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GUIDELINES

to AmpliSens® Ascaridosis-FRT PCR kit

for detection of *Ascaris* spp. DNA in 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® Ascaridosis-FRT** PCR kit for detection of *Ascaris* spp. DNA in biological material (feces and sputum) 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);
- iCycler iQ5 (Bio-Rad, USA);
- Mx3000P (Stratagene, USA).

Correspondence of fluorophores and detection channels

Channel for the fluorophore	Detection channel name for different instrument models[1]
FAM	FAM/Green
JOE	JOE/HEX/R6G/Yellow/Cy3

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 (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 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). Program the instrument.

NOTE: Well 1 must be filled with any test tube except for an empty one

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

3. Click the ***New*** button in the software main menu.
4. Select the reaction mixture volume and the rotor type:

   Reaction volume – 25 µl

   Rotor type – 36-Well Rotor or ***72-Well Rotor*** according to the rotor type.
5. Set the amplification program. To do this click the ***Edit profile*** button.

Table 1

Amplification program

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	**95**	15 min	–	1
2	**95**	10 s	–	45
	60	25 s	FAM/Green, JOE/Yellow	
	72	10 s	–	

Other channels are enabled if several tests are simultaneously carried out in a single run).

6. Set calibration parameters (activate ***Calibrate/Gain Optimisation…*** in a new experiment module):
   – perform the fluorescence detection in FAM/Green, JOE/Yellow channels (activate ***Calibrate Acquiring/Optimise Acquiring***);
   – perform calibration before the first measurement (activate the ***Perform Calibration Before 1st Acquisition/Perform Optimisation Before 1st Acquisition*** button);
   – set the channel calibrations from **5 Fl** to **10Fl** for all channels (activate ***Edit…,*** the ***Auto gain calibration channel settings*** window).
7. Start the amplification program by activating the ***Start Run*** button, and name the experiment.
8. During or after amplification, it is necessary to program the position of test samples, the Negative Control of extraction, the Positive Control of Amplification, the Negative Control of Amplification.

   To do this, it is necessary to enter the data into the grid of the samples (it opens automatically after amplification has been started). Enter the names/numbers of the test samples in the ***Name*** column. Define the Negative control of amplification as NCA, the Positive control of amplification as C+. Set the ***Unknown*** type opposite all the test samples, the ***Positive control*** type – for the Positive control of amplification, the ***Negative Control*** type next to the Negative Control of extraction and the ***NTC*** type next to the Negative Control of Amplification. Set the ***None*** type for the cells matching with the corresponding empty tubes.

Data analysis

1. Activate the ***Analysis*** button in the menu, select the mode of the analysis ***Quantitation***, activate ***Cycling A. FAM/Cycling A. Green, Show, Cycling A. JOE/Cycling A. Yellow***, ***Show*** or ***Cycling A. ROX/Cycling A. Orange, Show*** buttons.
2. Cancel the automatic choice of the threshold line level ***Threshold*** for every opened main window (FAM/Green, JOE/Yellow).
3. Activate ***Dynamic tube*** and ***Slope Correct*** buttons in the menu of main window (***Quantitation analysis***) and set ***More settings*** as ***10 %***.
4. In the ***CT Calculation*** menu, set ***Threshold = 0.05***.
5. In the results grid (***Quant. Results*** window), *Ct* values for each tested sample will appear. *Ct* values for test samples are taken into account 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 *Instruction manual* and *Important Product Information Bulletin* for the PCR kit).
6. 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. The samples are considered positive if *Ct* values do not exceed the boundary *Ct* value specified in the *Important Product Information Bulletin*.

AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ5 (Bio-Rad, USA)

INSTRUMENT

Carry out the sample pretreatment and reaction mixture preparation stages according to the PCR kit *Instruction Manual*. When working with the iCycler iQ5 instrument, it is recommended to use 0.2-ml polypropylene disposable tubes for PCR with domed caps (for example, Axygen, USA).

Programming the thermocycler

1. Turn on the instrument and the power supply unit of the optical block of the instrument.

NOTE: The lamp is to be warmed up during 15 min before starting the experiment.

2. Start the program iCycler iQ5.
3. Insert the tubes or strips into the reaction module of the thermocycler and program the instrument according to the *Instruction Manual* given by the manufacturer 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 tubes (strips) upside down while inserting them into the instrument.

4. Press the ***Create new*** button in the ***Workshop*** module to enter the mode of creating a new amplification protocol.
5. Set the amplification program (***Protocol***):

Table 2

Amplification program

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	**95**	15 min	–	1
2	**95**	10 s	–	45
	60	25 s	FAM, JOE/HEX	
	72	10 s	–	

Name the new protocol and save it.

6. Set the order of tubes in the ***Plate***. Select fluorophores in the ***Select/add fluorophores*** tab and press ***Fluorophore loading in Whole Plate mode*** to activate fluorophores. Set ***Sample Volume*** (***25 µl), Seal Type*** – ***Domed Cap***, ***Vessel Type*** – ***Tubes,*** and press ***Save/Exit Plate Editing*** to save the created protocol.
7. Add reagents for amplification and DNA samples into the tubes and insert them into the instrument.
8. Press the ***Run*** button, set ***Use Persistent Well Factors,*** press the ***Begin Run*** button, and save the experiment.

Data analysis

1. Data are analyzed in FAM and JOE/HEX channels.
2. Press the ***Data Analysis*** button in the menu.
3. Select ***20 – 25*** for ***Base line*** in ***Crossing Threshold User Defined***. Normally, the threshold line should cross only sigmoid curves of signal accumulation of positive samples and controls and should not cross the curves of different shapes. Otherwise, the threshold level should be raised.
4. *Ct* values for test samples are taken into account 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 *Instruction manual* and *Important Product Information Bulletin* for the PCR kit).
5. 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. The samples are considered positive if *Ct* values do not exceed the boundary *Ct* value specified in the *Important Product Information Bulletin*.

AMPLIFICATION AND DATA ANALYSIS USING Mx3000P (Stratagene, 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 0.2-ml disposable PCR tubes with domed caps (detection through the cap of the tube).

1. Switch the instrument on, start the program **Stratagene Mx3000P**.
2. Select ***Quantitative PCR (Multiple Standards)*** in the ***New Experiment Options*** window and check the ***Turn lamp on for warm-up*** box.

NOTE: The lamp is to be warmed up during 15 min before starting the experiment.

3. Insert the tubes into the instrument, lock the fixing arm and the door of the instrument.
4. Select ***Optics Configuration*** in ***Options*** menu and, in the ***Dye Assignment*** tab, set FAM parameter next to the ***FAM filter set*** item and JOE parameter next to the ***HEX/JOE filter set***.
5. Set the fluorescence detection parameters in the ***Plate Setup*** menu. To do this, select all the cells with the test tubes or strips and mark them as ***Unknown*** in the ***Well type*** field. Select FAM and JOE/HEX fluorophores in the ***Collect fluorescence data*** option.
6. Name each sample in the ***Well Information*** window.
7. Set amplification program in ***Thermal Profile Setup*** tab. To do this, use one of the following methods:
   - Press the ***Import…*** button. Select the fold containing the previous experimental file and open the fold. The needed thermocycling profile will appear in the ***Thermal Profile*** window;
   - Set the appropriate amplification program in ***Thermal Profile Setup*** menu.

Table 3

Amplification program

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	**95**	15 min	–	1
2	**95**	10 s	–	45
	60	25 s	FAM, JOE/HEX	
	72	10 s	–	

To set the parameter of the fluorescence signal detection at the set temperature it is necessary to select the ***All points*** option for the ***Data collection marker for dragging*** parameter and drag it with a mouse from the right part of the box to the field with the necessary temperature.

8. Start the amplification by clicking the ***Run*** and ***Start*** buttons, then name the experiment.

Data analysis

1. Select ***Analysis*** by clicking the corresponding button of the tool bar.
2. Make sure that all test samples in the ***Analysis Selection/Setup*** tab are active (the cells corresponding to the samples should be of a different colour).
3. Select the ***Results*** tab.
4. Make sure that the automatic selection of the threshold level for each channel is correct. Normally, the threshold line should cross only the sigmoid curves of signal accumulation of positive samples and controls and should not cross the baseline. Otherwise, the threshold level should be raised. To do this, activate each channel image separately in the lower ***Dyes shown*** panel. Check the threshold line location and change it, if necessary.
5. *Ct* values for test samples are taken into account 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 *Instruction manual* and *Important Product Information Bulletin* for the PCR kit).
6. 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. The samples are considered positive if *Ct* values do not exceed the boundary *Ct* value specified in the *Important Product Information Bulletin*.