AmpliSens® *TBEV, B.burgdorferi sl, A.phagocytophilum, E.chaffeensis / E.muris*-FRT PCR kit

RUO

For Professional Use Only

Instruction Manual

KEY TO SYMBOLS USED

REF	Catalogue number		Caution
LOT	Batch code	Σ	Contains sufficient for <n> tests
RUO	Research Use Only		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
IC	Internal control	**C+** *TBEV, B.b. sl, A.ph., E.ch. / E.m. /* STI	Positive control of amplification

1. INTENDED USE

AmpliSens® *TBEV, B.burgdorferi sl, A.phagocytophilum, E.chaffeensis / E.muris*-FRT PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of RNA of *Tick-borne encephalitis virus* (*TBEV), Borrelia burgdorferi sl* (Ixodes tick-borne borreliosis (ITB) pathogen), *Ehrlichia chaffeensis* and *Ehrlichia muris* (human monocytic ehrlichiosis (HME) pathogens) and DNA of *Anaplasma phagocytophilum* (human granulocytic anaplasmosis (HGA) pathogen) in the biological material (ticks, whole venous blood, cerebrospinal fluid, and tissue material) using real-time hybridization-fluorescence detection of amplified products. The material for PCR is RNA/DNA samples extracted from biological material and undergone reverse transcription (for RNA).

NOTE: For research use only. Not for diagnostic procedures

2. PRINCIPLE OF PCR DETECTION

Principle of testing is based on the RNA/DNA extraction from the samples of test material with the exogenous internal control sample (Internal Control STI-87-rec (IC)), RNA reverse transcription and amplification of DNA/cDNA fragments of the detected microorganisms and cDNA of the internal control with hybridization-fluorescence detection. Exogenous internal control (Internal Control-FL (IC)) allows to control all PCR-analysis stages of each individual sample and to identify possible reaction inhibition.

RNA reverse transcription with the Revertase (MMlv) enzyme and amplification of DNA/cDNA fragments with the use of specific primers and Taq-polymerase enzyme are performed with the RNA/DNA samples obtained at the extraction stage.

In real-time PCR, the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes, which bind specifically to the amplified product during thermocycling. The real-time monitoring of fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run.

AmpliSens® *TBEV, B.burgdorferi sl, A.phagocytophilum, E.chaffeensis / E.muris*-FRT PCR kit uses "hot-start", which greatly reduces the frequency of nonspecifically primed reactions. "Hot-start" is guaranteed by using a chemically modified polymerase (TaqF). Chemically modified polymerase (TaqF) is activated by heating at 95 °C for 15 min.

DNA/cDNA detection of the microorganisms for a single sample is performed in two tubes, with 3 pathogens differentiated in one tube and one pathogen and Internal Control STI-87-rec in the other.

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

Table 1

Channel for fluorophore	FAM	JOE	ROX
PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E. m.*			
DNA/cDNA-target	*TBEV* cDNA	*A.phagocytophilum* DNA	*E.chaffeensis/ E.muris* cDNA
Target gene	C gene	msp2 gene	16S RNA
PCR-mix-1-FRT *B.b. sl* / IC			
DNA/cDNA-target	Internal Control STI-87-rec (IC) cDNA	*B.burgdorferi sl* cDNA	—
Target gene	Artificially synthesized sequence	16S RNA	—

3. CONTENT

AmpliSens® *TBEV, B.burgdorferi sl, A.phagocytophilum, E.chaffeensis / E.muris*-FRT PCR kit is produced in 2 forms:

variant FRT-100 F, REF R-V59(RG,iQ,Mx,Dt)-CE;

variant FRT-100 F in bulk[1], REF R-V59(RG,iQ,Mx,Dt)-CE-B.

Variant FRT-100 F includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E. m.*	clear liquid from colorless to light lilac colour	0.6	2 tubes
PCR-mix-1-FRT *B.b. sl* / IC	clear liquid from colorless to light lilac colour	0.6	2 tubes
PCR-buffer-C	colorless clear liquid	0.6	2 tubes
Polymerase (TaqF)	colorless clear liquid	0.06	2 tubes
Positive Control cDNA *TBEV, B.b. sl, A.ph., E.ch. / E.m.* / STI (C+ *TBEV, B.b. sl, A.ph., E.ch. / E.m. /* STI**)**	colorless clear liquid	0.2	2 tubes
TE-buffer	colorless clear liquid	0.2	2 tubes
Internal Control STI-87-rec (IC)*	colorless clear liquid	0.6	2 tubes

* add **10 µl** of **Internal Control STI-87-rec (IC)** during the RNA/DNA extraction directly to the sample/lysis mixture (see **RIBO-prep** protocol).

Variant FRT-100 F is intended for 120 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

For sampling and pretreatment

- 0.9 % of sodium chloride (sterile saline solution) or phosphate buffered saline (PBS) (137 mM sodium chloride; 2,7 mM potassium chloride; 10 mM sodium monophosphate; 2 mM potassium diphosphate; pH=7,5±0,2).
- 96 % ethanol for pretreatment of oil treated ticks,
- Glycerin for the storage of pretreated ticks.
- Vacuum tubes for sampling, storage and transportation of blood samples.
- Sterile bilateral needles for vacuum tubes intended for venous blood collection.
- Sterile plastic container (50-60 ml) for storage and transportation of biological samples.
- Disposable tightly closed polypropylene 1.5- and 2.0-ml tubes for sampling and pretreatment.
- Sterile pipette tips with aerosol filters (up to 100; 200; 1,000 µl).
- Tube racks.
- Sterile tools (individual for each sample) for homogenization (porcelain mortar and pestle) or homogenizer for pretreatment of tissue material and ticks.
- Desktop centrifuge with rotor for 2-ml reaction tubes.
- Pipettes (adjustable).
- Refrigerator for 2–8 °C.
- Deep-freezer at the temperature from minus 24 to minus 16 °C.
- Reservoir throw off and inactivate the material.
- Disposable powder-free gloves and laboratory coat.

For RNA/DNA extraction, reverse transcription and amplification

- RNA/DNA extraction kit or Automated station for RNA/DNA extraction based on magnetic beads with MAGNO-sorb Nucleic Acid Extraction kit
- Set of consumables for the used automated station according to the manufacturer's recommendations.
- Reverse transcription kit.
- Sterile pipette tips with aerosol filters (up to 100 and 200 µl).
- Tube racks.
- Vortex mixer.
- PCR box.
- Real-time instruments (for example, Rotor-Gene 6000 (Corbett Research, Australia); Rotor-Gene Q (QIAGEN, Germany); CFX 96 (Bio-Rad, USA)).
- Disposable polypropylene PCR tubes (0.1- or 0.2-ml):
  a) screwed or tightly closed 1.5-ml tubes for reaction mixture preparation.
  b) thin-walled 0.2-ml PCR tubes with optical transparent domed or flat caps or strips of eight 0.2-ml tubes with optical transparent caps if a plate-type instrument is used;
  c) thin-walled 0.2-ml PCR tubes with flat caps or strips of four 0.1-ml Rotor-Gene PCR tubes if a rotor-type instrument is used.
- Pipettes (adjustable).
- Refrigerator for 2–8 °C.
- Deep-freezer at the temperature from minus 24 to minus 16 °C.
- Reservoir for used tips.
- Disposable powder-free gloves and laboratory coat.

[1] In bulk form contains unlabeled tubes. Tubes with identical reagent are packed in one bag with label.

5. GENERAL PRECAUTIONS

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol filters 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 inhalation of vapors, samples and reagents contact with the skin, eyes, and mucous membranes. Harmful if swallowed. If these solutions come into contact, rinse the injured area immediately with water and seek medical advice immediately.
- While observing the conditions of transportation, operation and storage, there are no risks of explosion and ignition.
- 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 are described in the manufacturer’s handbook [1]. It is recommended that this handbook is read before starting work.

AmpliSens® *TBEV, B.burgdorferi sl, A.phagocytophilum, E.chaffeensis / E.muris*-FRT PCR kit is intended to analyze RNA/DNA extracted with RNA/DNA extraction kits from the biological material (ticks, whole venous blood, cerebrospinal fluid, and autopsy material):

Sampling

Whole venous blood

To obtain a bacterial pellet of blood, whole blood should be taken on an empty stomach or in 3 hours after a meal by a disposable 0.8-1.1 mm diameter needle into a tube (special vacuum system) with 6% EDTA solution (final concentration after blood collection is 0.3%) or 3.2% sodium citrate solution as an anticoagulant. After blood collection, the tube should be gently turned upside down several times to ensure that the blood in the tube is thoroughly mixed with the anticoagulant. (Otherwise, the blood will clot and RNA/DNA extraction will not be possible!). Place the tube in a rack after mixing.

The blood samples can be stored until the leukocytes are obtained and prepared for RNA/DNA extraction:

- at the temperature from 20 to 25 °C - for 2 hours;
- at the temperature from 2 to 8 °C - for 12 hours.

Freezing of whole blood samples is not allowed!

Prepare the samples no later than the specified time.

Tissue material

The material is taken from the area of suspected location of the infectious agent, from the lesional tissue or from the area surrounding the lesional area, using a sterile tool (e.g. tweezers) into a sterile 50-ml plastic container with a tightly closing cap or a 2.0-ml tube. The tube is tightly closed.

Tissue samples can be stored before pretreatment:

- at room temperature - for 6 hours;
- at the temperature from 2 to 8 °C - for 3 days;
- at the temperature from minus 24 to minus 16 °C - for 1 week;
- at the temperature not more than minus 68 °C - for a long time.

Only one freeze-thawing cycle is acceptable.

Cerebrospinal fluid is collected in an amount no less than 1 ml by disposable needles into disposable 2.0-ml tubes.

The cerebrospinal fluid samples can be stored before the PCR analysis:

- at the temperature from 2 to 8 °C - for 1 day;
- at the temperature from minus 24 to minus 16 °C - within 1 week;
- at the temperature not more than minus 68 °C - for a long time.

Only one freeze-thawing cycle is acceptable.

Ticks

The collected material is sorted in the laboratory by species, sex, location and date of collection and placed in dry sterile 2.0-ml tubes. The number of ticks in one pool should not exceed 10.

The material can be stored after sorting and samples formation:

- at the temperature from minus 24 to minus 16 °C - for 1 month;
- at the temperature not more than minus 68 °C or in a Dewar vessel with liquid nitrogen - for a long time.

Only one freeze-thawing cycle is acceptable.

The above-mentioned material can be transported at 2-8 °C for 1 day.

Pretreatment

Whole venous blood

To obtain a bacterial pellet of blood, pre-preparation of blood samples is required.

Using a filter tip transfer 1.5 ml of blood with EDTA solution into a sterile disposable 2.0-ml tube. Centrifuge at 40 g (e.g., 800 rpm for a MiniSpin Eppendorf microcentrifuge) for 10 min. Using a new one filter tip transfer 500-600 µl of supernatant (plasma with leucocytes) into sterile disposable 1.5-ml tube (do not take the pellet with erythrocytes). Centrifuge at 10,000 g (for example, 12,000 rpm for the MiniSpin Eppendorf microcentrifuge) for 10 min. Use the pellet and 200 µl of supernatant for the RNA/DNA extraction.

The bacterial pellet of blood can be stored before the PCR analysis:

- at the temperature from minus 24 to minus 16 °C - for 1 week;
- at the temperature not more than minus 68 °C - for a long time.

Tissue material is to be pretreated.

For RNA/DNA extraction take 30-50 mg (µl) of the material and homogenize it by grinding using pre-cooled sterile porcelain mortars and pestle or using a homogenizer. Prepare 10% suspension using grinded tissue and precooled 0.9 % sodium chloride solution (sterile physiological solution) or phosphate buffer solution (PBS). For this purpose, add 9 volumes of saline solution or phosphate buffer per 1 volume of grinded tissue. Use 50 µl of the obtained suspension for RNA/DNA extraction.

The pretreated tissue material samples can be stored before the PCR analysis:

- at the temperature from minus 24 to minus 16 °C - for 1 week;
- at the temperature not more than minus 68 °C - for a long time.

Cerebrospinal fluid is to be pretreated.

Centrifuge 1-1.5 ml of cerebrospinal fluid at 10,000 g (e.g. 12,000 rpm for an Eppendorf microcentrifuge) for 10 min. Discard the supernatant into a waste disposal container. Use the cells pellet in 200 µl of supernatant for RNA/DNA extraction.

The cerebrospinal fluid samples can be stored before the PCR analysis:

- at the temperature from minus 24 to minus 16 °C - for 1 week;
- at the temperature not more than minus 68 °C - for a long time.

Freeze-thawing of the material is not allowed.

Tick suspension

Ticks are to be pretreated. Tick pools of no more than 10 specimens or a single tick (preferably for the Dermacentor genus) can be used for analysis. Place ticks into 1.5-ml Eppendorf tubes, add 500 µl of 96 % ethanol, and vortex. then remove liquid using a vacuum aspirator. Add 500 µl of 0.9% NaCl or phosphate buffer, vortex, and centrifuge to remove drops from the inner surface of the tubes caps. Remove liquid with a vacuum aspirator.

Use a sterile porcelain mortar and a pestle to prepare tick suspension. Homogenize ticks in 300 µl (a single *Ixodes* tick), 500 µl (a single *Dermacentor* tick), or 1 ml (tick pool) of 0.9% NaCl or phosphate buffer then centrifuge at 5,000 rpm for 2 min. Take 100 µl of the supernatant for RNA/DNA extraction from *Ixodes* ticks or 50 µl of the supernatant for RNA/DNA extraction from *Dermacentor* ticks.

Add glycerol (10% by volume) to the tube with the remained suspension, stir, and freeze at the temperature from minus 24 to minus 16 °C for further use.

The pretreated ticks can be stored before the PCR analysis:

- at the temperature from minus 24 to minus 16 °C - for 1 week;
- at the temperature not more than minus 68 °C or in the Dewar flask with liquid nitrogen - for a long time.

Only single freezing-thawing of the material is allowed.

Interfering substances and limitations of using test material samples

In order to control the RNA/DNA extraction efficiency and possible reaction inhibition the Internal Control (Internal Control STI-87-rec (IC)) is used in the PCR kit. The Internal Control is added in each biological sample at the extraction stage. The presence of internal control signal after the amplification testifies the effectiveness of nucleic acid extraction and the absence of PCR inhibitors.

Limitations of using test material samples

The samples that are inapplicable for analysis are the whole blood samples, collected in the tubes with heparin as anticoagulant.

Potential interfering substances

Endogenous and exogenous substances that may be present in the biological material (whole blood) used for the study were selected to assess potential interference.

Samples of whole venous blood without adding and with the addition of endogenous and exogenous potential interfering substances were tested. Concentration of every potential interfering substance in a sample is presented in Table. 2

Model samples with added quality control sample (QCS) containing *Tick-borne encephalitis virus* (*TBEV), Borrelia burgdorferi sl*, *Ehrlichia chaffeensis* and *Ehrlichia muris* RNA and DNA of *Anaplasma phagocytophilum* at concentration $5x10^4$ copies/ml were tested.

Table 2

Type of potential interferent	Potential interferent	Tested concentration in a sample	Interference presence
Endogenous substances	Hemoglobin	250 g/l (upper limit of normal - 170 g/l)	Not detected
	Total bilirubin	210 µmol/l (upper limit of normal - 21 µmol/l)	
	Total cholesterol	7.8 mmol/l (upper limit of normal - 7.8 mmol/l)	
	Tryglicerides	37.0 mmol/l (upper limit of normal - 3.7 mmol/l)	
Exogenous substances	Lithium heparin	from 12 to 30 IU/ml	Detected
	Potassium EDTA	from 1.2 to 2.0 mg/ml	Not detected

7. WORKING CONDITIONS

AmpliSens® *TBEV, B.burgdorferi sl, A.phagocytophilum, E.chaffeensis / E.muris*-FRT PCR kit should be used at the temperature from 20 to 28 °C and relative humidity from 15 to 75 %.

8. PROTOCOL

8.1. RNA/DNA extraction

It is recommended to use the following nucleic acid extraction kits:

- **RIBO-prep** – for RNA/DNA extraction from ticks, whole venous blood, cerebrospinal fluid and tissue material,
- **MAGNO-sorb** (can be used in combination with "open-type" automated nucleic acid extraction stations) – for RNA/DNA extraction from ticks and tissue material.

The RNA/DNA extraction of each test sample is carried out in the presence of **Internal Control STI-87-rec (IC)**.

Extract the RNA/DNA according to the manufacturer’s protocol.

The volumes of reagents and samples when the RNA/DNA is extracted by the RIBO-prep reagent kit:

Add **10 µl** of **Internal Control STI-87-rec (IC)** to each tube.

The volume of the test sample:

- for analysis of blood and cerebrospinal liquor - the pellet and **200 µl** of supernatant,
- for analysis of tissue material - **50 µl** of suspension,
- for analysis of ticks - **100 µl** of suspension of *Ixodes* ticks or **50 µl** of suspension of *Dermacentor* ticks.

Do not add **Negative Control (C–)** to the tube labeled C– (Negative Control of Extraction).

The volume of elution is **50 µl** (in case of RNA/DNA extraction from the homogenate of tissues or tick suspension) or **100 µl** (in case of RNA/DNA extraction from the concentrated cell pellet of CSF or white cell pellet).

The volumes of reagents and samples when the RNA/DNA is extracted by the MAGNO-sorb reagent kit:

Add **10 µl** of **Internal Control STI-87-rec (IC)** to each tube.

The volume of the test sample:

- for analysis of tissue material - **50 µl** of suspension,
- for analysis of ticks - **100 µl** of tick suspension.

Do not add **Negative Control (C–)** to the tube labeled C– (Negative Control of Extraction).

The volume of elution is **100 µl**.

NOTE: It is recommended to carry out the reverse transcription just after obtaining the RNA/DNA samples. It is allowed to store the RNA/DNA samples at the temperature from 2 to 8 °C for 30 min, at the temperature from minus 24 to minus 16 °C for 1 week and at the temperature not more than minus 68 °C for 1 year. Only one freeze-thawing cycle is required.

8.2. Reverse transcription

It is recommended to use the following kit for the complementary DNA (cDNA) synthesis from the RNA:

- **REVERTA-L**.

NOTE: Carry out the reverse transcription according to the manufacturer's protocol.

8.3. Preparing PCR

8.3.1 Preparing tubes for PCR

The total reaction volume is **25 µl**, the volume of the **cDNA/DNA** is **10 µl**.
The type of tubes depends on the PCR instrument used for analysis. Use disposable filter tips for adding reagents, DNA and control samples into tubes.

NOTE: All obtained cDNA/DNA samples should be examined in two tubes – one with **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.*** and the other one with **PCR-mix-1-FRT *B.b. sl* / IC.**

Calculate the required quantity of each reagent for reaction mixture preparation. For one reaction:

- **10 µl of PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.*** or **PCR-mix-1-FRT *B.b. sl* / IC,**
- **5 µl of PCR-buffer-C,**
- **0.5 µl of polymerase (TaqF).**

Prepare the reaction mixture for the total number of test and control samples plus one extra reaction. See numbers of control samples in item 7.

NOTE: Prepare the reaction mixture just before use.

1. Thaw the tubes with **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.*** and **PCR-mix-1-FRT *B.b. sl* / IC**. Thoroughly vortex the tubes with **polymerase (TaqF) and PCR-buffer-C** and sediment the drops by vortex.
2. In two new tubes prepare two reaction mixtures. Mix the required quantities of **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.*, polymerase (TaqF),** and **PCR-buffer-C** in one tube and **PCR-mix-1-FRT *B.b. sl* / IC, polymerase (TaqF),** and **PCR-buffer-C** in the other tube. Sediment the drops by vortex.
3. Take the required (two-fold) number of the tubes or strips taking into account the number of test samples and control samples.
4. Transfer **15 µl** of one of the prepared mixtures to each tube of the first tubes' row, and 15 ml of another reaction mixture to each tube of the second row.
5. Add **10 µl** of **cDNA/DNA samples** to the prepared tubes with two reaction mixtures.
6. Carry out the control amplification reactions:

NCA – Add **10 µl** of **TE-buffer** to 2 tubes with different reaction mixtures labeled NCA (Negative Control of Amplification).

C+$_{TBEV,\ B.b.\ sl,\ A.ph.,\ E.ch.\ /\ E.m.\ /\ STI}$ – Add **10 µl** of **Positive Control cDNA *TBEV, B.b. sl, A.ph., E.ch. / E.m.* / STI** to 2 tubes with different reaction mixtures labeled C+$_{TBEV,\ B.b.\ sl,\ A.ph.,\ E.ch.\ /\ E.m.\ /\ STI}$ (Positive Control of Amplification).

C– – Add **10 µl of cDNA obtained by extraction and reverse transcription of the Negative control of Extraction (containing the Internal Control STI-87-rec (IC) reagent only)** to 2 tubes with different reaction mixtures labeled C– (Negative control of Extraction).

NOTE: Perform the amplification reaction immediately after cDNA samples and controls are added to the reaction mixture.

8.3.2. Amplification

1. Create a temperature profile on your instrument as follows:

Table 3

Amplification program

	Rotor-type Instruments[2]			Plate-type Instruments[3]		
Step	**Temperature, °C**	**Time**	**Cycles**	**Temperature, °C**	**Time**	**Cycles**
1	**95**	**15 min**	**1**	**95**	**15 min**	**1**
2	**95**	**10 s**	**5**	**95**	**10 s**	**5**
	60	**30 s**		**60**	**35 s**	
	72	**15 s**		**72**	**15 s**	
3	**95**	**10 s**	**40**	**95**	**10 s**	**40**
	56	**30 s fluorescence detection**		**56**	**35 s fluorescence detection**	
	72	**15 s**		**72**	**15 s**	

Fluorescent signal detection is assigned in the channels for the FAM, JOE, and ROX fluorophores for the tubes with the **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.*** and in the channels for the FAM and JOE fluorophores for the tubes with the **PCR-mix-1-FRT *B.b. sl* / IC**.

2. Adjust the fluorescence channel sensitivity according to the *Important Product Information Bulletin.*
3. Insert the tubes into the reaction module of the instrument. If amplification is carried out simultaneously for both PCR-mixes-1, the tubes with **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.*** should be inserted first.
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 according to the Table 4.

Table 4

Channel for fluorophore	FAM	JOE	ROX
PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E. m.*			
DNA/cDNA-target	*TBEV* cDNA	*A.phagocytophilum* DNA	*E.chaffeensis/ E.muris* cDNA
PCR-mix-1-FRT *B.b. sl* / IC			
DNA/cDNA-target	Internal Control STI-87-rec (IC) cDNA	*B.burgdorferi sl* cDNA	—

Results are interpreted by the crossing (or not-crossing) the S-shaped (sigmoid) 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/DNA sample.
Principle of interpretation is the following:

Table 5

Results interpretation for the test samples

***Ct* value in the channel for the fluorophore**			**Result**
FAM	**JOE**	**ROX**	
PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.*			
< boundary value	determined or absent	determined or absent	***TBEV* cDNA is detected**
determined or absent	< boundary value	determined or absent	***A.phagocytophilum* DNA is detected**
determined or absent	determined or absent	< boundary value	***E.chaffeensis/E.muris* cDNA is detected**
absent or > boundary value	determined or absent	determined or absent	***TBEV* cDNA is NOT detected***
determined or absent	absent or > boundary value	determined or absent	***A.phagocytophilum* DNA is NOT detected***
determined or absent	determined or absent	absent or > boundary value	***E.chaffeensis/E.muris* cDNA is NOT detected**
PCR-mix-1-FRT *B.b. sl* / IC			
determined or absent	< boundary value	–	***B.burgdorferi sl* cDNA is detected**
< boundary value	absent or > boundary value	–	***B.burgdorferi sl* cDNA is NOT detected**
absent or > boundary value	absent or > boundary value	–	**Invalid****

* If the *Ct* value for this test sample is less than the boundary value in the channel for FAM fluorophore with **PCR-mix -1-FRT *B.b. sl* / IC**.
** If the *Ct* value for this test sample is greater than the boundary value or absent in the channels for FAM, JOE, ROX fluorophores with **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.***

In case of **invalid** result, the PCR analysis should be repeated for the corresponding test sample.

NOTE: Boundary *Ct* values are specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

The result of the analysis is considered reliable only if the results of both Positive and Negative Controls of amplification as well as Negative Control of extraction are correct (see Table 6).

Table 6

Results for controls

Control	**Stage for control**	***Ct* value in the channel for fluorophore**		
		FAM	**JOE**	**ROX**
PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.*				
C–	RNA/DNA extraction	Absent	Absent	Absent
NCA	PCR	Absent	Absent	Absent
C+	PCR	< boundary value	< boundary value	< boundary value
PCR-mix-1-FRT *B.b. sl* / IC				
C–	RNA/DNA extraction	< boundary value	Absent	–
NCA	PCR	Absent	Absent	–
C+	PCR	< boundary value	< boundary value	–

10. TROUBLESHOOTING

Results of analysis are not taken into account in the following cases:

1. The *Ct* value determined for the Positive Control of amplification (C+$_{TBEV,\ B.b.\ sl,\ A.ph.,\ E.ch.\ /\ E.m.\ /\ STI}$) is absent or greater than the specified boundary *Ct* value in the channels for FAM, JOE, or ROX fluorophores (with the use of **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.***) or in the channels for FAM and JOE fluorophores (with the use of **PCR-mix-1-FRT *B.b. sl* / IC**). The amplification and detection should be repeated for all samples in which specific cDNA/DNA was not detected in the appropriate channel.
2. The *Ct* value is determined for the Negative Control of extraction (C-) in the channels for FAM, JOE, ROX fluorophores (with the use of **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.)*** and in the channel for the JOE fluorophores (with the use of **PCR-mix-1-FRT *B.b. sl* / IC**) and/or Negative Control of amplification (NCA) (in all channels). The contamination of laboratory with amplification fragments or contamination of reagents, test samples is probable at any stage of PCR analysis. Measures for detecting and elimination of contamination source must be taken. PCR analysis should be repeated for all samples in which specific cDNA/DNA was detected in the appropriate channel.

11. TRANSPORTATION

AmpliSens® *TBEV, B.burgdorferi sl, A.phagocytophilum, E.chaffeensis / E.muris*-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® *TBEV, B.burgdorferi sl, A.phagocytophilum, E.chaffeensis / E.muris*-FRT** PCR kit are to be stored at 2–8 °C when not in use (except for PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.*, PCR-mix-1-FRT *B.b. sl* / IC, polymerase (TaqF), and PCR-buffer-C). All components of the **AmpliSens® *TBEV, B.burgdorferi sl, A.phagocytophilum, E.chaffeensis / E.muris*-FRT** PCR kit are stable until the expiry date on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

NOTE: PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.*, PCR-mix-1-FRT *B.b. sl* / IC, polymerase (TaqF), and PCR-buffer-C are to be stored at temperature from minus 24 to minus 16 °C.

NOTE: PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.*, and PCR-mix-1-FRT *B.b. sl* / IC are to be kept away from light.

[2] For example, Rotor-Gene 6000 (Corbett Research), Rotor-Gene Q (QIAGEN).

[3] For example, CFX96 (Bio-Rad).

13. SPECIFICATIONS

13.1. Analytical sensitivity (limit of detection)

Table 7

Microorganism	Biological material	Nucleic acid extraction kit	Reverse transcription kit	Analytical sensitivity (limit of detection), copies/ml
TBEV, Borrelia burgdorferi sl, *Ehrlichia chaffeensis* and *Ehrlichia muris*, *Anaplasma phagocytophilum*	Ticks of *Ixodes* and *Dermacentor* genera	RIBO-prep	REVERTA-L	5×10^3
		MAGNO-sorb		
	Whole venous blood	RIBO-prep		
	Cerebrospinal fluid	RIBO-prep		
	Autopsy material	RIBO-prep		
		MAGNO-sorb		

The claimed features are achieved while respecting the rules specified in the section "*Sampling and Handling"*.

13.2. Analytical specificity

The analytical specificity of **AmpliSens**® ***TBEV, B.burgdorferi sl, A.phagocytophilum, E. chaffeensis / E.muris*-FRT** PCR kit is ensured by selection of specific primers and probes as well as by selection of strict reaction conditions. The primers and probes were checked for possible homologies to all sequences deposited in gene banks by sequence comparison analysis.

The reagent kit detects RNA/DNA fragments of the reported microorganisms.

The analytical specificity of the reagent kit was proved when examining RNA/DNA of the strains listed in Table 8, as well as human genomic DNA, DNA of ticks *Ixodes persulcatus*, *Ixodes ricinus, Dermacentor reticulatus, Dermacentor marginatus*, DNA of *Clethrionomys glareolus* and *Apodemus agrarius* rodents.

Table 8

Microorganism	Strain	Concentration
Leptospira kirschneri (*bataviae* serogroup)	M20 (Copenhageni)	$8x10^7$ m.c./ml
Leptospira borgpetersenii (*Tarassovi* serogroup)	Poi	$8x10^7$ m.c./ml
Rickettsia heilongjiangensis	Primorye-25/81	10^6 copies/ml
Rickettsia conorii subsp. *conorii*	M1	$1,6x10^8$ copies/ml
Borrelia miyamotoi	Izh-4	$1,5x10^8$ copies/ml
Japanese encephalitis virus (*JEV*)	Pekin-1	Titre 10^6 $TCID_{50}$/ml
West Nile virus (*WNV*)	Leiv-VLG99-27889 human	$3x10^8$ copies/ml
Langat virus (*LGTV*)	TP-21	10^7 copies/ml
Powassan virus (*POWV*)	Baers	10^7 copies/ml

There were no nonspecific responses in tests with RNA/DNA of the above-mentioned organisms, ticks, rodents, as well as human DNA.

The information about interfering substances is specified in the *Interfering substances and limitations of using test material samples*.

13.3. Reproducibility and repeatability

Repeatability and reproducibility were determined by testing positive and negative model samples. Positive samples were a mixture of quality control sample (QCS) containing *TBEV* RNA, *Borrelia burgdorferi sl, Ehrlichia chaffeensis, Anaplasma phagocytophilum* DNA with a concentration of $5x10^3$ copies/ml each, and an NCA sample was used as a negative sample. Repeatability conditions included testing in the same laboratory, by the same operator, using the same equipment within a short period of time. Reproducibility conditions included testing in two independent laboratories, by different operators, on different days, on different equipment and different reagent kit series. The results are presented in Table 9.

Table 9

Sample type	Repeatability		Reproducibility	
	Number of samples	Agreement of results, %	Number of samples	Agreement of results, %
Positive	10	100	30	100
Negative	10	100	30	100

14. REFERENCES

1. European Centre for Disease Prevention and Control. Annual Epidemiological Report 2016 – Tick-borne encephalitis. [Internet]. Stockholm: ECDC; 2016.
2. Makenov, M.; Karan, L.; Shashina, N.; Akhmetshina, M.; Zhurenkova, O.; Kholodilov, I.; Karganova, GSmirnova, N.; Grigoreva, Y.; Yankovskaya, Y.; Fyodorova, M., First detection of tick-borne encephalitis virus in Ixodes ricinus ticks and their rodent hosts in Moscow, Russia. Ticks Tick Borne Dis 2019, 10, (6), 101265.

15. QUALITY CONTROL

In accordance with Federal Budget Institute of Science "Central Research Institute for Epidemiology" ISO 13485-Certified Quality Management System, each lot of **AmpliSens**® ***TBEV, B.burgdorferi sl, A.phagocytophilum, E.chaffeensis / E.muris*-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
30.01.24 BA	Through the text	Corrections according to the template
		Names of reagents are changed: 'RT-PCR-mix-2-FEP/FRT' to 'PCR-buffer-C', 'DNA-buffer' to 'TE-buffer'
	1. Intended use	Name of biological material was changed: blood to whole venous blood and autopsy material to tissue material
	3. Content	The volume and quantity of PCR-buffer-C, TE-buffer, Polymerase (TaqF), Internal Control STI-87-rec (IC) were changed
	4. Additional requirements	The section was actualized and updated with materials and instruments. Automated station for RNA/DNA extraction, CFX 96 were added. Rotor-Gene 3000, Mx3000P and iCycler iQ5 were deleted
	6. Sampling and handling	The information about sampling and handling was expanded. The subsection *Interfering substances and limitations of using test material samples* was added
	8. Protocol	In the subsection "8.1. DNA/RNA extraction" MAGNO-sorb was added. The section was supplemented with information on the volumes of reagents and samples when using RIBO-prep and MAGNO-sorb reagent kits
	9. Data analysis	Information on the correspondence of the amplification product and channels for the fluorophore, the principle of results interpretation for the test samples and controls are presented in tables
	10. Troubleshooting	The section was rewritten
	13. Specifications	The "13.1. Analytical sensitivity (limit of detection)" and "13.2. Analytical specificity" subsections were actualized. The "13.3. Reproducibility and repeatability" was added
	14. References	The section was actualized

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