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GUIDELINES

to AmpliSens® *HCV*-genotype-FRT PCR kit

for differentiation of *hepatitis C virus* (*HCV*) genotypes in the biological material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

AmpliSens®

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INTENDED USE

The guidelines describe the procedure of using **AmpliSens® *HCV*-genotype-FRT** PCR kit for differentiation of *hepatitis C virus* (*HCV*) genotypes in the biological material (peripheral 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),
- CFX96 (Bio-Rad, USA),
- DTprime (“DNA-Technology”, Russia)

The table below shows *HCV* subtypes detected using AmpliSens® *HCV*-genotype-FRT:

***HCV* genotype**	***HCV* subtypes detected using AmpliSens® *HCV*-genotype-FRT**
1	1a, 1b
2	2a, 2b, 2c, 2f, 2k
3	3a
4	4a, 4c
5	5a
6	6a, 6b, 6f, 6j, 6m, 6n

REVERSE TRANSCRIPTION USING Rotor-Gene 3000/6000 (Corbett Research, Australia) AND Rotor-Gene Q (QIAGEN, Germany) INSTRUMENTS

When working with Rotor-Gene 3000 one should use the Rotor-Gene version 6.1 and higher 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/Q.

Carry out the RNA extraction and reaction mixture preparation for reverse transcription reaction stages according to the PCR kit *Instruction Manual*. 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).

Insert tubes into the Rotor-Gene 3000/6000/Q instrument, place the rotor into the instrument, and close the lid.

Programming the instrument

1. Click ***New*** in the program main menu.
2. In the opened window select ***Advanced*** menu and ***Dual Labeled Probe/Hydrolysis probes***. Activate the ***New*** button.
3. Select ***36-Well Rotor*** and ***No Domed 0.2 ml Tubes***/***Locking ring attached***. Click the ***Next*** button.
4. Set the operator and specify the ***Reaction volume*** as **20 µl** in the opened window. Click the ***Next*** button.
5. Select the ***Edit profile*** button and enter the following parameters in the opened window:
   - select ***Hold*** and set ***37 °C*** and ***30 min***;
   - select ***Cycling*** and remove it using the ***Remove*** button
6. Click ***OK***. Click the ***Calibrate/Gain Optimisation…*** button in the ***New Run Wizard*** window. Make sure that calibration is not selected (no checkmark) in the opened window; otherwise, disable the calibration process. Click the ***Remove All*** button if fluorescence channels were selected. Click ***Close***.
7. Click ***Next***, then ***Start run***.
8. Name the experiment and save it to the disc.

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

When working with Rotor-Gene 3000 one should use the Rotor-Gene version 6.1 and higher 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/Q.

Carry out the RNA extraction, reverse transcription and reaction mixture preparation for PCR 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).

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 following programming tube order).

NOTE: The well no. 1 must be filled with a test tube from the current experiment which contain “1b/3a” reaction mixture.

Programming the instrument

1. Press the ***New*** button in the main menu 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 **25 µl**. Activate the ***15 µl oil layer volume*** function. Click the ***Next*** button.
5. Select the ***Edit profile*** button and set the amplification program (see Table 1).

Table 1

AmpliSens-1 program for rotor-type instruments

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Cycles***
Hold	95	15 min	–	1
Cycling 1	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling 2	95	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow	
	72	15 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: Samples indicated as ***None*** will not be analyzed.

Data analysis

Data analysis in the FAM/Green channel

1. Activate the ***Analysis*** button then select ***Quantitation*** button and activate the ***Cycling A. FAM/Cycling A. Green*** button.
2. Cancel ***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 **8 %.**
6. In the results grid (***Quant. Results*** window) the *Ct* (cycle threshold) values will appear.

Data analysis in the JOE/Yellow channel.

1. Activate the ***Analysis*** button then select ***Quantitation*** button and activate the ***Cycling A. JOE/Cycling A. Yellow*** button.
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* = 0.035.**
5. Select the ***More Settings/Outlier Removal*** parameter and set the ***NTC threshold*** value **8 %.**
6. In the results grid (***Quant. Results*** window) the *Ct* (cycle threshold) values will appear.

REVERSE TRANSCRIPTION USING DTprime ("DNA-Technology LLC", Russia)

INSTRUMENT

Carry out the RNA extraction and reaction mixture preparation for reverse transcription reaction stages according to the PCR kit *Instruction Manual*. 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).

1. Switch on the device and start the RealTime_PCR v.7.3 program. In the start window, select an existing operator or add a new operator and select ***Device operation*** mode.
2. In the dialog window ***List of devices***, select the required instrument and click the ***Connect*** button.
3. In the ***Test*** menu, choose the ***Create new test*** command, enter the name of the new test – ***RT-37*** and click the ***OK*** button. In the ***Test*** window that appears, set the following parameters:
   - ***Type*** – qualitative;
   - ***Method*** – threshold (Ct);
   - ***Tubes*** – check with a checkmark the ***Sample*** box;
   - ***Controls*** – no;
   - ***PCR mix volume*** – 20 µl;
   - ***Fluorophores*** – leave unchanged;
   - Set the amplification program using the ***Create new program/edit program*** command and click ***OK***. In the ***Template*** window, select the button with the one-step program and click the ***Apply*** button. Set the name of the RT-37 program and enter the following parameters in the corresponding columns: temperature 37° C, time 30 min, 00 sec. Remove detection and click ***OK***.
4. Select the ***Add test*** button and select the name RT-37 in the appeared window, specify the number of samples and click ***OK***.
5. Specify the sample names in the ***Identificator*** column of the appeared ***Protocol*** tab. Specify the location of test tubes in the working block of the device in the ***Free filling*** window. Click the ***Apply*** button.

NOTE: Monitor the tubes. There must not be drops left on the walls and caps of the tubes. Don't turn the strips upside down while inserting them into the instrument.

6. Specify the volume of working mixture – 20 µl and press the ***Start run*** button.
7. Select the ***Start run*** tab, check the test parameters. Press the ***Open thermal unit*** button and place the tubes in strict accordance with the indicated arrangement of tubes in the

working block of the instrument.

8. Sequentially press the buttons ***Close thermal unit*** and ***Start run***. Save the experiment.

AMPLIFICATION AND DATA ANALYSIS USING DTprime ("DNA-Technology LLC", Russia) INSTRUMENT

Carry out the RNA extraction, reverse transcription and reaction mixture preparation for PCR stages according to the PCR kit *Instruction Manual*. 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).

1. Switch on the device and start the RealTime_PCR v.7.3 program. In the start window, select an existing operator or add a new operator and select ***Device operation*** mode.
2. In the dialog window ***List of devices***, select the required instrument and click the ***Connect*** button.
3. In the ***Test*** menu, choose the ***Create new test*** command, enter the name of the new test – ***HCVgFL*** and click the ***OK*** button. In the ***Test*** window that appears, set the following parameters:
   - ***Type*** – qualitative;
   - ***Method*** – threshold (Ct);
   - ***Tubes*** – check with a checkmark the ***Sample*** box;
   - ***Controls*** – no;
   - ***PCR mix volume*** – 25 µl;
   - ***Fluorophors*** – Fam – IC; Hex – specific target;
   - Set the amplification program (see table 2) using the ***Create new program/edit program*** command and click ***OK***.

Table 2

AmpliSens-1 program for plate-type instruments

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Cycle repeats***
1	95	15 min	–	1
2	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
3	95	5 s	–	40
	60	30 s	Fam, Hex	
	72	15 s	–	

4. Select the ***Add test*** button and select the name ***HCVgFL*** in the appeared window, specify the number of samples and click ***OK***.
5. Specify the sample names in the ***Identificator*** column of the appeared ***Protocol*** tab. Specify the location of test tubes in the working block of the device in the ***Free filling***

window. Click the ***Apply*** button.

6. Specify the volume of working mixture – 25 µl and press the ***Start run*** button.
7. Select the ***Start run*** tab, check the test parameters. Press the ***Open thermal unit*** button and place the tubes in strict accordance with the indicated arrangement of tubes in the working block of the instrument.

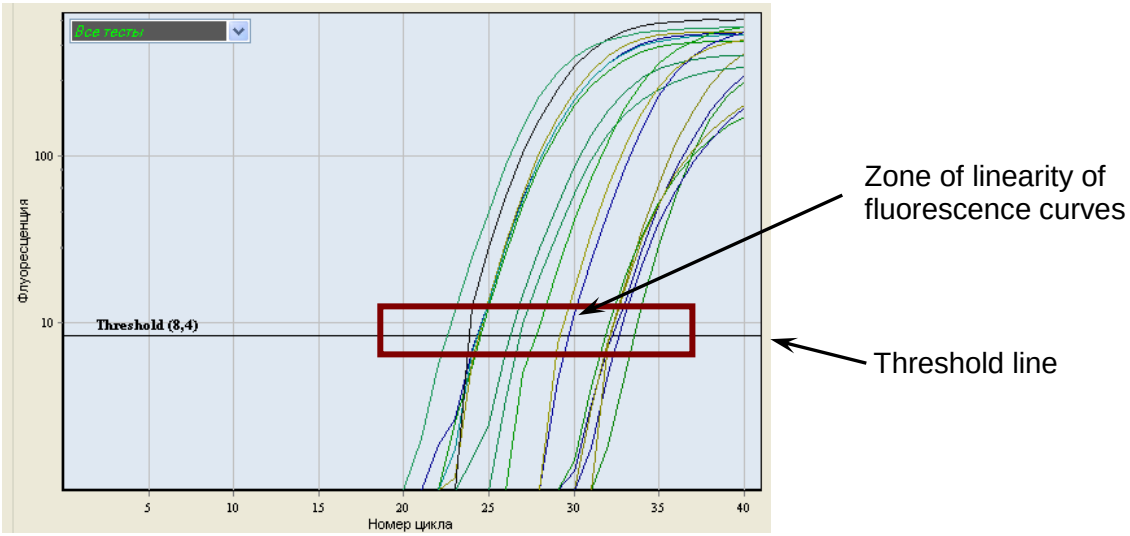
NOTE: Monitor the tubes. There must not be drops left on the walls of the tubes as falling drops during the amplification process may lead to the signal failure and complicate the results analysis. Don't turn the strips upside down while inserting them into the instrument.

8. Sequentially press the buttons ***Close thermal unit*** and ***Start run***. Save the experiment.

Data analysis

1. Switch to the ***Archive viewer*** mode and open the saved data file.
2. Specify the ***Type of analysis***: Ct (Cp) for all channels in the drop-down list.
3. Specify the ***Method***: Threshold (Ct) in the drop-down list.
4. Switching off fitting (smoothing) of data using the ***Φ*** button (turn off the button).
5. Click the **Change parameters of data analysis** button. In the opened tab set the ***Criterion of the PCR positive result – 70 %, Criteria of validity: lower line/threshold for the positive result – 20 %, upper line/threshold for normalization – 30 %***. Do not use the ***Normalization*** option (there should be no checkmark in the corresponding window). Click the ***Apply*** button.
6. For each channel, check the correctness of the automatic selection of the threshold line, for this switch to the logarithmic scale by putting a tick in the log_Y box located at the bottom of the monitor screen. Set the threshold line level with the left mouse button at the level where the fluorescence curves are linear (see Fig. 1).

Figure 1 – Setting threshold line



7. Click the ***Report*** button. Click the ***Save report as...*** button (it is recommended to save the report to the ***My Documents*** folder), select the ****MS Word/Acrobat Reader/JPEG/HTML format***, select the folder to save, name the file and click the ***Save*** button.

REVERSE TRANSCRIPTION USING CFX96 (Bio-Rad, USA) INSTRUMENT

Carry out the RNA extraction and reaction mixture preparation for reverse transcription reaction stages according to the PCR kit *Instruction Manual*. 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).

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. Select ***Protocol*** and click the ***Create new…*** button in the ***Run Setup*** window. In the opened ***Protocol Editor–New*** window set the following parameters: ***time – 30 min***, ***temperature – 37 °C***. Set ***Sample Volume – 20 µl***.
4. In the ***Protocol Editor New*** window select ***File***, then ***Save As***, and name the protocol. This protocol can be selected 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. Place the reaction tubes in the wells of the instrument. Click the ***Start Run*** button in the ***Start Run*** tab then save the file of the experiment.

NOTE: Monitor the tubes. There must not be drops left on the walls and caps of the tubes. Don’t turn the strips upside down while inserting them into the instrument.

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

Carry out the RNA extraction, reverse transcription and reaction mixture preparation for PCR 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).

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 3). Set ***Sample Volume – 25 µl.***

Table 3

AmpliSens-1 program for plate-type instruments

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

NOTE! Set ***Ramp Rate* 2,5 °C/sec** 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 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.

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.

NOTE: The well no. 1 must be filled with a test tube from the current experiment which contain "1b/3a" reaction mixture.

8. Proceed to the analysis of results after the end of the run.

Analysis of results

1. Start the program and open the saved file of the experiment.
2. Open the ***Quantification*** tab (opens by default) which displays fluorescence curves, tube setup, and the table with *Ct* values.
3. For each channel indicate ***Log Scale*** with a checkmark. Set the threshold line at the level where fluorescence curves are linear (see fig. 2).
4. Click the ***View/Edit Plate*** button on the toolbar and enter sample names in the opened window if required.
5. Click ***Tools*** on the toolbar, then ***Reports…***, and then save the generated report.

Figure 2. Setting threshold line