

GUIDELINES

to AmpliSens[®] *Vibrio cholerae*-FRT PCR kit

for detection of *Vibrio cholerae* DNA and identification of pathogenic strains of *Vibrio cholerae* in biological material and environmental samples 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® *Vibrio cholerae*-FRT** PCR kit for detection of *Vibrio cholerae* DNA and identification of pathogenic strains of *Vibrio cholerae* in the biological material and environmental samples 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).

Correspondence of names of fluorophores and detection channels

Channel for the fluorophore	Detection channel name for different instrument models ¹
FAM	FAM/Green
JOE	JOE/HEX/R6GYellow/Cy3
ROX	ROX/Orange/TxR

DNA identification of all serogroups *Vibrio cholerae* is performed according to the presence of the *Hly* sequence, identification of pathogenic *Vibrio cholerae* strains is performed according to by the presence of the main virulence factors, *CtxA* and *tcpA*, species identification to serogroups O1 is performed according to by the presence of amplification of the *wbeT* target and species identification to serogroups O139 is performed according to by the presence of amplification of the *wbfR* target. The reaction is conducted in “multiplex” format in two tubes with PCR-mix-1-FRT *Vibrio cholerae* screen and PCR-mix-1-FRT *Vibrio cholerae* type.

Table 1

Correspondence of PCR-mix-1-FRT and detection channels

PCR-mix-1-FRT	Detection channel		
	FAM/Green	JOE/Yellow	ROX/Orange
PCR-mix-1-FRT <i>Vibrio cholerae</i> screen	CtxA	Internal Control	tcpA
PCR-mix-1-FRT <i>Vibrio cholerae</i> type	wbeT	Hly	wbfR

¹ The detection channels names in each section of the guidelines are specified in accordance with the described instrument.

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 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.

Insert the tubes or strips into the rotor of the Rotor-Gene 3000/6000/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.

If "screen" and "type" tests are performed simultaneously, calibration should be performed using the tube that contains NCA with PCR-mix-1-FRT *Vibrio cholerae* screen (insert it into well No. 1 of the rotor).

Programming the thermocycler

1. Click the **New** button in the software main menu.
2. Select the **Advanced** tab and **Dual Labeled Probe/Hydrolysis probes** template in the opened window. Click the **New** button.
3. In the opened window select the **36-Well Rotor** and tick the **No Domed 0,2ml Tubes / Locking Ring Attached** option.
4. Click the **Next** button.
5. Select the reaction volume: **Reaction volume - 25 µl**. Tick the **15 µl oil layer volume** option if it doesn't ticked by advanced for Rotor-Gene 6000 instrument.
6. Click the **Next** button.
7. Click the **Edit profile** button.
8. Set the amplification program:

Amplification program

Step	Temperature, °C	Time	Fluorescence detection	Cycles
Hold	95	5 min	–	1
Cycling	95	10 s	–	10
	60	25 s	–	
	72	10 s	–	
Cycling 2	95	10 s	–	35
	56	25 s	FAM, JOE, ROX	
	72	10 s	–	

9. After setting up the temperature profile click the **OK** button.
10. Click the **Calibrate/Gain Optimisation...** button. In the opened window click the **Calibrate Acquiring/Optimise Acquiring** button. Tick the box in the **Perform Calibration Before 1st Acquisition/Perform Optimisation Before 1st Acquisition** line. Indicate **5** in the **Min Reading** box and **10** in the **Max Reading** box for all the channels (**FAM/Green, JOE/Yellow** and **ROX/Orange**). In the **Tube position** specify the tube number for automatically identification of the **gain/** parameter. (By default this is the 1st position in the rotor, therefore there must be a tube with a reaction mixture in the well No. 1 of the rotor).
Click the **Close** button to close the **Auto Gain Calibration Setup** window. Click the **Next** button.
11. Insert the prepared tubes in the rotor. Start the amplification program by activating the **Start Run** button.
12. Name the experiment and save it to the disc (the experiment results will be automatically saved in this file).

During the work of the thermocycler or after it has stopped working it is necessary to program the tube position in the rotor. To do this it is necessary to click the **Edit samples** button (in the lower right part of the main window). Set the **Unknown** type for all test samples and controls in the **Samples** menu. Click the **Finish** button.

Data analysis

NOTE: The results analysis should be performed separately for each PCR-mix-1 by marking the area with the tubes applied to specified PCR-mix-1

Amplification data analysis for PCR-mix-1-FRT *Vibrio cholerae* screen:

1. Activate the button **Analysis** in the menu, select the mode of the analysis **Quantitation**, activate the **Cycling A. FAM/Cycling A. Green, Show** buttons.
2. Cancel the automatic choice of the threshold line level **Threshold**.
3. Activate the **Dynamic tube** button in the menu of main window (**Quantitation analysis**).
4. Click the **Linear scale** button (at the bottom of the window) (if this sale is activated by default, one can see the **Log scale** button instead of the **Linear scale** button).
5. Choose the parameter **More settings/Outlier Removal** and set **5 %** for the value of negative samples threshold (**NTC/Threshold**).
6. In the **CT Calculation** menu (in the right part of the window) indicate the threshold line level **0.05** in the **Threshold** box.

7. In the results grid (the **Quantitation Results** window) one will be able to see the *Ct* values.
8. Perform the same operations for **JOE/Yellow** and **ROX/Orange** channels, according to the items 1-5.
9. In the **CT Calculation** menu (in the right part of the window) indicate the threshold line level **0.05** in the **Threshold** box for **JOE/Yellow** channel.
10. In the **CT Calculation** menu (in the right part of the window) indicate the threshold line level **0.1** in the **Threshold** box for **ROX/Orange** channel.

Amplification data analysis for PCR-mix-1-FRT *Vibrio cholerae* type:

1. Activate the button **Analysis** in the menu, select the mode of the analysis **Quantitation**, activate the **Cycling A. FAM/Cycling A. Green, Show** buttons.
2. Cancel the automatic choice of the threshold line level **Threshold**.
3. Activate the **Dynamic tube** button in the menu of main window (**Quantitation analysis**).
4. Click the **Linear scale** button (at the bottom of the window) (if this sale is activated by default, one can see the **Log scale** button instead of the **Linear scale** button).
5. Choose the parameter **More settings/Outlier Removal** and set **5 %** for the value of negative samples threshold (**NTC/Threshold**).
6. In the **CT Calculation** menu (in the right part of the window) indicate the threshold line level **0.05** in the **Threshold** box.
7. In the results grid (the **Quantitation Results** window) one will be able to see the *Ct* values.
8. Perform the same operations for **JOE/Yellow** and **ROX/Orange** channels, according to the items 1-5.
9. In the **CT Calculation** menu (in the right part of the window) indicate the threshold line level **0.05** in the **Threshold** box for **JOE/Yellow** channel.
10. In the **CT Calculation** menu (in the right part of the window) indicate the threshold line level **0.1** in the **Threshold** box for **ROX/Orange** channel.

Results interpretation

Results are interpreted by the crossing (or not-crossing) the fluorescence curve with the threshold line set at the specific level that corresponds to the presence (or absence) of a *Ct* value of the cDNA sample in the corresponding column of the results grid.

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