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GUIDELINES

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

for differentiation of *hepatitis B virus* (*HBV*) genotypes A, B, C and D 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® *HBV*-genotype-FRT** PCR kit for differentiation of *hepatitis B virus* (*HBV*) genotypes A, B, C and D in the 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);
- CFX96 (Bio-Rad, USA);
- DTprime (“DNA-Technology”, Russia).

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 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 DNA extraction 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 so that the first tube is in No. 1 well (the carousel cells are numbered, the numbers are used for the further programming of the samples' position in the thermocycler). Program the instrument.
3. Click the ***New*** button in the main program menu.
4. In the opened window, select the template of experiment run ***Advanced*** and ***Dual Labeled Probe/Hydrolysis probes***. Click the ***New*** button.
5. Select ***36-Well Rotor*** (or ***72-Well Rotor***) and ***No Domed 0.2 ml Tubes/Locking ring attached***. Click ***Next***.
6. Select the operator and set the ***Reaction volume*** as **25 µl**. Tick the ***15 µl oil layer volume*** for Rotor-Gene 6000/Q. Click **Next**.
7. Select the ***Edit profile*** button and set the temperature profile of the experiment (see Table 1).

Table 1

The AmpliSens-1 amplification program for rotor-type instruments

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Number of cycles***
Hold	95	15 min	–	1
Cycling	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling 2	95	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red	
	72	15 s	–	

8. Click ***OK***.
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);
   - set channels calibration from 5Fl to 10Fl for all dyes (the ***Edit*** button of the ***Auto gain calibration channel settings***). Click ***Close***.
10. Click ***Next.*** Start the amplification program by activating the ***Start Run*** button.
11. Name the experiment and save it to the disk (results of the run will be automatically saved in this file).
12. Enter the data into the grid of the samples (it opens automatically after the amplification has been started). Indicate the names/numbers of the test and control samples in the box ***Name***. Set the type ***None*** for the cells matching with the corresponding empty tubes.

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

Data analysis in the FAM/Green channel

1. Activate the button ***Analysis*** in the menu, select the mode of the analysis ***Quantitation***, activate the button ***Cycling A. FAM*** or the ***Cycling A. Green*** button. Click ***Show***.
2. Cancel the ***Threshold*** automatic choice.
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), set ***Threshold = 0.03*****.**
5. Choose the parameter ***More settings/Outlier Removal*** and set **20 %** 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 in the JOE/Yellow channel

1. Activate the button ***Analysis*** in the menu, select the mode of the analysis ***Quantitation***, activate the button ***Cycling A. JOE*** or ***Cycling A. Yellow*** button. Click ***Show***.
2. Cancel the ***Threshold*** automatic choice.
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), set ***Threshold = 0.03*****.**
5. Choose the parameter ***More settings/Outlier Removal*** and set **10 %** for the value of negative samples threshold (***NTC/Threshold***).
6. In the results grid (***Quant. Results*** window one will be able to see the *Ct* values.

Data analysis in the ROX/Orange channel

1. Activate the button ***Analysis*** in the menu, select the mode of the analysis ***Quantitation***, activate the button ***Cycling A. ROX*** or the ***Cycling A. Orange*** button. Click ***Show***.
2. Cancel the ***Threshold*** automatic choice.
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), set ***Threshold = 0.03*****.**
5. Choose the parameter ***More settings/Outlier Removal*** and set **10 %** for the value of negative samples threshold (***NTC/Threshold***).
6. In the results grid (***Quant. Results*** window one will be able to see the *Ct* values.

Data analysis in the Cy5/Red channel

1. Activate the button ***Analysis*** in the menu, select the mode of the analysis ***Quantitation***, activate the button ***Cycling A. Cy5*** or the ***Cycling A. Red*** button. Click ***Show***.
2. Cancel the ***Threshold*** automatic choice.
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), set ***Threshold = 0.03*****.**
5. Choose the parameter ***More settings/Outlier Removal*** and set **15 %** for the value of negative samples threshold (***NTC/Threshold***).
6. In the results grid (***Quant. Results*** window one will be able to see the *Ct* values.

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

Carry out the DNA extraction and reaction mixture preparation stages according to the PCR kit *Instruction Manual*. When carrying out the amplification it is recommended to use thin-walled PCR tubes (0.2 ml) with optically transparent domed or flat caps (detection through the cap of the tube).

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 – ***HBVgFL*** 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;
   - ***Fluorophores*** – Fam – IC; Hex – specific target; Rox – specific target; Cy5 – 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***
Hold	95	15 min	–	1
Cycling	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling 2	95	5 s	–	40
	60	30 s	Fam, Hex, Rox, Cy5	
	72	15 s	–	

4. Select the ***Add test*** button and select the name ***HBVgFL*** 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 ***F*** 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 – 10 %, 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. View data separately for each channel.
7. Set the threshold line (***Threshold***) for each channel at a time at the specific level in accordance with the table 3. ***Threshold*** value is set at the specific level of maximum fluorescence obtained for the Positive Control sample in the last amplification cycle in the respective channel. To set the threshold line at the specific level, drag it with a cursor while pressing the left mouse button.
8. 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.

Table 3

Channel	***Threshold line level***
Fam	25%
Hex	15%
Rox	15%
Cy5	25%

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

Carry out the DNA extraction and reaction mixture preparation stages according to the PCR kit *Instruction Manual*. When carrying out the amplification it is recommended to use thin-walled PCR tubes (0.2 ml) with optically transparent domed or flat caps (detection through the cap of the tube).

Program the instrument according to the *Instruction 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 4). Set ***Sample Volume – 25 µl.***

Table 4

The AmpliSens-1 amplification program for plate-type instruments

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Number of cycles***
Hold	95	15 min	–	1
Cycling	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling 2	95	5 s	–	40
	60	30 s	FAM, HEX, ROX, Cy5	
	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.

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

Data analysis

1. Start the program and open the saved file.
2. Open the ***Quantification*** tab (opens by default) with the fluorescence curves, plate setup, and results grid with *Ct* values.
3. Set the threshold line (***Threshold***) for each channel at a time at the specific level in accordance with the table 5. ***Threshold*** value is set at the specific level of maximum fluorescence obtained for the Positive Control sample in the last amplification cycle in the respective channel. To set the threshold line at the specific level, drag it with a cursor while pressing the left mouse button.
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.

Table 5

Channel	***Threshold line level***
FAM	25 %
HEX	15 %
ROX	15 %
Cy5	25 %