AmpliSens® *Leptospira*-FRT PCR kit

IVD

For Professional Use Only

Instruction Manual

KEY TO SYMBOLS USED

REF	Catalogue number	⚠	Caution
LOT	Batch code	Σ	Sufficient for
IVD	*In vitro* diagnostic medical device		Use-by Date
VER	Version		Consult instructions for use
	Temperature limit		Keep away from sunlight
	Manufacturer	NCA	Negative control of amplification
	Date of manufacture	C–	Negative control of extraction
EC REP	Authorized representative in the European Community	C+	Positive control of amplification
PCE	Positive control of extraction	IC	Internal control

1. INTENDED USE

AmpliSens® *Leptospira*-FRT PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of 16S RNA of pathogenic *Leptospira* genospecies in the clinical material (blood and cerebrospinal fluid), autopsy material (brain, kidney, liver, lung tissue, and mesenterial lymph nodes) and biological material (material obtained from died animals (lung, brain, and kidney tissue) and animals suffering from acute leptospirosis (blood) or *Leptospira* persisting in kidneys (urine)) using real-time hybridization-fluorescence detection of amplified products.

NOTE: The results of PCR analysis are taken into account in complex diagnostics of disease.

2. PRINCIPLE OF PCR DETECTION

Detection of 16S RNA of pathogenic *Leptospira genospecies* by the polymerase chain reaction (PCR) is based on the amplification of the pathogen genome specific region using specific primers. In the real-time PCR, the amplified product is detected with the use of fluorescent dyes. These dyes are linked to oligonucleotide probes, which bind specifically to the amplified product during thermocycling. The real-time monitoring of the fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run.

AmpliSens® *Leptospira*-FRT PCR kit is a qualitative test that contains the Internal Control (Internal Control STI-87-rec (IC)). It must be used in the isolation procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition.

AmpliSens® *Leptospira*-FRT PCR kit uses "hot-start", which greatly reduces the frequency of nonspecifically primed reactions. "Hot-start" is guaranteed by separation of nucleotides and Taq-polymerase using chemically modified polymerase (TaqF). Chemically modified polymerase (TaqF) is activated by heating at 95 ºC for 15 min.

The results of amplification are registered in the following fluorescence channels:

Table 1

Channel for fluorophore	FAM	JOE
DNA-target	IC STI-87-rec cDNA	*Leptospira* cDNA
Target gene	Artificially synthesized sequence	16S RNA

3. CONTENT

AmpliSens® *Leptospira*-FRT PCR kit is produced in 1 form:
variant FRT REF R-B49(RG,iQ)-CE.
Variant FRT includes:

Reagent	Description	Volume, ml	Quantity
RT-G-mix-2	colorless clear liquid	0.01	2 tubes
RT-PCR-mix-1-FRT *Leptospira*	colorless clear liquid	0.6	1 tube
RT-PCR-mix-2-FEP/FRT	colorless clear liquid	0.3	1 tube
Polymerase (TaqF)	colorless clear liquid	0.03	1 tube
TM-Revertase (MMlv)	colorless clear liquid	0.015	1 tube
Positive Control cDNA *Leptospira* (C+$_{Leptospira}$)	colorless clear liquid	0.1	1 tube
RNA-eluent	colorless clear liquid	0.07	2 tubes
Negative Control (C–)*	colorless clear liquid	1.6	2 tubes
Positive Control *Leptospira*-rec	colorless clear liquid	0.03	5 tubes
Internal Control STI-87-rec (IC)**	colorless clear liquid	0.12	5 tubes

* must be used in the extraction procedure as Negative Control of Extraction

** add 10 µl of Internal Control during the DNA extraction procedure directly to the sample/lysis mixture (see **RIBO-sorb** REF K2-1-Et-50-CE, **RIBO-zol-C** REF K2-13-50-CE, **RIBO-prep** REF K2-9-Et-50-CE protocols).

Variant FRT PCR kit is intended for 60 reactions, including controls.

4. ADDITIONAL REQUIREMENTS

- RNA extraction kit.
- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).
- Sterile RNase-free pipette tips with aerosol filters (up to 200 µl).
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with a rotor for 2 ml tubes.
- PCR box.
- Real-time instruments (for example, Rotor-Gene 3000/6000 (Corbett Research, Australia); iCycler iQ5 (Bio-Rad, USA); or equivalent).
- Disposable polypropylene PCR tubes (0.2-ml)
- Refrigerator for 2 to 8 °C.
- Deep-freezer at the temperature from minus 24 to minus 16 °C.
- Reservoir for used tips.

5. GENERAL PRECAUTIONS

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use a new tip for every procedure.
- Store all extracted positive material (specimens, controls and amplicons) away from all other reagents and add it to the reaction mix in a distantly separated facility.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable protective gloves and laboratory cloths, and protect eyes while samples and reagents handling. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use the PCR kit if the internal packaging was damaged or its appearance was changed.
- Do not use the PCR kit if the transportation and storage conditions according to the Instruction Manual were not observed.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local regulations.
- Samples should be considered potentially infectious and handled in biological cabinet in compliance with appropriate biosafety practices.
- Clean and disinfect all samples or reagents spills using a disinfectant, such as 0.5 % sodium hypochlorite or another suitable disinfectant.
- Avoid samples and reagents contact with the skin, eyes, and mucous membranes. If these solutions come into contact, rinse the injured area immediately with water and seek medical advice immediately.
- Safety Data Sheets (SDS) are available on request.
- The PCR kit is intended for single use for PCR analysis of specified number of samples (see the section "Content").
- The PCR kit is ready for use in accordance with the Instruction Manual. Use the PCR kit strictly for intended purpose.
- Use of this product should be limited to personnel trained in DNA amplification techniques.
- Workflow in the laboratory must be one-directional, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment and reagents in the area where the previous step was performed.

⚠ Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

6. SAMPLING AND HANDLING

NOTE: Obtaining samples of biological materials for PCR-analysis, transportation and storage is described in manufacturer's handbook [1]. It is recommended that this handbook is read before starting work.

AmpliSens® *Leptospira*-FRT PCR kit is intended for analysis of RNA extracted with RNA extraction kits from the following material.

Human material
- clinical material (blood, cerebrospinal fluid);
- autopsy material (brain, kidney, liver, and lung tissue and mesenterial lymph nodes).

Animal material (biological material)
- urine;
- blood;
- brain, kidney, and lung tissue.

The material can be stored at 2-8 °C for 1 day. The autopsy material can be stored at the temperature not more than minus 16 °C for 1 week, at the temperature not more than minus 68 °C for a long time.

Sampling and pretreatment

6.1. *Blood and cerebrospinal fluid.* Whole blood is taken in the morning after overnight fasting into the tube with 6 % EDTA solution in the ratio 1 : 20. The closed tube with whole peripheral blood should be rotated several times. The tube with blood should be centrifugated at 1,000 g for 10 min to obtain blood plasma (if the blood was stored at 2–8 °C more than 1 hour, it should be mixed carefully by inverting the tube). Transfer 1 ml of plasma into two tubes. Two tubes with 1.0 ml of plasma should be centrifuged at 13,000 rpm for 10 min to concentrate bacterial cells. Then, 900 µl of the supernatant plasma should be removed with a filter tip into the container with disinfectant. The pellet and 100 µl of the supernatant are tested for the presence of *Leptospira* 16S RNA. The second pellet prepared in the same way should be stored at the temperature not more than minus 16 °C for repeated extraction (if any technological procedure is performed incorrectly). The pellet obtained from blood plasma can be stored at the temperature not more than minus 16 °C for 1 week or at the temperature not more than minus 68 °C for a long time.

When cerebrospinal fluid is analyzed, the pellet is obtained by the same procedure by centrifugation at 13,000 rpm for 10 min. The pellet and 100 µl of the supernatant are analyzed.

6.2. *Urine* for analyses is taken into a sterile container. If there is no chance to test material within 24 h after sampling, urine is transferred to a centrifuge tube or an Eppendorf tube. The contents of the tube is mixed with glycerol (~10 % v/v) and frozen. It can be stored at the temperature not more than minus 16 °C for 1 week or at the temperature not more than minus 68 °C for a long time.

If a cooling centrifuge (4 ºC) with a speed of 9,000–10,000 g intended for 30-ml tubes is available, the following sample preparation procedure is used. The sample is centrifuged at 9,000–10,000 g for 10 min, the supernatant is transferred to a container with disinfectant. Leave ~1 ml of the supernatant over the pellet in the tube and resuspend it. Transfer the suspension to a new tube and concentrate it by centrifugation at 13 000,rpm for 10 min. Then, 900 µl of the supernatant is transferred to the container with disinfectant, and the pellet and 100 µl of the supernatant is used for RNA isolation. In case of large quantities of salts and mucus, 100 µl of the supernatant and the upper layer of cells should be carefully taken from the salt pellet and transferred into a new tube for RNA isolation.

If you have no centrifuge for 30-ml tubes and a speed of 9,000–10,000 g, bacteria are concentrated from 1 ml of urine as described above using 1.5-ml tubes and a microcentrifuge for Eppendorf tubes. The remaining urine should be decontaminated in a disinfectant.

6.3. *Animal internal organs and autopsy material* is to be homogenized in sterile porcelain mortars with pestles. Then, 10 % suspension in sterile saline or phosphate buffer is prepared; 30 µl of the suspension is taken for RNA extraction.

7. WORKING CONDITIONS

AmpliSens® *Leptospira*-FRT PCR kit should be used at 18–25 °C.

8. PROTOCOL

8.1. RNA Extraction

It is recommended to use the following nucleic acid extraction kits:
- **RIBO-prep**, REF K2-9-Et-50-CE,
- **RIBO-zol-C**, REF K2-13-50-CE,
- **RIBO-sorb**, REF K2-1-Et-50-CE.

NOTE: Extract the RNA according to the manufacturer's protocol.

NOTE: **If using the RIBO-prep kit** extract the RNA/DNA according to the manufacturer's protocol taking into account next additions and improvements:
- If RNA is extracted from the tissues homogenates. Add **10 µl** of **Internal Control STI-87-rec (IC)** to each tube and then add **300 µl** of **Solution for Lysis**. Label test tubes. Then, add **50 µl** of test suspensions to the tubes with **Solution for Lysis** and **Internal Control STI-87-rec (IC).**
- If RNA is extracted from the cell pellets after *Leptospira* concentration, **300 µl** of **Solution for Lysis** and **10 µl** of **Internal Control STI-87-rec (IC)** should be added directly into the tubes with the pellets.
- Add only **10 µl** of **Internal Control STI-87-rec (IC)** and **300 µl** of **Solution for Lysis** into the tube for the Negative Control of extraction (C–).
- Add **300 µl** of **Solution for Lysis,** and then add **90 µl** of **Negative Control (C–), 10 µl** of **Internal Control STI-87-rec (IC)** and **10 µl** of **Positive Control *Leptospira*-rec** into the tube for the Positive Control of extraction (PCE).
- **40 µl** of **RNA-buffer** is to be used.

NOTE: **If using the RIBO-zol-C and RIBO-sorb kit** extract the RNA/DNA according to the manufacturer's protocol taking into account next additions and improvements:
- Add **300 µl** of **Solution D**, **10 µl** of **Internal Control STI-87-rec (IC)** and **100 µl** of **Negative control (C–)** into the tube for the Negative Control of extraction (C–).
- Add **300 µl** of **Solution D,** and then add **90 µl** of **Negative Control (C–), 10 µl** of **Internal Control STI-87-rec (IC)** and **10 µl** of **Positive Control *Leptospira*-rec** into the tube for the Positive Control of extraction (PCE).
- After the first stage of RNA extraction with **RIBO-zol-C** kit the top phase of obtained samples (**400 µl** when extracting RNA from cerebrospinal fluid, urine pellets and tissues homogenates, **200 µl** – from blood pellets, 450 µl – from C– and PCE samples) should be transferred into new 1.5-ml tubes with **400 µl** of **Lysis Solution (**from RIBO-sorb kit).
- **40 µl** of **RNA-buffer** is to be used.

8.2. Preparing reverse transcription and PCR

The total reaction volume is **25 µl**, the volume of DNA sample is **10 µl**.

NOTE: Only RNase-free, DNase-free disposable plastic consumables must be used when working with RNA.

8.2.1 Preparing tubes for reverse transcription and PCR

1 Prepare the required number of tubes for amplification of cDNA obtained from clinical and control samples. The type of tubes depends on the PCR instrument used for analysis. For carrying out N reactions with 2 controls, N+2 tubes are required.
2 Prepare the reaction mixture, calculating per one reaction:
- **10 µl** of **RT-PCR-mix-1-FRT *Leptospira***
- **5 µl** of **RT-PCR-mix-2-FEP/FRT**
- **0.5 µl** of **polymerase (TaqF)**
- **0.25 µl** of **TM-Revertase (MMlv)**
- **0.25 µl** of **RT-G-mix-2**

3 Transfer **15 µl** of the prepared mixture to each tube. Discard the unused mixture.
4 Using filter tips add **10 µl** of **RNA** samples obtained at the RNA extraction stage into prepared tubes.
5 Carry out the control amplification reactions:

NCA – Add **10 µl** of **RNA-eluent** to the tube labeled NCA (Negative Control of Amplification).

C+ – Add **10 µl** of **Positive Control cDNA *Leptospira* (C+$_{Leptospira}$)** to the tube labeled C+ (Positive Control of Amplification).

C– – Add **10 µl** of **the sample extracted from the Negative Control of Extraction sample** to the tube labeled C– (Negative control of Extraction).

PCE – Add **10 µl** of **the sample extracted from the Positive control of Extraction sample** to the tube labeled PCE (Positive control of Extraction).

NOTE: Amplification is to be carried immediately after mixing the reaction mixture, RNA-sample and controls. The time period between addition of RNA-samples into the reaction mixture and amplification starting is to be not more than 10-15 min.

8.2.2. Amplification

1 Create a temperature profile on your instrument as follows:

Table 2

Amplification program for *Leptospira* cDNA

	Rotor-type instruments[1]			Plate-type instruments[2]		
Step	**Temperature, °C**	**Time**	**Cycles**	**Temperature, °C**	**Time**	**Cycles**
1	**50**	**30 min**	**1**	**50**	**30 min**	**1**
2	**95**	**15 min**	**1**	**95**	**15 min**	**1**
3	**95**	**20 s**	**10**	**95**	**20 s**	**10**
	65	**50 s**		**65**	**50 s**	
	72	**20 s**				
4	**95**	**20 s**	**38**	**95**	**20 s**	**40**
	61	**50 s Fluorescence acquiring**		**61**	**50 s Fluorescence acquiring**	
	72	**20 s**		**65**	**20 s**	

Fluorescent signal is detected in the channels for the FAM and JOE fluorophores.
2 Adjust the fluorescence channel sensitivity according to the Guidelines [2].
3 Insert tubes into the reaction module of the device.
4 Run the amplification program with fluorescence detection.
5 Analyze results after the amplification program is completed

9. DATA ANALYSIS

Analysis of results is performed by the software of the real-time PCR instrument used by measuring fluorescence signal accumulation in two channels:
- The signal of the IC cDNA amplification product is detected in the channel for the FAM fluorophore.
- The signal of the *Leptospira* cDNA amplification product is detected in the channel for the JOE fluorophore.

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.

Principle of interpretation is the following:
- *Leptospira* cDNA is **detected** if the *Ct* value determined in the results grid in the channel for the JOE fluorophore is less than the boundary *Ct* value specified in the Guidelines.
- *Leptospira* cDNA is **not detected** in a sample if the *Ct* value is not determined (absent) in the channel for the JOE fluorophore, whereas the *Ct* value determined in the channel for the FAM fluorophore is less than the boundary *Ct* value specified in the Guidelines.
- The result is **invalid** if the *Ct* value is not determined (absent) in the channel for the JOE fluorophores whereas the *Ct* value in the channel for the FAM fluorophore is greater than the specified boundary *Ct* value. In such cases, the PCR analysis of this sample should be repeated starting from the RNA extraction stage.
- The result is **equivocal** if the *Ct* value determined in the channel for the JOE fluorophore is greater than the boundary *Ct* value specified in the Guidelines, whereas the *Ct* value determined in the channel for the FAM fluorophore is less than the boundary *Ct* value specified in the Guidelines. In such cases, the PCR analysis of this sample should be repeated two times starting from the RNA extraction stage.

NOTE: Boundary *Ct* values are specified in the Guidelines [2]

The result of the analysis is considered reliable only if the results obtained for the Positive and Negative Controls of amplification and extraction are correct (see Table 3).

Table 3

Results for controls

Control	Stage for control	*Ct* value in the channel for the fluorophore	
		FAM	JOE
PCE	DNA extraction	Present	< boundary value
C–	DNA extraction	Present	Absent
NCA	PCR	Absent	Absent
C+	PCR	Absent	< boundary value

[1] For example, Rotor-Gene 3000/Rotor-Gene 6000 (Corbett Research, Australia).
[2] For example, iCycler iQ5 (Bio-Rad, USA).

10. TROUBLESHOOTING

Results of analysis are not being registered in the following cases:

1. If the *Ct* value is determined for the Negative Control of extraction (C–) in the channel for the JOE fluorophore and/or for the Negative Control of amplification (NCA) in the channels for the FAM and JOE fluorophores 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 channel for the FAM fluorophore and/or for the Positive Control of extraction (PCE) in the channels for the FAM and JOE fluorophores, 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 channel for the JOE fluorophore, the results of analysis are considered invalid. Analysis of all samples should be repeated starting from the RT-PCR stage..

If you have any further questions or if encounter problems, please contact our Authorized representative in the European Community.

11. TRANSPORTATION

AmpliSens® *Leptospira*-FRT PCR kit should be transported at 2–8 ºC for no longer than 5 days.

12. STABILITY AND STORAGE

All components of the **AmpliSens® *Leptospira*-FRT** PCR kit are to be stored at 2–8 ºC when not in use (except for RT-G-mix-2, RT-PCR-mix-1-FRT *Leptospira*, RT-PCR-mix-2-FEP/FRT, Polymerase (TaqF), and TM-Revertase (MMlv)). All components of the **AmpliSens® *Leptospira*-FRT** PCR kit are stable until the expiry date stated on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

NOTE: RT-G-mix-2, RT-PCR-mix-1-FRT *Leptospira*, RT-PCR-mix-2-FEP/FRT, Polymerase (TaqF), and TM-Revertase (MMlv) are to be stored at the temperature from minus 24 to minus 16 °C.

NOTE: RT-PCR-mix-1-FRT *Leptospira* is to be stored away from light

13. SPECIFICATIONS

13.1. Sensitivity

Analytical Sensitivity of **AmpliSens® *Leptospira*-FRT** PCR kit is not less than $5x10^3$ copies per 1 ml of sample (copies/ml).

NOTE: The claimed analytical features of **AmpliSens® *Leptospira*-FRT** PCR kit are guaranteed only when additional reagents kits **RIBO-sorb** and **RIBO-zol-C** or **RIBO-prep** (manufactured by Federal Budget Institute of Science "Central Research Institute for Epidemiology") are used.

13.2. Specificity

The analytical specificity of **AmpliSens® *Leptospira*-FRT** PCR kit is ensured by the selection of specific primers and probes as well as stringent reaction conditions. The primers and probes have been checked for possible homologies to all sequences published in gene banks by sequence comparison analysis.

The clinical specificity of **AmpliSens® *Leptospira*-FRT** PCR kit was confirmed in laboratory clinical trials.

14. REFERENCES

1. Handbook "Sampling, Transportation, and Storage of Clinical Material for PCR diagnostics", developed by Federal Budget Institute of Science "Central Research Institute for Epidemiology" of Federal Service for Surveillance on Consumers' Rights Protection and Human Well-Being.
2. Guidelines to the **AmpliSens® *Leptospira*-FRT** PCR kit for qualitative detection of 16S RNA of pathogenic *Leptospira* genospecies in the clinical material, autopsy material and biological material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection developed by Federal Budget Institute of Science "Central Research Institute for Epidemiology".

15. QUALITY CONTROL

In compliance with Federal Budget Institute of Science "Central Research Institute for Epidemiology" ISO 13485-Certified Quality Management System, each lot of the **AmpliSens® *Leptospira*-FRT** PCR kit has been tested against predetermined specifications to ensure consistent product quality.

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
13.12.10	Cover page	The phrase "For Professional Use Only" was added
	Intended use	The phrase "The results of PCR analysis are taken into account in complex diagnostics of disease" was added
	Content	New sections "Working Conditions" and "Transportation" were added
		The "Explanation of Symbols" section was renamed to "Key to Symbols Used"
	Stability and Storage	The information about the shelf life of reagents before and after the first use was added
		Information that RT-PCR-mix-1-FRT *Leptospira* is kept away from light was added
	Key to Symbols Used	The explanation of symbols was corrected
	Footer	Reference number was changed from R-B49(RG)-CE to R-B49(RG,iQ)-CE
	Sampling and Handling	Duration of the urine sample storage was changed
		Information about storage and disposal of urine samples was added
01.07.11 RT	Cover page, text	The name of Institute was changed to Federal Budget Institute of Science "Central Research Institute for Epidemiology"
16.10.15 ChA	3. Content	Quantity of Negative Control (C-) tubes was changed from 1 to 2
	8.1. RNA Isolation	For RIBO-prep the procedure of positive control of extraction preparation was changed: 90 µl of Negative Control (C-) is to be added additionally
17.01.17 ME	Text	Corrections according to the template
	1. Intended use	Types of biological material was specified
	8.1. RNA extraction	The sections were rewritten
	8.2.2. Amplification	
	9. Data analysis	
	14. References	The reference to Guidelines was added
23.04.20 KK	Through the text	The text formatting was changed
	2. Principle of PCR detection	The table with targets was added.
	Footer	The phrase "Not for use in the Russian Federation" was added
16.03.21 MA	—	The name, address and contact information for Authorized representative in the European Community was changed

AmpliSens®

EC REP

Ecoli Dx, s.r.o., Purkyňova 74/2
110 00 Praha 1, Czech Republic
Tel.: +420 325 209 912
Cell: +420 739 802 523

Federal Budget Institute of Science "Central Research Institute for Epidemiology"
3A Novogireevskaya Street
Moscow 111123 Russia