

GUIDELINES

to **AmpliSens[®] *Leptospira*-FRT PCR kit**

for qualitative detection of 16S RNA of pathogenic *Leptospira* genospecies in the biological and autopsy material from humans and biological material from animals by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

AmpliSens[®]



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INTENDED USE

Guidelines describe the procedure of using **AmpliSens® *Leptospira*-FRT** PCR kit for qualitative detection of 16S RNA of pathogenic *Leptospira* genospecies in the biological material (blood and cerebrospinal fluid), autopsy material (brain, kidney, liver, lung tissue, and mesenterial lymph nodes) and material obtained from died animals (lung, brain, and kidney tissue) and animals suffering from acute leptospirosis (blood) or *Leptospira* persisting in kidneys (urine) by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using the following instruments:

- Rotor-Gene 3000/6000 (Corbett Research, Australia);
- iCycler iQ, 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 6.1 and higher 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.

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 (the rotor wells are numbered, the numbers are used for the further programming of the samples' order in the thermocycler).

NOTE: Well 1 must be filled with any test tube.

3. Set the amplification program:

Table 1

Leptospira cDNA amplification program

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
1	50	30 min	–	1
2	95	15 min	–	1
3	95	20 s	–	10
	65	50 s	–	
	72	20 s	–	
4	95	20 s	–	38
	61	50 s	FAM/Green, JOE/Yellow	
	72	20 s	–	

4. Adjust fluorescence channel sensitivity. Click the **Calibrate/Gain Optimisation... button** in the **New Run Wizard** window. In the opened window:
 - perform the measurement of the fluorescence in the FAM/Green, JOE/Yellow channels (activate the **Calibrate Acquiring/Optimise Acquiring**);
 - perform the calibration in the selected channels before the first detection (tick the **Perform Calibration Before 1st Acquisition/ Perform Optimisation Before 1st Acquisition** option). Click the **Close** button;
 - for signal measurement optimisation for the selected channels set calibration of the FAM/Green channel from **3FI** to **7FI**, the JOE/Yellow channel – from **10FI** to **20FL**.
5. Start the amplification program by activating the **Start run** button. Name the experiment.
6. 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 controls, the type **Negative control** – for the Negative control of extraction, the type **NTC** – for the Negative control of amplification. Set the type **None** for the cells matching with the corresponding empty tubes.

Data analysis

Amplification data analysis in the JOE/Yellow channel (*Leptospira* DNA):

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**.
2. Cancel the automatic choice of the threshold line level **Threshold**.
3. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of main window (**Quantitation analysis**).
4. In the **Calculation** menu (in the right part of the window) indicate the threshold line level **0.04** in the **Threshold** box.
5. Choose the parameter **More settings/Outlier Removal** and set **10 %** for the value of negative samples threshold (**NTC/Threshold**).
6. In the results grid (the **Quantitation Results** window) one will be able to see the **Ct** values.
7. For the Positive Control of Extraction (PCE) – **Positive Control *Leptospira*-rec** – the **Ct** value should be less than the boundary value 26.
8. For the Positive Control of Amplification (C+) – **Positive Control cDNA *Leptospira* (C+*Leptospira*)** – **Ct** value should be less than the boundary value 25.
9. For Negative Control of Extraction (C-) – **Negative Control (C-)** – **Ct** values should be

absent.

10. For Negative Control of Amplification (NCA) – **RNA-eluent** – Ct values should be absent.

11. The samples are positive if the determined Ct value is less than 32. If the Ct value in a sample is greater than this boundary value, then the sample is equivocal. The analysis of this sample should be repeated in two repeats.

Amplification data analysis in the FAM/Green channel (IC):

1. Activate the button **Analysis** in the menu, select the mode of the analysis **Quantitation**, activate the buttons **Cycling A. FAM/Cycling A. Green, Show**.
2. Cancel the automatic choice of the threshold line level **Threshold**.
3. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of main window (**Quantitation analysis**).
4. In the **Calculation** menu (in the right part of the window) indicate the threshold line level **0.03** in the **Threshold** box.
5. Choose the parameter **More settings/Outlier Removal** and set **10 %** for the value of negative samples threshold (**NTC/Threshold**).
6. In the results grid (the **Quantitation Results** window) the Ct values should appear for **Internal Control STI-87-rec (IC)** in each test sample, Positive control of extraction (PCE) and Negative control of extraction (C–).
7. For Negative Control of Amplification (NCA) – **RNA-eluent** and Positive control of amplification (C+) – **Positive Control cDNA Leptospira (C+*Leptospira*)** – Ct values should be absent.
8. The Ct values determined for the test samples should not exceed the value 25 for the blood sediment and cerebrospinal fluid and the value 27 for the tissues homogenates. If the Ct value in a sample exceeds this boundary value, then the result is considered invalid in case of negative result in the JOE/Yellow channel. It is necessary to repeat the analysis of this sample from RNA extraction stage.

Boundary Ct values for control and test samples

Sample	FAM/Green	JOE/Yellow
	Detection of IC	Detection of <i>Leptospira</i>
PCE	Present	< 26
C–	Present	Absent
NCA	Absent	Absent
C+	Absent	< 25
Test samples	<24.5 (Blood sediment and cerebrospinal fluid) <26.5 (Tissues homogenates) <27 (Urine sediment)	<32

Troubleshooting

1. If the Ct value is determined for the Negative Control of Extraction (C–) in the JOE/Yellow channel and/or for the Negative Control of Amplification (NCA) in the FAM/Green and JOE/Yellow channels in the results grid, it indicates contamination of reagents or samples. In such cases, the results of analysis are considered to be irrelevant. Analysis should be repeated and measures to detect and eliminate the source of contamination should be taken.
2. If no signal is detected for the Negative Control of extraction (C–) in the FAM/Green channel and/or for the Positive Control of Extraction (PCE) in the FAM/Green and JOE/Yellow channels, the results of analysis are considered invalid. Analysis of all samples should be repeated starting from the extraction stage.
3. If no signal is detected for Positive Control of Amplification (C+) in the JOE/Yellow channel, analysis results are considered invalid. Analysis of all samples should be repeated starting from the RT-PCR stage.

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

INSTRUMENT

Programming the thermocycler

1. Turn on the instrument and the power supply unit of the optical block of the instrument. Start measurements not earlier than 30 min after turning on the optic part of the instrument.
2. Set the amplification program

Table 3

Leptospira cDNA amplification program

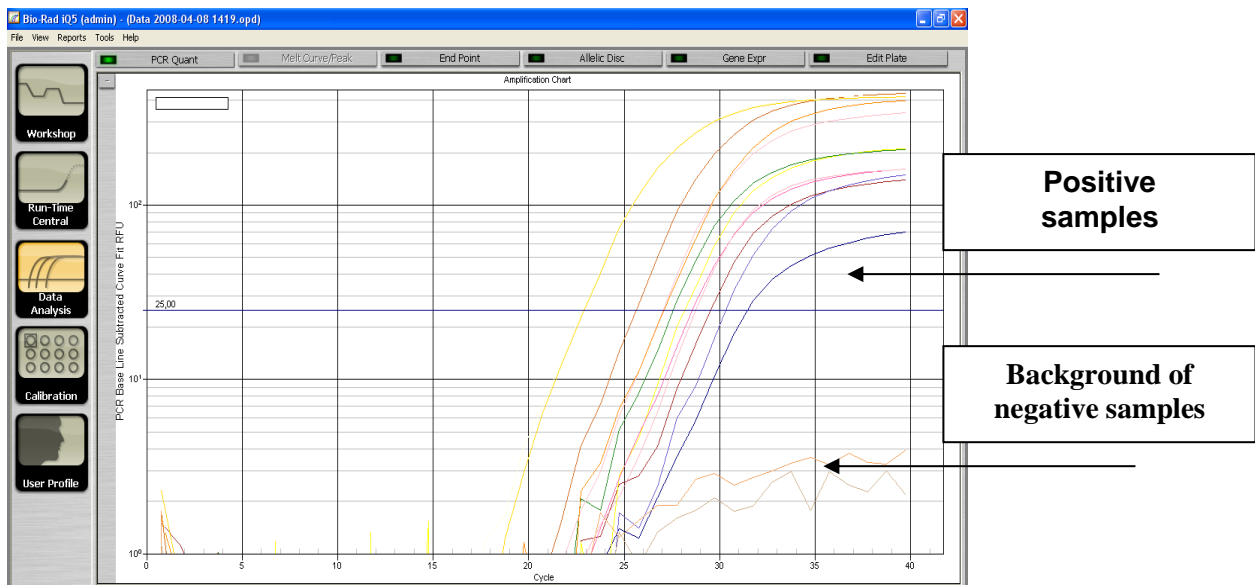
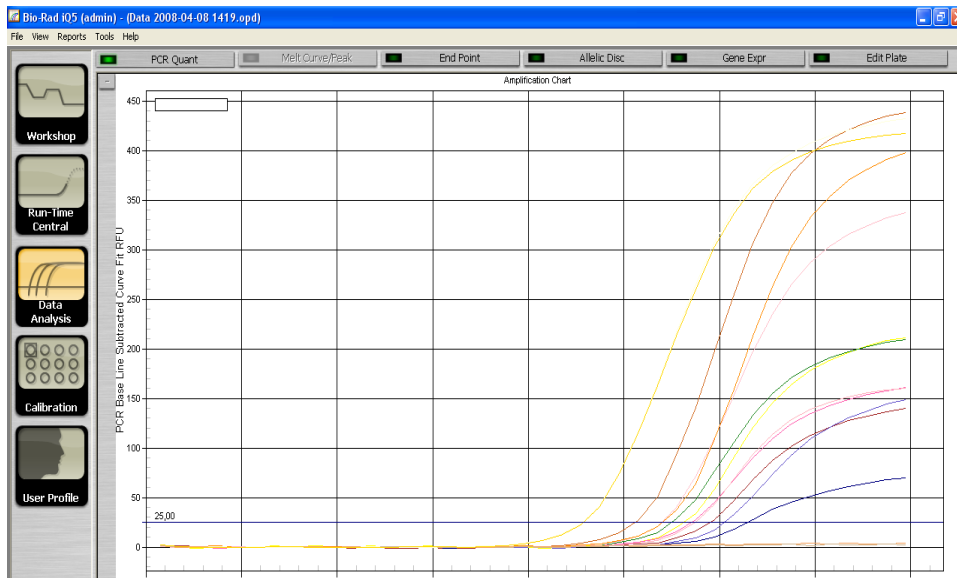
Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
1	50	30 min	–	1
2	95	15 min	–	1
3	95	20 s	–	10
	65	50 s	–	
4	95	20 s	–	40
	61	50 s	FAM, JOE/HEX	
	65	20 s	–	

3. Set the plate setup (set the order of the tubes in the reaction chamber and the detection of fluorescent signal). Select the fluorophores (**Select/add fluorophores** button). Activate the fluorophores for the samples in the created protocol by the **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**. Then save the created protocol by clicking the **Save&Exit Plate Editing** button.
4. After that add the reagents and RNA samples into the tubes, insert the tubes into the instrument.
5. Run the experiment (**Run** button). Select **Run Persistent Plate**. Save the experiment.

Data analysis

Amplification data analysis in the JOE/Yellow channel (*Leptospira* DNA):

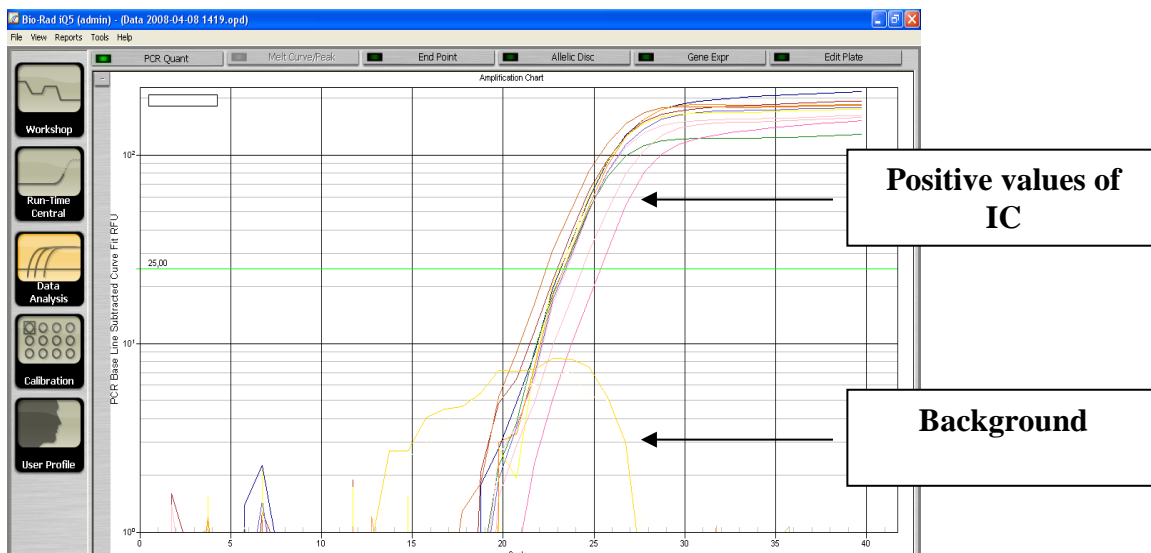
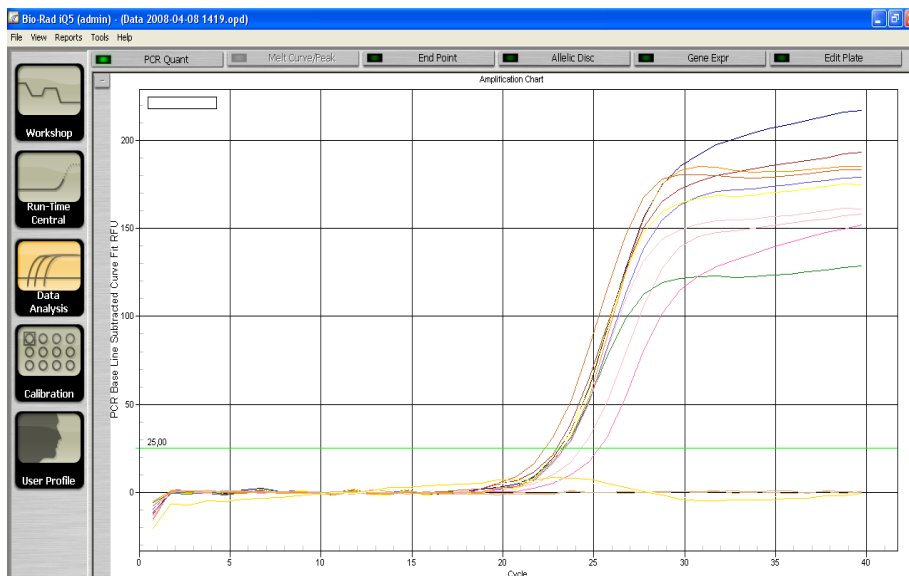
1. Click the **Data Analysis** button.
2. Select the **Base line** in the range 20-25 in the **Crossing Treshold User Defined** window. The threshold line is to cross only with sigmoid curves describing the accumulation of the signal detecting positive samples and controls. The threshold line is not to cross the curves of other shapes. If it happens, it is necessary to raise the threshold line level.



3. For the Positive Control of Extraction (PCE) – **Positive Control *Leptospira*-rec** – the *Ct* value should be less than the boundary value 27.
4. For the Positive Control of Amplification (C+) – **Positive Control cDNA *Leptospira* (C+*Leptospira*)** – *Ct* value should be less than the boundary value 26.
5. For Negative Control of Extraction (C-) – **Negative Control (C-)** – *Ct* values should be absent.
6. For Negative Control of Amplification (NCA) – **RNA-eluent** – *Ct* values should be absent.
7. The samples are positive if the determined *Ct* value is less than 32. If the *Ct* value in a sample is greater than this boundary value, than the sample is equivocal. The analysis of this sample should be repeated in two repeats.

Amplification data analysis in the FAM/Green channel (IC):

1. Select the **Base line** in the range 20-25 in the **Crossing Treshold User Defined** window.



2. The C_t values should appear for **Internal Control STI-87-rec (IC)** in each test sample, Positive control of extraction (PCE) and Negative control of extraction (C-).
3. For Negative Control of Amplification (NCA) – **RNA-eluent** and Positive control of amplification (C+) – **Positive Control cDNA *Leptospira* (C+*Leptospira*)** – C_t values should be absent.
4. The C_t values determined for the test samples should not exceed the value 27 for the blood sediment and cerebrospinal fluid and the value 29 for the tissues homogenates. For urine sediment samples the C_t value can be up to 29 cycle. If the C_t value in a sample exceeds this boundary value, then the result is considered invalid in case of negative result in the JOE/HEX channel. It is necessary to repeat the analysis of this sample from RNA extraction stage.


Boundary Ct values for control and test samples

Sample	FAM/Green	JOE/Yellow
	Detection of IC	Detection of <i>Leptospira</i>
PCE	Present	< 27
C–	Present	Absent
NCA	Absent	Absent
C+	Absent	< 26
Test samples	<27 (Blood sediment and cerebrospinal fluid) <27 (Tissues homogenates) <29 (Urine sediment)	<32

Troubleshooting

1. If the Ct value is determined for the Negative Control of Extraction (C–) in the JOE/HEX channel and/or for the Negative Control of Amplification (NCA) in the FAM and JOE/HEX channels in the results grid, it indicates contamination of reagents or samples. In such cases, the results of analysis are considered to be irrelevant. Analysis should be repeated and measures to detect and eliminate the source of contamination should be taken.
2. If no signal is detected for the Negative Control of extraction (C–) in the FAM channel and/or for the Positive Control of Extraction (PCE) in the FAM and JOE/HEX channels, the results of analysis are considered invalid. Analysis of all samples should be repeated starting from the extraction stage.
3. If no signal is detected for Positive Control of Amplification (C+) in the JOE/HEX channel, analysis results are considered invalid. Analysis of all samples should be repeated starting from the RT-PCR stage.

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
21.10.15 ME	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) instruments	Text was corrected with the template. Amplification program was added. The interpretation of results was added. It was added that for the data analysis the Slope Correct button should be activated
	Amplification and data analysis using iCycler iQ5 (Bio-Rad, USA) instrument	Information about work with the iCycler iQ instrument was deleted. The chapter was rewritten
13.07.17 PM	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) instruments	In the table “Boundary <i>Ct</i> values for control and test samples” the column “Designation in the instruction manual” was deleted and the detection channels were indicated
	Amplification and data analysis using iCycler iQ5 (Bio-Rad, USA) instrument	
31.01.19 EM	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) instruments	The <i>Ct</i> values for the blood sediment, cerebrospinal fluid and tissues homogenates were changed, the specification about urine sediment samples was deleted
	Amplification and data analysis using iCycler iQ5 (Bio-Rad, USA) instrument	The <i>Ct</i> values for the tissues homogenates were changed
01.06.21 EM	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-B49(RG,iQ)-CE was added