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For Professional Use Only

GUIDELINES

to AmpliSens® *Dengue virus* type-FRT PCR kit

for qualitative detection of RNA of *Dengue virus* type 1-4 in the human clinical (blood plasma, blood serum) and autopsy material (brain, liver, spleen tissues), in animal material (brain, spleen tissues), in mosquitoes by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

AmpliSens®

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TABLE OF CONTENTS

INTENDED USE 3
AMPLIFICATION AND DATA ANALYSIS USING Rotor-Gene 6000 (Corbett Research, Australia) AND Rotor-Gene Q (QIAGEN, Germany) INSTRUMENTS 3
AMPLIFICATION AND DATA ANALYSIS USING CFX96 (Bio-Rad, USA) 5
TROUBLESHOOTING 9

INTENDED USE

The guidelines describe the procedure of using **AmpliSens® *Dengue virus* type-FRT** PCR kit for detection and differentiation of RNA of *Dengue virus* type 1-4 in the human clinical (blood plasma, blood serum) and autopsy material (brain, liver, spleen tissues), in animal material (brain, spleen tissues), in mosquitoes by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using the following instruments:

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

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 instruments 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/Rotor-Gene 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 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 rotor of the thermocycler so that the first tube could get into well 1, put the rotor into the instrument (the rotor cells are numbered, the numbers are used for the further programming of the samples' position in the thermocycler).

NOTE: Balance the rotor of the instrument if it is not loaded entirely. Fill the spare wells with empty tubes (don't use the tubes left after previous experiments). Well 1 must be filled with any studied tube except for an empty one.

3. Click the ***New*** button in the main menu of the program.
4. In the open window select the template of the run setting ***Advanced*** and mark ***Dual Labeled Probe/Hydrolysis probes***. Click the ***New*** button.
5. In the open window choose ***36-Well Rotor*** and mark that the locking ring is fixed. Click the ***Next*** button.
6. In the open window set the operator and select the volume of the reaction mix: ***Reaction volume – 25 µl***. Mark the ***15 µl oil layer volume*** function with a tick. Click

the ***Next*** button.

7. In the open window it is necessary to set the temperature profile of the experiment. Click the ***Edit profile*** button and set the following amplification parameters:

The amplification program for rotor-type instruments

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Number of cycles***
1	50	30 min	–	1
2	95	15 min	–	1
3	95	10 s	–	5
	56	35 s	–	
	72	15 s	–	
4	95	10 s	–	40
	54	35 s	FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red, Cy5.5/Crimson	
	72	15 s	–	

8. As soon as the temperature profile of the experiment is chosen click the ***OK*** button.
9. Click the ***Calibrate/Gain Optimisation…*** button in the ***New Run Wizard*** window.
   - perform the calibration in the FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red, and Cy5.5/Crimson channels (click the ***Calibrate Acquiring/Optimise Acquiring*** button);
   - perform the calibration in the FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red, Cy5.5/Crimson channels before the first detection (activate the ***Perform Calibration Before 1st Acquisition/ Perform Optimisation Before 1st Acquisition*** option);
   - set the calibrations for all the channels from **5Fl** to **10Fl** (activate the ***Edit…*** button in the ***Auto gain calibration channel settings*** window). Click the ***Close*** button.
10. Click the ***Next*** button and start the amplification by clicking the ***Start run*** button.
11. Name the experiment and save it on the disc (the results of the experiment will be saved automatically in this file).
12. Enter the data into the samples grid (it opens automatically after the amplification has been started). Mark the names/numbers of the test and control samples in the ***Name*** column. Set the ***None*** type for empty wells.

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

Data analysis:

The results are interpreted according to the crossing (or not-crossing) of the fluorescence curve with the threshold line that correspond to the presence (or absence) of the *Ct* (threshold cycle) value in the results grid.

Amplification results analysis through the FAM/Green channel:

1. Activate the ***Analysis*** button, select the ***Quantitation*** analysis mode; activate the

Cycling A. FAM/Cycling A. Green, ***Show*** button.

2. Cancel the automatic choice of the threshold line level (the ***Threshold*** button).
3. The ***Dynamic tube, Slope Correct*** buttons are to be activated in the menu of the main window (***Quantitation analysis***).
4. In the ***CT Calculation*** menu (in the right part of the window) set the threshold line level ***Threshold = 0.03***.
5. Select the ***More settings/Outlier Removal*** parameter and set the value of the threshold of negative samples (***NTC Threshold***) as 10 ***%***.
6. ***Eliminate Cycles before – 5.***
7. In the results grid (the ***Quant. results*** window) the ***Ct*** values will appear**.**

The analysis in the JOE/Yellow, ROX/Orange, Cy5/Red, and Cy5.5/Crimson channels is carried out in a similar way to the results analysis in the FAM/Green channel in accordance with the settings given in the table below:

Channel	Threshold	More Settings/ Outlier Removal	Slope Correct	Eliminate Cycles before
FAM/Green	0.03	10 %	on	5
JOE/Yellow	0.03	10 %	on	5
ROX/Orange	0.03	10 %	on	5
Cy5/Red	0.03	15 %	on	5
Cy5.5/Crimson	0.03	5 %	on	5

NOTE: If the fluorescence curves in the FAM/Green channel do not correspond the exponential growth set the value of the threshold of the negative samples (***NTC threshold***) by 20 %.
In case when the fluorescence curves in the JOE/Yellow, ROX/Orange, Cy5/Red, and Cy5.5/Crimson channels do not correspond the exponential growth set the value of the threshold of the negative samples (***NTC threshold***) by 15 – 20 %.

Results analysis:

The result of the analysis is considered reliable only if the results obtained for Positive and Negative Controls of amplification as well as for the Negative Control of extraction are correct (see the “Results for control” table in the *Instruction Manual* and the *Ct* values given in the *Important Product Information Bulletin* enclosed to the PCR kit).

The interpretation of test samples should be performed in accordance with the *Instruction Manual* and the *Important Product Information Bulletin* enclosed to the PCR kit.

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 optically transparent domed or flat 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.

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*** in the start window (or select ***New***, and then ***Run…*** in the ***File*** menu).
3. Select the ***Protocol*** tab in the ***Run Setup*** window and click the ***Create new…*** button. Set the amplification parameters in the ***Protocol Editor – New*** window. Set the volume of the reaction mix ***Sample Volume – 25 µl.***

Amplification program for plate-type instruments

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Number of cycles*
Hold	50	30 min	–	1
Hold	95	15 min	–	1
Cycling 1	95	10 s	–	5
	56	40 s	–	
	72	20 s	–	
Cycling 2	95	10 s	–	40
	54	40 s	FAM, HEX, ROX, Cy5, Quasar 705	
	72	20 s	–	

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

4. Save the protocol by selecting ***File*** and then ***Save As*** in the ***Protocol Editor New*** window and name the file. One can select the file with this program for further runs in the ***Protocol*** tab by clicking the ***Select Existing…*** button.
5. As soon as the needed program is selected or edited click the ***OK*** button in the low part of the window.
6. Click the ***Create new…*** button in the ***Plate*** tab. Set the position of the tubes in the module in the ***Plate Editor - New*** window. Select ***Unknown*** in the ***Sample type*** menu by clicking the ***Select Fluorophores*** button***.*** Mark all the fluorophores (FAM, HEX, ROX, Cy5, Quasar705) with a tick and click ***OK***, and then tick the fluorescence signal detection through the needed channels in the chosen tubes. Set the name of the

samples in the ***Sample name*** window.

7. Save the plate scheme by choosing ***File*** and then ***Save As*** in the ***Plate Editor New*** window, and set the name of the file. As soon as the needed plate scheme is selected or edited click the ***OK*** button in the low part of the window.
8. Insert the reaction tubes into the thermocycler wells in accordance with the plate scheme that was programmed beforehand. Start the selected program with the set plate scheme from the ***Start Run*** tab by clicking the ***Start Run*** button, select the directory for saving the file of the run.
9. After the program is finished proceed to the data analysis.

Data analysis:

The results are interpreted using the software of the instrument according to the crossing of the fluorescence curve with the threshold line set on the corresponding level (which corresponds to the *Ct* value shown in the appropriate column of the results grid).

1. Fluorescence curves, the tubes' position in the module, and a grid with *Ct* values are shown in the ***Quantification*** tab.
2. Set the threshold line level for each channel in turn (drag it with a cursor while pressing the left mouse button) in accordance with the table.

   The threshold line level is set as a percentage from the maximum fluorescence level of Positive Control (C+) samples in the last amplification cycle registered on the appropriate channel.

Channel	Threshold
FAM, Cy5	Set the threshold line level at 20 % from the maximum fluorescence level of the Positive Control samples in the last amplification cycle
HEX, ROX, Cy5.5	Set the threshold line level at 10 % from the maximum fluorescence level of the Positive Control samples in the last amplification cycle

The fluorescence curve of Positive Control (C+) samples should cross the threshold line in the area of typical exponential growth which becomes a linear growth.

3. Select ***Tools*** on the tool bar, then click ***Reports…*** and save the document.

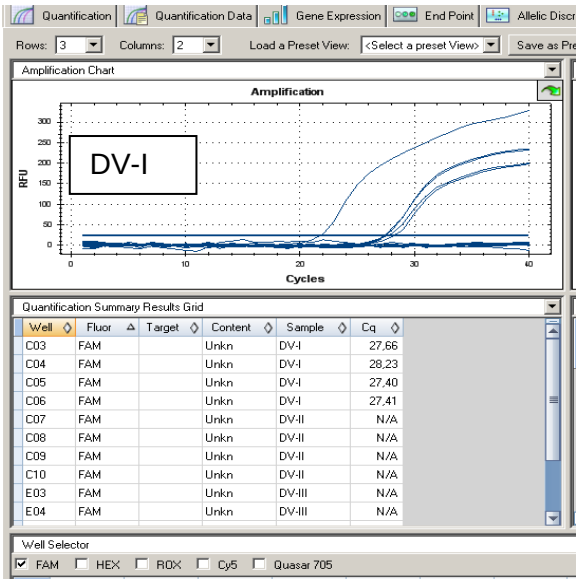
Results analysis:

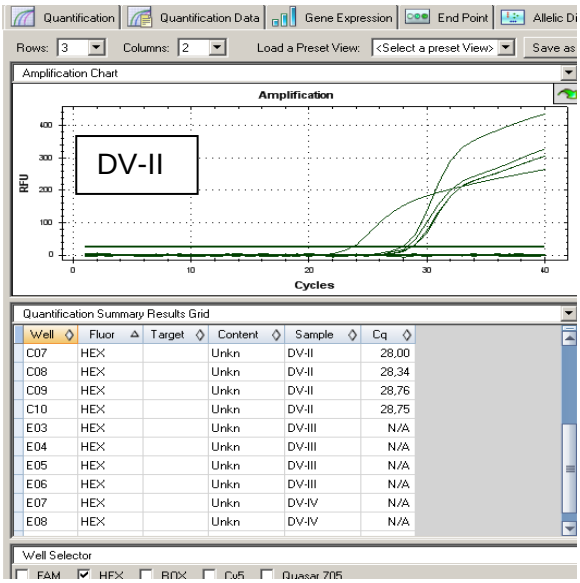
The result of the PCR analysis is considered reliable if the results obtained for Positive and Negative Controls of amplification as well as for the Negative Control of the RNA extraction are correct (see the "Results for control" table in the *Instruction Manual* and the *Ct* values given in the *Important Product Information Bulletin* enclosed to the PCR kit).

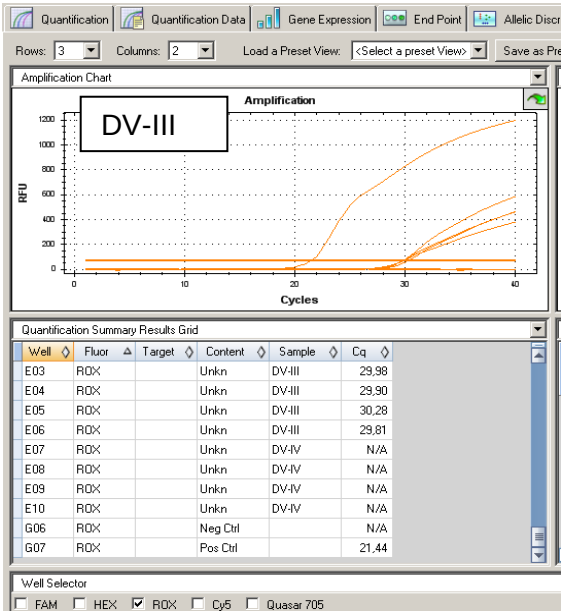
The interpretation of test samples should be performed in accordance with the *Instruction Manual*

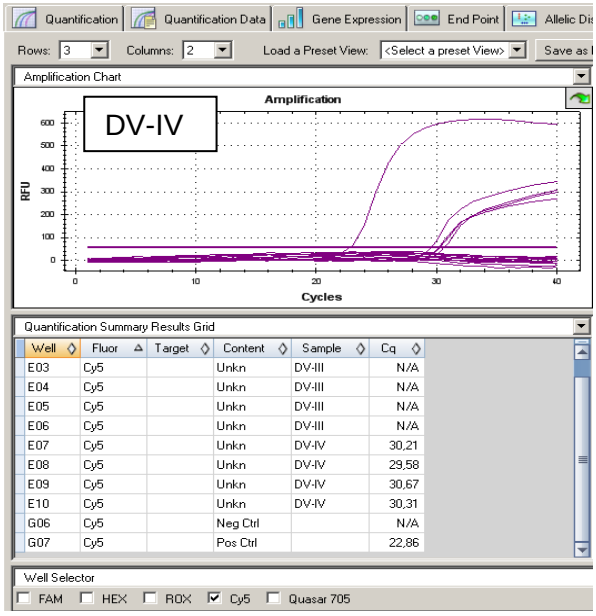
and the *Important Product Information Bulletin* enclosed to the PCR kit.

Example of amplification using CFX96









TROUBLESHOOTING

1. If the *Ct* value for the Positive Control of amplification (C+) in the **FAM/Green, JOE/HEX/Yellow, ROX/Orange, Cy5/Red** channels is absent or higher than the boundary value the PCR should be repeated for all samples in which the specific cDNA was not detected.
2. If the ***Ct*** value is defined for the Negative Control of extraction (C–) in any channel (**FAM/Green, JOE/HEX/Yellow, ROX/Orange, Cy5/Red**) it is necessary to repeat the PCR for all the samples in which the cDNA in the given channel or channels is detected.
3. If the *Ct* value is defined for the Negative Control of amplification (NCA) in any channel (**FAM/Green, JOE/HEX/Yellow, ROX/Orange, Cy5/Red, Cy5.5/Quasar705**) it is necessary to repeat the amplification for all the samples in which the cDNA in any channel is detected. NCA is to be carried out at least three times.

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
11.11.19 SK	Amplification and data analysis using Rotor-Gene 6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany) instruments	«Eliminate Cycles before» was added to the table in Data analysis; the values of the threshold were changed in the note
	Amplification and data analysis using CFX96 (Bio-Rad, USA)	The values of the threshold line level were changed in Data analysis
29.12.20 KK	Through the text	The symbol  was changed to NOTE:
	Cover page	The phrase “Not for use in the Russian Federation” was added
18.03.21 VA	Front page	The name, address and contact information for Authorized representative in the European Community was changed