Cancel the automatic choice of the threshold line level for each of the main open windows (by activating the ***Threshold*** button.
3. Activate the ***Dynamic tube*** and ***Slope Correct*** buttons in the main window menu (***Quantitation analysis***).
4. In ***CT Calculation*** menu (in the right part of the window) indicate the threshold line level **0.05** in the ***Threshold*** box.

5. Choose the parameter ***More settings/Outlier Removal*** and set **3 %** for the value of negative samples threshold (***NTC/Threshold***).
6. In the results grid (the ***Quant. Results*** window) one will be able to see the *Ct* values.

Data analysis for *HBV* cDNA (ROX/Orange channel)

1. Activate the button ***Analysis*** in the menu, select the mode of the analysis ***Quantitation***, activate the button ***Cycling A. ROX/Cycling A. Orange, Show***.
2. Cancel the automatic choice of the threshold line level for each of the main open windows (by activating the ***Threshold*** button.
3. Activate the ***Dynamic tube*** and ***Slope Correct*** buttons in the main window menu (***Quantitation analysis***).
4. In ***CT Calculation*** menu (in the right part of the window) indicate the threshold line level **0.05** in the ***Threshold*** box.
5. Choose the parameter ***More settings/Outlier Removal*** and set for the value of negative samples threshold (***NTC/Threshold***) specified in *the Important Product Information Bulletin* of reagent kit.
6. In the results grid (the ***Quant. Results*** window) one will be able to see the *Ct* values.

Data analysis for *HIV*-2 cDNA (Cy5.5/Crimson channel)

1. Activate the button ***Analysis*** in the menu, select the mode of the analysis ***Quantitation***, activate the button ***Cycling A. Cy5,5/Cycling A. Crimson, Show***.
2. Cancel the automatic choice of the threshold line level for each of the main open windows (by activating the ***Threshold*** button.
3. Activate the ***Dynamic tube*** and ***Slope Correct*** buttons in the main window menu (***Quantitation analysis***).
4. In ***CT Calculation*** menu (in the right part of the window) indicate the threshold line level **0.05** in the ***Threshold*** box.
5. Choose the parameter ***More settings/Outlier Removal*** and set for the value of negative samples threshold (***NTC/Threshold***) specified in the *Important Product Information Bulletin* of reagent kit.
6. In the results grid (the ***Quant. Results*** window) one will be able to see the *Ct* values for IC.

Data analysis for IC (Cy5/Red channel)

1. Activate the button ***Analysis*** in the menu, select the mode of the analysis ***Quantitation***, activate the button ***Cycling A. Cy5/Cycling A. Red, Show***.
2. Cancel the automatic choice of the threshold line level for each of the main open

windows (by activating the ***Threshold*** button.

3. Activate the ***Dynamic tube*** and ***Slope Correct*** buttons in the main window menu (***Quantitation analysis***).
4. In ***CT Calculation*** menu (in the right part of the window) indicate the threshold line level **0.05** in the ***Threshold*** box.
5. Choose the parameter ***More settings/Outlier Removal*** and set for the value of negative samples threshold (***NTC/Threshold***) specified in the *Important Product Information Bulletin* of reagent kit.
6. In the results grid (the ***Quant. Results*** window) one will be able to see the *Ct* values for IC.

AMPLIFICATION AND DATA ANALYSIS USING CFX96 (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 caps (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. Don't turn the strips upside down while inserting them into the instrument.

Programming the thermocycler

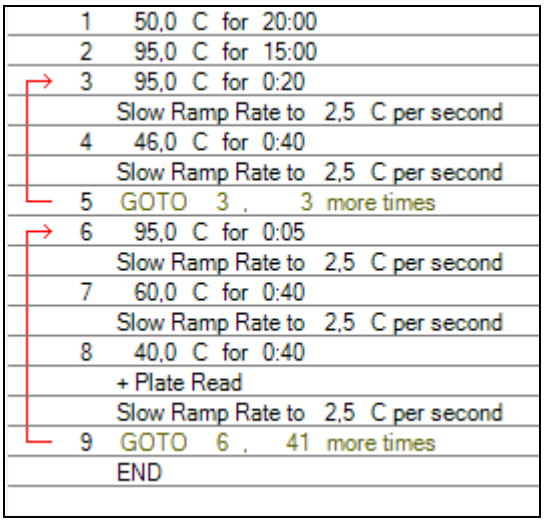
1. Turn on the instrument and start the ***Bio-Rad CFX Manager*** program.
2. Select ***Create a new Run*** (or select ***New*** and then ***Run…*** in the ***File*** menu).
3. In the ***Run Setup*** window, select ***Protocol*** and click the ***Create new…*** button. Set amplification parameters (time, temperature, cycles, and fluorescence acquiring cycle) in the opened ***Protocol Editor – New*** window (see Table 4). Set ***Sample Volume – 50 µl.***

Table 4

Amplification program for AmpliSens *HBV / HCV / HIV* for plate-type instruments

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Number of cycles***
Hold	50	20 min	–	1
Cycling	95	15 min	–	1
Cycling 2	95	20 s	–	4
	46	40 s	–	
Cycling 3	95	5 s	–	42
	60	40 s	–	
	40	40 s	FAM, HEX, ROX, Cy5, Quasar 705	

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. 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 tubes 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.

Data analysis

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).

The fluorescence curves, plate setup, and results grid with *Ct* values are displayed in the ***Quantification*** tab.

The curves of accumulation of fluorescence signals are analyzed in five channels:

- FAM – *HCV*;
- HEX– *HIV*-1;
- ROX – *HBV*;
- Cy.5 – IC;
- Quasar 705 – *HIV*-2.

It is necessary to indicate ***Log Scale*** with a checkmark for each channel for threshold level setting. Set the threshold line at the level where fluorescence curves are linear (use the left mouse button). Deselect checkmark for ***Log Scale.***

Click the ***View/Edit Plate*** button on the toolbar and enter sample names in the opened window.

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
04.03.14 ME	Amplification and data analysis using CFX96 (Bio-Rad, USA) instrument	The amplification program was corrected
	Text	The use of the channel Cy5.5/Crimson/Quasar 705 was deleted
10.02.15 ME	Footer	REF R-V62(RG,Dt)-CE was added
	Text	The use of the channel Cy5.5/Crimson/Quasar 705 for the *HIV*-2 RNA detection was added
20.03.15 ME	Amplification and data analysis using Rotor-Gene 6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany) instruments	For the Cy5,5/Crimson channel the threshold was changed from 0.03 to 0.05, the *More settings/Outlier Removal* parameter was changed from 5 to 10 %
15.05.15 PM	Through the text	The clinical material was changed to biological
15.07.15 ME	Footer	REF R-V62-Q(RG,Dt)-CE was added
31.05.16 PM	Amplification and data analysis using Rotor-Gene 6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany) instruments	The procedure of programming the thermocycler was corrected according to the template
24.05.17 DV	Amplification and data analysis using Rotor-Gene 6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany) instruments	The value of the outlier removal threshold was deleted. Added a reference to the Important Product Information Bulletin about the value of negative samples threshold.
16.11.20 KK	Footer	The **REF** R-V50-4x(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
04.08.23 BA	Footer	The **REF** R-V62(RG,Dt)-CE-B was added