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GUIDELINES

to **AmpliSens® Florocenosis / *Candida*-FRT**

PCR kit for simultaneous detection and quantitation of *Candida* genus fungi DNA (*C.albicans, C.glabrata, C.krusei, C.parapsilosis* and *C.tropicalis*) in the biological material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

AmpliSens®

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TABLE OF CONTENTS

INTENDED USE

The guidelines describe the procedure of using **AmpliSens® Florocenosis / *Candida*-FRT** PCR kit for simultaneous detection and quantitation of *Candida* genus fungi DNA (*C.albicans, C.glabrata, C.krusei, C.parapsilosis* and *C.tropicalis*) in the biological material (urogenital swabs, oral and oropharyngeal swabs and urine samples) by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using the following instruments:

- Rotor-Gene 6000 (five channels, six channels)(Corbett Research, Australia);
- Rotor-Gene Q (five channel, six channel)(QIAGEN, Germany);
- CFX96 (Bio-Rad, 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/R6GYellow/Cy3
ROX	ROX/Orange/TxR
Cy5	Cy5/Red
Cy5.5	Cy5.5/Crimson/Quasar705

[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 6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN GmbH, Germany)

One should use 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, or PCR tubes (0.1 ml) with caps from the four-pieces-strips (detection through the bottom of the tube).

Creating the template for the run

1. Click the ***New*** button in the software main menu. To create the template select the ***Advanced*** tab in the opened window ***New run***. For programming and creating a new template the mode ***Advanced*** in ***New Run*** window should be selected.
2. Select the ***TwoStep/Hidrolysis Probes*** template in the tab for edition and click the ***New*** button.
3. In the opened window select the ***36-Well Rotor*** and tick the ***No Domed Tubes / Locking Ring Attached*** option. Click the ***Next*** button.
4. In the opened window enter the operator name, select the reaction volume – 25 µl. Tick the ***15 µL oil layer volume*** option. Click the ***Next*** button.
5. In the ***New Run Wizard*** window set the temperature profile of the experiment. To do this click the ***Edit profile*** button and set the amplification program:

AmpliSens-1 amplification program for rotor-type instruments

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Cycles*
1	95	15 min	–	1
2	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
3	95	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red, Cy5.5/Crimson	
	72	15 s	–	

NOTE: **AmpliSens-1** program is a universal amplification program for carrying out tests with the help of AmpliSens kits to identify the DNA of STI pathogens. All the tests or any tests combinations can be carried out in one instrument simultaneously including the tests for identification and genotyping *human papilloma virus* (*HPV* HCR).

6. After setting up the temperature profile click the ***OK*** button.
7. Set the automatic calibration for selecting the ***Gain*** parameter. Click ***Calibrate/Gain Optimisation*** in the ***Channel Setup*** window. In the opened window ***Auto Gain Calibration Setup*** click ***Calibrate Acquiring/Optimize Acquiring.*** For the ***FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red*** and ***Cy5.5/Crimson*** channels value **5** should be put into the ***Min Reading*** column and value **10** in ***Max Reading*** column (it is acceptable to set ***Calibrate/Gain Optimisation…*** value from 4 Fl to 8 Fl for ***JOE/Yellow, ROX/Orange, Cy5/Red*** and ***Cy5.5/Crimson*** channels). In the column ***Tube position*** the number of tube - **1** should be set, the parameter ***Gain*** will be selected automatically according to the signal. Tick the ***Perform Calibration Before 1-st Acquisition/Perform Optimisation Before 1-st Acquisition*** option. Close the ***Auto Gain Calibration Setup*** window. Click the ***Close*** button.
8. Click the ***Next*** button. For saving the programmed template it is necessary to click the ***Save Template*** button enter the template file name, corresponding to the amplification program – **AmpliSens**. Save the file into a proposed folder: ***Templates\Quick Start Templates***; close the ***New Run Wizard window.*** After that the template will appear in the list of templates in ***New Run*** window.

The template **AmpliSens-1** programmed in such a manner can be used for amplification and detection in any tests for indication of DNA of the infectious agents of STI with the help of kits produced by Federal Budget Institute of Science “Central Research Institute for Epidemiology”.

<u>Using the ready template for the run</u>

1. Insert the tubes into the rotor. One of the prepared tubes with the reaction mixture must be inserted to the first. Attach the locking ring, insert the rotor by clicking it into place using the locating pin on the rotor hub. Close the lid of the instrument.

NOTE: The first tube in the rotor is used for automatic optimization of the signal level, so at the first position of the rotor the tube with the reaction mixture should be inserted. During the simultaneous tests using MULTIPRIME kits the tube with the reaction mixture of the kit, for which the supreme number of channel is used, should be inserted at the first place

NOTE: The additional requirements for setting the channels’ calibration ranges are specified in the information list “Calibration priorities for the AmpliSens kits for Rotor-Gene 3000/6000 (Corbett Research, Australia) and Rotor-Gene Q

(QIAGEN, Germany) thermocyclers"

NOTE: It is forbidden to use the tubes with PCR-mix passed through amplification earlier for filling of the rotor. The cells of rotor can stay empty.

2. Click the ***New*** button in the software main menu. In the opened ***New Run*** window select the ***Advanced*** tab. Then select the **AmpliSens** template (which is programmed as described in the "Creating the template for the run" section) in the template list.
3. In the opened window select the ***36-Well Rotor (or 72-Well Rotor)*** and tick the ***No Domed 0,2ml Tubes / Locking Ring Attached*** option. Click the ***Next*** button.
4. In the opened window check that the reaction volume is 25 µl and the ***15 µl oil layer volume*** option is activated. Click the ***Next*** button.
5. In the next window the correctness of the amplification and detection program and auto-optimization conditions of signal level given in the template can be checked.
6. Start the amplification by the ***Start run*** button. Herewith, the rotor with the samples should be already fixed and the lid must be closed. Name the experiment and save it to the disc (the results of the experiment will be automatically saved in this file).
7. Enter the data into the grid of the samples (it opens automatically after the 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 type ***Unknown*** opposite all the test samples, the type ***Positive control*** – for the Positive control of amplification, the type ***Negative control*** – for the Negative control of amplification. Set the type ***Standard*** and enter the values from the *Important product information bulletin* enclosed to the given lot of the PCR kit into the ***Given conc.*** column. Set the type ***None*** for the cells matching with the corresponding empty tubes. Click the ***Finish/OK*** button.

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

NOTE: The table of samples can be edited before the start. For this select the ***Edit Samples Before Run Started*** in the ***File*** menu in submenu ***User preferences.***

8. After the amplification program is finished the results can be analysed.

NOTE: After the amplification program has finished, the tubes are to be removed from the rotor and utilized

Data analysis

The results analysis is performed separately (consequently) for each channel according to the instruction manual and given description. Further calculation of concentrations and obtained results interpretation can be performed manually or automatically with the use of the software in Microsoft Excel format.

The results are interpreted according to the crossing (or not-crossing) of the S-shaped (sigmoid) fluorescent curve with the threshold line set at the specific level, that corresponds to the presence (or absence) of the *Ct* The calibration curve plotting and calculation of ***Candida*** spp. DNA concentration are performed automatically in accordance with the *Ct* values of DNA calibrators.

NOTE: The concentrations values of DNA calibrators (CND1 and CND2) are specified in the *Important Product Information Bulletin* enclosed in the PCR kit.

Amplification data analysis in the FAM/Green channel:

1. Check that the DNA-calibrators are marked in the sample table (***Standard*** type) and their concentrations are set according to the *Important Product Information Bulletin* enclosed to the PCR kit.
2. Activate the button ***Analysis*** in the menu, select the mode of the analysis ***Quantitation***, activate the buttons ***Cycling A. FAM/Cycling A. Green***, ***Show****.*
3. Cancel the automatic choice of the threshold line level ***Threshold.***
4. Make sure that the ***Dynamic tube*** and ***Slope Correct*** buttons are activated in the main window menu (***Quantitation analysis***).
5. In the ***CT Calculation*** menu (in the right part of the window) indicate the threshold line level **0.1** in the ***Threshold*** box.
6. Choose the parameter ***More settings/Outlier Removal*** and set the values of negative samples threshold (***NTC/Threshold***): from **20** to **30 %** for **Cy5.5/*Crimson*** channel and from **10%** to **20** % for other channels.
7. In the results grid (the ***Quantitation Results*** window) one will be able to see the *Ct* values and DNA concentration values (***Calc Conc (copies/reaction)***)***.***

Results analysis in the JOE/Yellow, ROX/Orange, Cy5/Red, Cy5.5/Crimson is carried out similarly to results analysis in the FAM/Green channel in accordance with the settings in the table below.

Channel	Detecting DNA target	Threshold	Dynamic tube	Slope Correct	More Settings/ Outlier Removal
FAM/Green	*C.albicans* DNA	0.1	on	on	10-20 %
JOE/Yellow	*C.glabrata* DNA	0.1	on	on	10-20 %
ROX/Orange	*C.krusei* DNA	0.1	on	on	10-20 %
Cy5/Red	*C.parapsilosis* and *C.tropicalis* DNA	0.1	on	on	10-20 %
Cy5.5/Crimson	IC DNA	0.1	on	on	20-30 %

Results interpretation

The result of PCR is considered reliable only if the results obtained for the negative controls of amplification and extraction as well as for DNA calibrator CND2 are correct in accordance with the table of assessment of results for controls (see the *Instruction manual*), boundary values specified in the *Important Product Information Bulletin* enclosed to the PCR kit and the value of Efficiency coefficient ***E*** falls within the range specified in the *Important Product Information Bulletin* enclosed to the PCR kit. Otherwise, see section "Troubleshooting".

For the sample in which the DNA of *Candida* species is not detected or the amount of DNA copies is less than 100, the result is considered to be valid only if the *Ct* value determined in the **Cy5.5/Crimson** channel (the channel for detection of the IC DNA amplification results) does not exceed the boundary value specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

The obtained values of DNA-target copies number in the results grid for the given channel are used for calculation of genome equivalents number of *Candida* type detected in this channel in 1 ml of initial biological material according to the formula:

[Number of genome equivalents] per 1 ml (GE/ml) = K x [Number of copies] of *Candida* DNA

NOTE: **K** coefficient for calculation of result in GE/ml is specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

If the obtained result is greater than $2x10^5$ GE/ml then the result "greater than $2x10^5$ GE/ml" is specified, if the obtained result is less than 200 GE/ml then the result "less than 200 GE/ml" is specified (taking into account the linear range of the kit). If the obtained result is less than 10 GE/ml then the result "is not detected" is specified.

The example of data analysis is given in the table below.

Name	***Result***	***Comment***
1	The DNA of *Candida* group is not detected	*Ct* value in Cy5.5/Crimson channel is less than the boundary value, the result is valid
2	*Candida albicans* DNA *(*$6x10^3$ GE/ml) is detected	–
3	*Candida glabrata* DNA (700 GE/ml) is detected, *Candida albicans* DNA (less than 200 GE/ml) is detected	–
4	*Candida parapsilosis* DNA and/or *Candida tropicalis* (320 GE/ml) is detected	–
5	Invalid result	The *Ct* value is absent in the Cy5.5/Crimson channel and the amount of *Candida* DNA is less than 100 copies

AMPLIFICATION AND DATA ANALYSIS USING CFX96 (Bio-Rad Laboratories, Inc., 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 domed or flat optically transparent caps, or tubes (0.2 ml) with transparent caps from the eight-pieces-strips (detection through the cap of the tube).

Programming the thermocycler

1. Turn on the instrument and start the ***Bio-Rad CFX Manager*** software.
2. Program the instrument according to the *Instruction Manual* provided by the manufacturer.

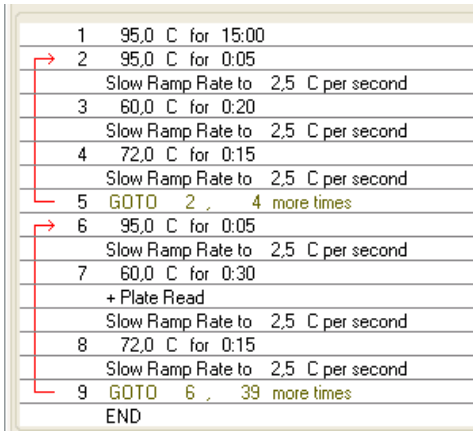
Creating the template for the run

1. In the ***Startup Wizard*** window it is necessary to select the ***Create a new Run/Experiment*** (or select ***New*** in the ***File*** menu and then select ***Run…/Experiment…***). Click ***OK***.
2. In the ***Run Setup*** window, select ***Protocol*** tab and click the ***Create new…***. Set the amplification parameters (time, temperature, cycles, and fluorescence acquiring cycle) in the opened ***Protocol Editor – New*** window. Set ***Sample Volume* – 30 µl.**

AmpliSens-1 amplification program for plate-typed instrument

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Number of cycles***
1	**95**	15 min	-	1
2	**95**	5 s	-	5
	60	20 s	-	
	72	15 s	-	
3	**95**	5 s	-	40
	60	30 s	FAM, HEX, ROX, Cy5, Quasar705	
	72	15 s	-	

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



NOTE: **AmpliSens-1** program is a universal amplification program for carrying out tests with the help of AmpliSens kits to identify the DNA of STI pathogens. All the tests or any tests combinations can be carried out in one instrument simultaneously including the tests for identification and genotyping *human papilloma virus* (*HPV* HCR).

3. Save the protocol: in the ***Protocol Editor New*** window select ***File***, then ***Save As***, name the file and click ***Save***.
4. Set the plate setup: in the ***Plate*** tab click the ***Create new…*** button. Set the tube order in the opened ***Plate Editor – New*** window. Click the ***Select Fluorophores…*** button and click the ***Selected*** checkbox next to the **FAM, HEX, ROX, Cy5** and **Quasar705** fluorophores and click ***OK***. In the ***Sample type*** menu select ***Unknown*** for all the samples, except for the DNA-calibrators. Then in the ***Load*** column (in the right part of the window) tick the fluorescence signal acquiring for all the samples in the required channels. Define sample names in the ***Sample name*** window, moreover the ***Load*** function is to be ticked. Mark ***Sample type*** of DNA-calibrators CND1 and CND2 in all the channels as ***Standard*** and specify their concentration in the ***Concentration*** field according to the *Important Product Information Bulletin* enclose to the PCR kit, moreover the ***Load*** function is to be ticked.
5. Save the plate setup: select ***File*** and then ***Save as*** in the ***Plate Editor New*** window. Enter the file name, click ***Save***.
6. Select the ***Start Run*** tab. Open the lid of the instrument by the ***Open Lid*** button. Insert the reaction tubes in the wells of the instrument in accordance with the entered plate setup. Close the lid of the instrument using ***Close Lid*** button.

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.

7. Click ***Start Run*** button to start the **AmpliSens-1** program according to the plate setup. Select the directory for the file saving, name the file, click ***Save***.

Using the ready template for the run

The test parameters and the plate setup set earlier can be used for the further runs. To do this:

- click the ***Select Existing…*** button in the ***Run Setup*** window of the ***Protocol*** tab. Select the needed file with the amplification program in the ***Select Protocol*** window. Click ***Open***.
- go to the ***Plate*** tab in the ***Run Setup*** window. Click the ***Select Existing…*** button. Select the needed file with the plate setup in the ***Select Plate*** window. Click ***Open***. Click the ***Edit selected*** button to edit the plate setup.

Data analysis

The obtained results are analysed by the software of the CFX96 instrument. Further calculation of concentrations and obtained results interpretation can be performed manually or automatically with the use of the software in Microsoft Excel format.

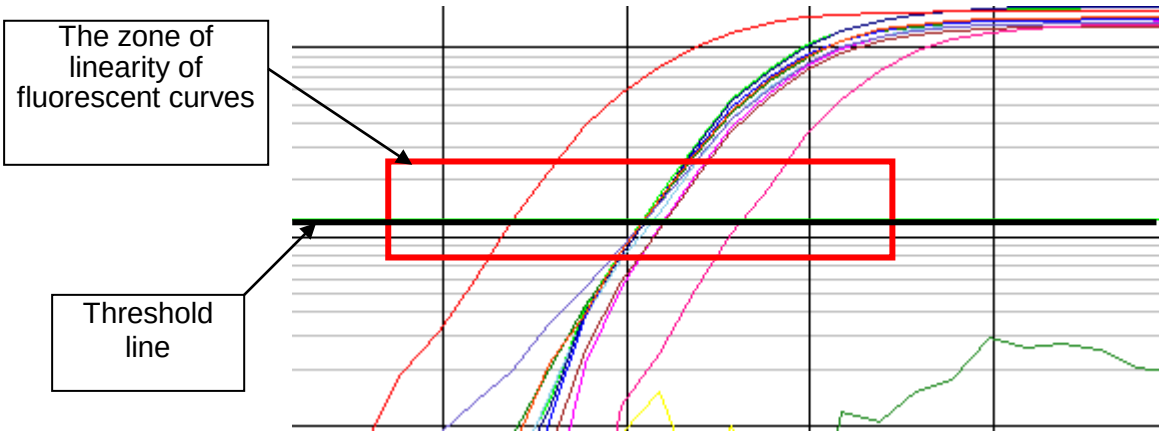
The results are interpreted according to the crossing (or not-crossing) of the S-shaped (sigmoid) fluorescence curve with the threshold at the level of exponential growth of the signal, that corresponds to the presence (or absence) of the *Ct (Cq)* value in the corresponding column of the results table. The calibration curve plotting and calculation of the **Candida** species DNA concentration are performed automatically in accordance with the *Ct* values.

NOTE: The concentration values of DNA calibrators (CND1 and CND2) are specified in the *Important Product Information Bulletin* enclosed to the PCR kit

1. Start the software and open the saved file. To do this, select ***File*** in the menu, then ***Open*** and ***Data file*** and select the needed file.
2. Analyze data separately for each channel turning off other channels (delete the ticks in the channel box under the main window with the ***Amplification*** curve).
3. Check the correctness of threshold line automatic choice for each channel. The threshold line is to cross only with S-shaped (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 set the threshold line level for each channel manually. To do this, tick the ***Log Scale*** item (logarithmic scale selection) and set (with the left mouse button) threshold line at the level, where the curves of DNA-calibrators are linear, and higher than the base line fluctuations. Otherwise, the level can be determined in the range of 10-20 % of maximum fluorescence obtained for the DNA-calibrator CND1 in the last amplification cycle (turning off the logarithmic scale) for each channel

except for the channel for the Cy5.5 fluorophore. In the channel for the Cy5.5 fluorophore, the threshold line is set at the level of 10-20 % of maximum fluorescence obtained for the C– sample.

Figure 1



4. ***Cq*** values and calculated concentrations **SQ** for the analysed channel will appear in the result grid.
5. Click the ***View/Edit Plate…*** button on the toolbar and set the samples names and calibrators concentrations in the opened window.
6. To continue work with data, the *Ct* values for each channel can be copied to the Excel from the results grid of the instrument software. To generate report of the run in ***.pdf*** format it is necessary to select the ***Tools*** on the toolbar, then select ***Reports…*** Save the generated report: select ***File*** and then ***Save as***, name the file and click ***Save.***

Results interpretation

The result of PCR is considered reliable only if the results obtained for the negative controls of amplification and extraction as well as for DNA calibrator CND2 are correct are correct in accordance with the table of assessment of results for controls (see the *Instruction manual*) boundary values specified in the *Important Product Information Bulletin* enclosed to the PCR kit and the value of amplification efficiency ***E*** is within the range specified in the *Important Product Information Bulletin* enclosed to the PCR kit. Otherwise see section "Troubleshooting".

For the sample in which the DNA of *Candida* species is not detected or the amount of DNA copies is less than 100, the result is considered to be valid only if the *Ct* value determined in the **Quasar705** channel (the channel for detection of the IC DNA amplification results) does not exceed the boundary value specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

The obtained values of DNA-target copies number in the results grid for the given channel are used for calculation of genome equivalents number of *Candida* type detected in this channel in 1 ml of initial biological material according to the formula:

[Number of genome equivalents] per 1 ml (GE/ml) = K x [Number of copies] of *Candida* DNA

NOTE: **K** coefficient for calculation of result in GE/ml is specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

If the obtained result is greater than $2x10^5$ GE/ml then the result "greater than $2x10^5$ GE/ml" is specified, if the obtained result is less than 200 GE/ml then the result "less than 200 GE/ml" is specified (taking into account the linear range of the kit). If the obtained result is less than 10 GE/ml then the result "is not detected" is specified.

The example of data analysis is given in the table below.

Name	***Result***	***Comment***
1	The DNA of *Candida* group is not detected	*Cq* value in the Quasar705 channel is less than the boundary value, the result is valid
2	*Candida albicans* DNA *(*$6x10^3$ GE/ml) is detected	–
3	*Candida glabrata* DNA $7x10^2$ GE/ml is detected, *Candida albicans* DNA *(*less than 200 GE/ml) is detected	–
4	*Candida parapsilosis* DNA and/or *Candida tropicalis* ($3x10^2$ GE/ml) is detected	–
5	Invalid result	The *Cq* value is absent in the Quasar705 channel and the amount of *Candida* DNA is less than 100 copies

CALCULATION OF DNA CONCENTRATION

The calculation of the results is recommended to carry out using the template of results calculation (the software) in Microsoft Excel format:

- enter the data from the *Important Product Information Bulletin* into the table in the ***Important Product Information Bulletin*** section*;*
- complete the columns in the ***Run Information*** section;
- copy the sample names and paste them into the corresponding column ***Sample Name*** of the ***Instrument Data*** section;
- copy and paste the *Ct* values for each of five channels consequently into the corresponding column of the ***Instrument Data*** section;
- click the ***Calculate*** button. The following data will be displayed automatically in the corresponding cells:
  1. calibration status
  2. concentrations of detected *Candida* spp. DNA (GE/ml),
  3. status of the samples,
  4. the results for each sample and its interpretation.

Calculate the DNA concentrations of the detected *Candida* spp. in GE/ml using the formula:

[Number of genome equivalents] per 1 ml = K x [Number of copies] of *Candida* DNA

NOTE: The values of the calibrators' concentrations and coefficient K are specified in the *Important Product Information Bulletin* enclosed to the given lot of the PCR kit and cannot be used for the calculation of results obtained with the use of another lot of the PCR kit.

TROUBLESHOOTING

1. The *Ct* value for the Negative control of extraction (C–) and/or Negative control of amplification (NCA) is determined in the channels for FAM and/or JOE and/or ROX and/or Cy5 fluorophores. The PCR analysis must be repeated for all samples in which the threshold cycle value was determined in the channels for FAM and/or JOE and/or ROX and/or Cy5 fluorophores.
2. The *Ct* values for DNA calibrators (CND1, CND2) are absent or greater than the boundary value specified in the *Important Product Information Bulletin* in channels for FAM and/or JOE and/or ROX and/or Cy5 fluorophores**.** The amplification must be repeated for all samples.
3. The Efficiency coefficient **E** does not fit into the range specified in the *Important Product Information Bulletin* when plotting the calibration curve. It is necessary to check the correctness of selected threshold line level and the correctness of DNA calibrators concentrations according to the *Important Product Information Bulletin* enclosed to the PCR kit. If the calibrators concentration values and threshold line level are correct and the efficiency value is out of required range, the amplification should be repeated for all calibrators and samples.
4. The *Ct* value is absent in the channels for FAM, JOE, ROX,Cy5 fluorophores or the number of *Candida* spp. DNA copies for the test sample is less than 100 and the *Ct* value in the channel for Cy5.5 fluorophore is absent or is greater than the boundary value specified in the *Important Product Information Bulletin*. The analysis should be repeated for this sample starting from the DNA extraction stage.

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
17.06.15 ME	Text	Corrections according to the template
	Amplification and data analysis using LineGene 9660 (BIOER TECHNOLOGY CO., LTD, China) instrument	The chapter was added
15.10.15 ME	Amplification and data analysis using Rotor-Gene 6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany), Amplification and data analysis using LineGene 9660 (BIOER TECHNOLOGY CO., LTD, China) Instrument, Amplification and data analysis using CFX96 (Bio-Rad, USA)	The sample CND1 in the tables “Results for controls” was changed to CND2. The software in Microsoft Excel format is recommended for calculation of concentrations
20.07.18 EM	Amplification and data analysis using LineGene 9660 (BIOER TECHNOLOGY CO., LTD, China) instrument	The chapter was deleted
	Text	All the chapters were corrected according to the Russian Guidelines
31.05.21 MM	Through the text	The symbol  was changed to NOTE:
	Cover page	The phrase “For research use only. Not for diagnostic procedures” was added
13.07.23 EM	Footer	**REF** R-F5-100-FT(RG,CFX)-CE was added
07.08.25 HM	Through the text	Corrections according to the template
	Conducting real-time PCR with the use of Rotor-Gene 3000, Rotor-Gene 6000, and Rotor-Gene Q instruments	The *Calibrate /Gain Optimisation…* value for JOE/Yellow, ROX/Orange, Cy5/Red and Cy5.5/Crimson channels was changed from "4 Fl to 8 Fl" to "5 Fl to 10 Fl" . The note “it is acceptable to set *Calibrate/Gain Optimisation…* value from 4 Fl to 8 Fl for the JOE/Yellow, ROX/Orange, Cy5/Red and Cy5.5/Crimson channels” was added
	Calculation of DNA concentration	The chapter was added
	Troubleshooting	The chapter was added