

# GUIDELINES

## to AmpliSens<sup>®</sup> *Cryptococcus neoformans*-FRT PCR kit

for qualitative detection of *Cryptococcus neoformans* DNA in the biological material (cerebrospinal fluid, bronchoalveolar lavage, sputum, blood, skin lesions aspirate, viscera biopsy and autopsy 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® Cryptococcus neoformans-FRT** PCR kit for qualitative detection of *Cryptococcus neoformans* DNA in the biological material (cerebrospinal fluid, bronchoalveolar lavage, sputum, blood, skin lesions aspirate, viscera biopsy and autopsy material) 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);
- iCycler iQ5 (Bio-Rad, USA).

## AMPLIFICATION AND DATA ANALYSIS USING Rotor-Gene 3000/6000 (Corbett Research, Australia) INSTRUMENTS

When working with Rotor-Gene 3000 one should use the Rotor-Gene Version 6 software and the Rotor-Gene 6000 versions 1.7 (build 67) software or higher for Rotor-Gene 6000.

**Hereinafter, all the terms corresponding to different instruments and software are indicated in the following order: for Rotor-Gene 3000 / for Rotor-Gene 6000.**

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 carousel of the Rotor-Gene 3000/6000/Q instrument (the carousel cells are numbered, the numbers are used for the further programming of the samples' position in the thermocycler). Program the instrument.

**NOTE:** Well 1 must be filled with any studied tube except for an empty one.

3. Program the amplification process for the rotor-type instruments (see Table 1).

## AmpliSens-1 amplification program

<b>Step</b>	<b>Temperature, °C</b>	<b>Time</b>	<b>Fluorescence detection</b>	<b>Number of cycles</b>
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	
	72	15 s	–	

4. Select the **Calibrate/Gain Optimisation** button in the **New Run Wizard** window:
  - perform the fluorescence detection in FAM/Green, JOE/Yellow channels (activate the **Calibrate Acquiring/Optimise Acquiring** button);
  - perform the calibration in FAM/Green, JOE/Yellow channels before the first detection (activate the **Perform Calibration Before 1<sup>st</sup> Acquisition/ Perform Optimisation Before 1<sup>st</sup> Acquisition** button);
  - to set FAM/Green, JOE/Yellow channels calibration, indicate **5** in the **Min Reading** box and **10** in the **Max Reading** box (activate **Edit...**, the window **Auto gain calibration channel settings**).
5. Start the amplification program by activating the **Start Run** button, and name the experiment.
6. During the work of the amplifier or after it has stopped working it is necessary to program the position of the test samples, negative control of the extraction, positive and negative controls of the DNA amplification.

To do this, it is necessary to 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 samples in the box **Name**. Mark the positive controls of amplification as C+<sub>1</sub> and C+<sub>2</sub>, negative control of amplification as NCA. Set the type **Unknown** opposite all the test samples, the type **Positive control** – for positive controls, the type **Negative control** – for the negative control of the extraction, and the type **NTC** – for the negative control of the PCR. Set the type **None** for the cells matching with the corresponding empty tubes.

**Data analysis:**

1. Activate the button **Analysis** in the menu, select the mode of the analysis **Quantitation**, activate the buttons **Cycling A. JOE/Cycling A. Yellow, Show, Cycling A. FAM/Cycling A. Green, Show**.

2. Cancel the automatic choice of the threshold line level for each of the main open windows (FAM/Green and JOE/Yellow) by activating the **Threshold** button.
3. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of each main window (**Quantitation analysis**).
4. In the **CT Calculation** menu (in the right part of the window) indicate the threshold line level **0.03** in the **Threshold** box. Choose the parameter **More settings/Outlier Removal** and set **10 %**.
5. In the results grid (the **Quant. Results** window) one will be able to see the *Ct* values.
6. The *Ct* values for the test samples are to be analyzed if only the results for the control samples (NCA, C+<sub>1</sub>, C+<sub>2</sub>, C–) are satisfying in accordance with boundary *Ct* values specified in *Important Product Information Bulletin*.

### AMPLIFICATION AND DATA ANALYSIS WITH THE USE OF iCycler iQ5 (Bio-Rad, USA) INSTRUMENTS

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 caps (e.g. Axygen, USA) (detection through the cap of the tube).

1. Switch on the instrument and the power supply unit of the optical part of the instrument.

**NOTE:** The lamp is to be warmed up during 20-30 min before starting the experiment.

2. Set the amplification program (see Table 2).

Table 2

**AmpliSens-1 amplification program**

<b>Step</b>	<b>Temperature, °C</b>	<b>Time</b>	<b>Fluorescence detection</b>	<b>Number of cycles</b>
Hold	<b>95</b>	15 min	–	1
Cycling	<b>95</b>	5 s	–	5
	<b>60</b>	20 s	–	
	<b>72</b>	15 s	–	
Cycling 2	<b>95</b>	5 s	–	40
	<b>60</b>	30 s	FAM, JOE/HEX	
	<b>72</b>	15 s	–	

3. Set the order of the tubes in the reaction chamber (**Plate**), select the fluorophores (**Select/add fluorophores**), activate the fluorophores for the samples in the created protocol by **Fluorophore loading in Whole Plate mode** button. Set the reaction volume (**Sample Volume**) as **25 µl**, the caps type (**Seal Type**) as **Domed Cap**, and the tubes type (**Vessel Type**) as **Tubes**. Save the created protocol by clicking the **Save&Exit Plate Editing** button.

4. Add the reagents for amplification and DNA samples to the tubes. Insert the tubes into the reaction module of the amplifier (thermocycler).
5. Start the instrument (**Run**), select **Run Persistent Plate** and save the experiment.

**Data analysis:**

1. Results analysis is carried out in the JOE/HEX and FAM channels.
2. Activate the **Data Analysis** button.
3. Check the correctness of automatic threshold line choice. Normally, the threshold line is to cross only with sigmoid curves describing the accumulation of the signal detecting positive samples and controls. The threshold line is not to cross the base line. If it happens, it is necessary to raise the threshold level.
4. The *Ct* values for the test samples are to be analyzed if only the results for the control samples (NCA, C+<sub>1</sub>, C+<sub>2</sub>, C-) are satisfying (see *Important Product Information Bulletin*).
5. The *Ct* value for the positive controls of amplification C+<sub>1</sub> and C+<sub>2</sub> in the respective channels is to be less than the boundary *Ct* value specified in *Important Product Information Bulletin*.

## CALCULATION OF *Cryptococcus neoformans* DNA CONCENTRATION

If it is necessary to calculate *Cryptococcus neoformans* DNA concentration, set the **Standard** type for C+1 and C+2 and specify their concentrations in the **Given Conc** column. Set the concentration for C+1 as 10,000, for C+2 as 100.

The calibration curve is automatically plotted on the basis of the Ct threshold cycle values (the fluorescence curve crossing with threshold line set at the respective level) and known C+1 and C+2 values, and DNA copies of *Cryptococcus neoformans* (JOE/Yellow/HEX channel) and IC (FAM/Green channel) are calculated. The obtained values are used for calculation of *Cryptococcus neoformans* DNA concentration in test samples using the formulas:

- calculation of *Cryptococcus neoformans* DNA concentration per ml of sample extracted from 100 µl:


number of *Cryptococcus neoformans* DNA copies in the PCR sample x 100 = copies/ml

- calculation of *Cryptococcus neoformans* DNA concentration per ml of sample extracted from volumes more than 100 µl:

number of *Cryptococcus neoformans* DNA copies in the PCR sample x 100 x N = copies/ml

$$N = \frac{100}{\text{Extraction volume, } \mu\text{l}}$$

### List of Changes Made in the Guidelines

<b>VER</b>	<b>Location of changes</b>	<b>Essence of changes</b>
28.12.20 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