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EliGene® Tick-borne diseases RT

CE

REF 90091-RT (for 50 samples)

Kit components:

5 x 150 µl **TBD Mix**
2 x 50 µl **PC DNA TBD**
5 x 200 µl **IAC DNA**
1 x 50 µl **Enzyme Mix**
Instruction for Use

Storage and shelf life after first opening:

All components of the kit must be transported and stored at -20 °C. Kit and remaining MasterMixes must be stored at -20 °C in a dark.

Intended use

EliGene® Tick-borne diseases RT kit is intended for DNA diagnostics of pathogenic species *Borrelia spielmanii*, *Borrelia afzelii Borrelia garinii, Borrelia valaisiana, Borrelia burgdorferi sensu stricto, Anaplasma phagocytophilum* and tick-borne encephalitis virus (TBEV) from isolated nucleic acid.

Principle of the method

This diagnostic kit is based on RealTime PCR method. In this kit primers and dual-labeled fluorescent probes for the detection of DNA are used.

Channel	FAM	HEX	Texas Red	Cy5
Target	Borrelia	IAC	Anaplasma	TBEV

Introduction

Lyme borreliosis is a disease caused by spirochete *Borrelia burgdorferi*. The detection of this bacterium could be made from any clinical material using the DNA diagnostics. There can be found five pathogenic species of *Borrelia* in Czech Republic: *Borrelia spielmanii*, *B. afzelii, B. garinii, B. valaisiana and B. b. sensu stricto* (Bonczek et al.,2015). EliGene® Tick-borne diseases RT Kit contains mix of primers and probes that are specific to all five mentioned *Borrelia* species with high sensitivity that allows detecting 1–10 Borrelia genomes in 5 µl of DNA sample. DNA diagnostics is useful in cases of *Borrelia* suspicion (anamnesis of tick or insect that suck the blood). For diagnostic purposes following clinical specimen are recommended:

a) whole EDTA blood (when the patient has a temperature and in acute period of the disease);

b) urine;

c) cerebrospinal fluid (in cases of neuroborreliosis);

d) synovial fluid;

e) tissue (skin biopsy).

Anaplasma phagocytophilum is obligate intracellular, gram-negative rickettsial organisms infecting human leukocytes. *Anaplasma* is transmitted to humans by tick bites primarily from the tick *Ixodes scapularis* and the

tick *Ixodes pacificus*. It causes human granulocytic anaplasmosis (HGA) previously known as human granulocytic ehrlichiosis (HGE). Typical symptoms include fever, headache, chills, and muscle aches and they usually occur within 1-2 weeks of a tick bite. The epidemiology of this infection is very much like that of Lyme disease (caused by *Borrelia burgdorferi*) and babesiosis (caused primarily by *Babesia microti*), which all have the same tick vector.

Primary sample collection, handling and storage

Clinical material:	Recommended DNA isolation procedure:
Blood, urine, CSF, synovial fluid, tissue	Manual: Quick-DNA/RNA Miniprep Plus Kit (Zymo Research)
	Automatic: **ZEPHYRUS Magneto** (ELISABETH PHARMACON)

Ticks

Blood:

It is recommended to take blood in patients with suspicion of *Borrelia* whose have the fever. Non-clotting blood takes into fluid EDTA. For the DNA diagnostic purposes of *Borrelia* and TBEV, it is necessary to isolate DNA from the sample during the day of blood taking. The blood sample is necessary to be transported and stored at 4 °C, in any event do not freeze the blood sample!

Always follow the manufacturer's isolation kit instructions when processing the above-mentioned samples.

Add 20 µl of internal control (IAC DNA) to each sample before isolation.

Additional required equipment

- Automatic pipette 5–20 µl and sterile tips with filter DNA-, RNA- free, DNase-, RNase- free (we recommend plastic with CE certificate for diagnostic purposes).
- Sterile stand DNA-, RNA- free, DNase-, RNase- free.
- Equipment for RealTime PCR – the kit is designed for Quant Studio 5 (Thermofisher Scientific), CFX96 Touch Real-Time PCR Detection System (Bio-Rad), MIC qPCR Cycler (Bio Molecular Systems).
- Sterile plastic (strips, plates, tubes) DNase-, RNase- free compatible with given RealTime PCR system.
- Lab safety gloves.

Configuration of Real Time instrument

Follow the cycler manufacturer's manual when using the kit. Below is a list of cyclers included settings that were used when testing the EliGene® Tick-borne diseases RT kit.

- For *Borrelia* DNA detection the probe labeled with FAM is used (exc. 494 nm – em. 518 nm).
- For *Anaplasma phagocytophilum* DNA detection the probe labeled with TexasRed is used (exc. 589 nm – em. 615 nm).
- For tick-borne encephalitis RNA detection the probe labeled with Cy5 is used (exc. 650 nm – em. 670 nm).
- For Internal control the probe labeled with HEX is used (exc. 520 nm – em. 548 nm).

QuantStudio 5 (ThermoFisher Scientific):

Use the Experiment type," Presence/Absence", Chemistry "TaqMan Probes", and Run Mode "Standard". As reporter dyes use FAM (Borrelia), VIC/HEX (IAC DNA), ROX (Anaplasma), Cy5 (TBEV) and as a passive reference dye use NONE.

Set up the following temperature profile:

Holding stage

55°C 15 min Ramp rate (1.6°C/s)

95°C 2 min Ramp rate (1.6°C/s)

Cycling stage – 45 cycles

95°C 10 s Ramp rate (1.6°C/s)

55°C 20 s Ramp rate (1.6°C/s) Data collection ON

67°C 30 s Ramp rate (1.6°C/s)

Post-Read Stage

40°C 1 min Ramp rate (1.6°C/s)

Collect emission signal at the second step at 55 °C.

The complete temperature profile can be up-loaded from Run Template "EliGene_TBD_QS5_v00.edt". The Run Template can be copied from the CD included in the kit.

CFX96 Touch (Bio-Rad):

In Startup Wizard Create a new Experiment for CFX96 instrument and Create New Protocol.

Set up the following temperature profile:

Step 1	55°C	15 min
Step 2	95°C	2 min
Step 3	95°C	10 s
Step 4	55°C	20 s + Plate Read
Step 5	67°C	30 s
Step 6	*GOTO Step 3*	*44x*
Step 7	40°C	60 s

Enter the Sample Volume 20 µl.

Collect emission signal at the Step 3 at 55° C.

For filter settings use the "Scan Mode" All Channels but in Plate Manager select for the samples only fluorophores FAM, HEX, TexasRed, Cy5. Then assign the samples with positions as an "Unknown" sample or "Standard".

The complete temperature profile can be up-loaded from Run Template "EliGene_TBD_CFX96_v00.pcrl". The Run Template can be copied from the CD included in the kit.

MIC qPCR (Bio Molecular Systems Pty Ltd):

In Run Setup Create a new Run Profile for the MIC instrument. Enter the Sample Volume of 20 µl and Temperature Control "Fast TAQ (v3)".

Set up the following temperature profile:

Holding stage

55°C 15 min

95°C 2 min

Cycling stage – 45 cycles

95°C 10 s

55°C 20 s Acquire on "Green", "Yellow", "Orange", "Red"

67°C 30 s

Holding stage

40°C 60 s

The complete temperature profile can be up-loaded from Run Template "EliGene_TBD_MIC_v00.mictemplate". The Run Template can be copied from the CD included in the kit.

Reagent preparation

- To avoid the contamination keep all tubes closed and follow the instructions.
- Before the usage, all reagents must be completely thawed, briefly mix on vortex and shortly spin.
- In the step of Proteinase K addition of Isolation protocol add 20 µl of Internal Control (IAC DNA) to isolated sample. In no case add the internal control to isolated nucleic acid (NA) just before the analysis.
- **If you do not use all the volume of MasterMix, store the tube at dark at temperature 4°C up to 14 days. For long-term storage use the freezer (-20 °C, dark). MasterMix should not go through more than five freeze- thaw cycles.**

WARNING: The contamination in laboratory space is also possible. Use separate pipette for MasterMixes, separate pipette for positive controls and separate pipette for samples. Follow all recommendations for laboratories of DNA analyses.

Preparation of Reaction Mix for the instruments QuantStudio 5, CFX96 and MIC qPCR

1. Detection: Take one microtube with TBD Mix and after the thawing pipette 14 µl of the mix to amplification microtube or plate and add 1 µl of Enzyme Mix and 5 µl of isolated NA. During the pipetting of samples be careful to avoid cross-contamination of samples.
2. Positive control: Take one microtube with TBD Mix and after the thawing pipette 14 µl of mix to amplification microtube or plate and add 1 µl of Enzyme Mix and 5 µl of PC DNA TBD. During the pipetting of positive control be careful to avoid contamination of other samples. Use separate pipette for positive controls!

Insert the micro tubes or plate with samples to the RealTime PCR instrument and run the program according to chapter "Configuration of Real Time instrument" above.

Result reading

QuantStudio 5 (ThermoFisher Scientific):

In "**Analyse Settings**" edit the original parameters for Ct for individual channels (FAM, VIC, ROX, Cy5). Enter the Threshold level for each channel to a value equal to **1/3 of the maximal intensity of positive control**, select "Automatic Baseline" option and analyze results by selecting "**Apply**".

Positive result for *Borrelia*: The positive result is characterized by the growth of fluorescence signal in FAM channel. In a case of negative results, the amplification will not occur.

Positive result for *Anaplasma phagocytophilum*: The positive result is characterized by the growth of fluorescence signal in ROX channel. In a case of negative results, the amplification will not occur.

Positive result for *TBE virus*: The positive result is characterized by the growth of fluorescence signal in Cy5 channel. In a case of negative results, the amplification will not occur.

The Internal Control (IAC DNA) must be amplified in each sample. The Internal Control amplification is characterized by the growth of fluorescence signal in VIC (HEX) channel.

CFX96 Touch Real-Time PCR Detection System (Bio-Rad):

In Data Analysis window choose "Quantification". In "Settings" menu choose option "Baseline Setting" and select "Baseline Subtracted Curve Fit" option and "Apply Fluorescence Drift Correction" option.

In Data Analysis window select a single fluorophore (FAM, HEX, TxRed, Cy5) by the clicking the box next to the fluorophore name located under the amplification chart and read the results for individual samples.

Positive result for *Borrelia*: The positive result is characterized by the growth of fluorescence signal in FAM channel (em. 518 nm). In a case of negative results, the amplification will not occur.

Positive result for *Anaplasma phagocytophilum*: The positive result is characterized by the growth of fluorescence signal in TxRed channel (em. 615 nm). In a case of negative results, the amplification will not occur.

Positive result for *TBE virus*: The positive result is characterized by the growth of fluorescence signal in Cy5 channel (em. 670 nm). In a case of negative results, the amplification will not occur.

The Internal Control (IAC DNA) must be amplified in each sample. The Internal Control amplification is characterized by the growth of fluorescence signal in HEX channel (em. 548 nm).

MIC qPCR (Bio Molecular Systems):

To view and analyze acquired data, click the small cross next to Cycling under Analysis module and then select the target – Non-Assay Green (FAM), Non-Assay Yellow (HEX), Non-Assay Orange (TxRed) and Non-Assay Red (Cy5). In the down right corner **increase** "**Fluorescence Cut-off Level**" **to 20 %**. A quantification cycle (Cq) value is displayed for each positive well. This is the cycle number at which the sample fluorescence rises above the threshold (the red horizontal line near the bottom of the amplification curve chart). In the Samples panel on the right, click sample names to hide or display the associated amplification curves and result data.

Positive result for *Borrelia*: The positive result is characterized by the growth of fluorescence signal in Green channel. In a case of negative results, the amplification will not occur.

Positive result for *Anaplasma phagocytophilum*: The positive result is characterized by the growth of fluorescence signal in Orange channel. In a case of negative results, the amplification will not occur.

Positive result for *TBE virus*: The positive result is characterized by the growth of fluorescence signal in Red channel. In a case of negative results, the amplification will not occur.

The Internal Control must be amplified in each sample. The Internal Control amplification is characterized by the growth of fluorescence signal in Yellow channel.

Interpretation of results

Negative result:

If the increasing of amplification signal in FAM, TxRed or Cy5 channel does not appear before cycle number 40, the result of test should be interpreted as probably *Borrelia* DNA, *Anaplasma phagocytophilum* DNA and *TBE*

virus RNA negative or with concentration of these below the detection limit of this kit (10 genomic DNA/reaction, respectively 50 copies virus RNA/reaction). The signal for Internal Control must be positive. This result does not exclude the occurrence of infection, because results of this test are dependent on proper sample collection and elaboration. Results are also dependent on enough quantity of analyzed DNA, respectively RNA.

Positive result:

Amplification signal in FAM channel (494 – 518 nm) for TBD Mix will appear before cycle number 40. *Borrelia* DNA was detected in the sample. The sample is *Borrelia* DNA positive.

Amplification signal in TexasRed channel (589 – 615 nm) for TBD Mix will appear before cycle number 40. *Anaplasma phagocytophilum* DNA was detected in the sample. The sample is *Anaplasma phagocytophilum* DNA positive.

Amplification signal in Cy5 channel (650 – 670 nm) for TBD Mix will appear before cycle number 40. *TBE virus* RNA was detected in the sample. The sample is *TBEV* RNA positive.

WARNING: The contamination in laboratory space is also possible. Use separate pipette for MasterMixes, separate pipette for positive controls and separate pipette for samples. Follow all recommendations for laboratories of DNA/RNA analyses.

Inhibited sample:

In the case that increasing of amplification signal specific for DNA, respectively RNA of above-mentioned pathogens, as well as increasing of amplification signal in HEX channel (specific for Internal Control) is not observed, it is necessary to repeat the analysis. The best would be to use new DNA prepared by new isolation process.

Control procedure

EliGene® Tick-borne disease RT Kit involves Internal Control (IAC DNA) and Positive Control (PC DNA TBD). Internal control follows the quality of NA isolation and detects mistakes in the isolation process. It also detects the occurrence of an inhibition of amplification process. In the case that the sample is negative, the Cp of internal control must be Cp < 35.

Positive control follows the proper function of MasterMix. Minimal Cp of positive control must be 35 or less. The Cp higher than 35 for positive control can't be accepted and NA detection must be repeated with new sample. In the case of repeatedly higher Cp contact manufacturer ELISABETH PHARMACON.

Use negative control for each run. As negative control use the water for molecular biology used in your laboratory. For negative control use the pipette for DNA/RNA samples.

Reference material:

To monitor all examination processes covering NA isolation and RealTime PCR detection it is possible to use sample positive for *Borrelia*, *Anaplasma* DNA and TBEV RNA. The commercial positive material is not available.

Troubleshooting:

1. If there is no amplification of Internal control (IAC DNA), there is some problem in the isolation of DNA/RNA or the kit is after the expiration date or there is RealTime instrument breakdown.
2. If there is no amplification of Positive Control, the kit is after the expiration date or there is RealTime instrument breakdown.

Performance characteristics

Analytical performance characteristics:

Kit EliGene® Tick-borne diseases RT has a very high sensitivity - detects 10 or more genomic DNA of *Borrelia spielmanii, B. afzelii, B. garinii, B. valaisiana, B. burgdorferi s. s.* and *Anaplasma phagocytophilum* added to the amplification mix and 50 copies of tick-borne encephalitis virus RNA.

Analytical sensitivity of the test was verified as follows. The insert with specific DNA concentration was prepared by cloning and subsequently diluted to desired concentration of target sequence. The addition of human DNA to sample in common concentration has no effect to sensitivity of the method. Simultaneous analysis of multiple pathogens in one reaction mixture did not affect the assay result.

Analytical specificity of method was verified by searching the DNA database NCBI (GenBank, http://www.ncbi.nlm.nih.gov/) for the sequences of primers and probes and by addition of human DNA to mastermix. 50 different samples of human DNA did not give false positive result. Further, false positive result was not observed after the addition of DNA from these organisms: *C. trachomatis*, *M. tuberculosis*, *M. bovis*, *M. cansasii*, *M. xenopii*, *M. avium*, *M. marinum*, *Lactobacillus*, *Enterococcus faecalis*, genus. *Pseudomonas*, *E. coli*, *A. niger*, *C. albicans*, *S. aureus*, *S. agalactiae*, *N. gonorrhoeae*, *U. urealyticum* Adenovirus, HBV, EBV, CMV, HSV1, HSV2, VZV.

Clinical specificity was tested on 250 clinical and environmental samples (blood, CSF, tissue, urine, synovial fluid and ticks). Of these, 20 were positive for TBE virus, 56 were positive for *Anaplasma phagocytophilum*, 100 were positive for *Borrelia burgdorferi sensu lato*. Compared to the reference methods, 100 % agreement was achieved in results.

Diagnostic performance characteristics:

The clinical sensitivity and specificity of EliGene® Tick-borne diseases RT kit is 100%.

Measuring interval

The kit enables the detection of 10^1–10^8 DNA molecules in reaction mix and 10^2–10^8 RNA molecules in reaction mix.

Internal control of quality

As an internal control of quality the Internal control (IAC DNA) for checking the process of NA isolation and amplification, together with Positive Control for functional control of MasterMix is used.

Limitation of the examination procedure

The sensitivity of kit depends on handling with sample (isolation of DNA/RNA). It is strictly recommended to use isolation kits and procedures mentioned above.

The sensitivity of NA detection depends on the sample collection, the way of storage (store at 4 °C) and the way of elaboration (NA isolation, day of taking the sample, detection immediately after the isolation).

Biological reference intervals

Not applicable information for this kit.

Warning

After mixing, MasterMix is stable at -20 °C. Do not freeze tubes with MasterMix more than 5 times! Do not mix components of the kits of different lots.

Warnings and general precautions

- Handle and dispose of all biological samples as if they were capable of transmitting infective agents. Avoid direct contact with the biological samples. Avoid splashing or spraying. The materials that come into contact with biological samples must be autoclaved at 121 °C for one hour before disposal.
- Handle and dispose of all reagents and all assay materials as if they were capable of transmitting infective agents. Avoid direct contact with the reagents. Avoid splashing or spraying. Waste must be treated and disposed of in compliance with the appropriate safety standards. Disposable combustible materials must be incinerated. Liquid waste containing acids or bases must be neutralized before disposal.
- Wear suitable protective clothing and gloves and protect eyes/face.
- Never pipette solutions by mouth.
- Do not eat, drink, smoke or apply cosmetic products in the work areas.
- Wash hands carefully after handling samples and reagents.
- Dispose of leftover reagents and waste in compliance with regulations in force.
- Read all the instructions provided with the kit before running the assay.
- Follow the instructions provided with the kit while running the assay.
- Do not use the kit after the expiry date.
- Only use the reagents provided in the kit and those recommended by the manufacturer.
- Do not mix reagents from different batches.
- Do not use reagents from other manufacturer´s kit.

Warnings and precautions for molecular biology

- Molecular biology procedures, such as NA isolation, reverse transcription, amplification and detection of nucleic acids, require qualified staff to prevent the risk of erroneous results, especially due to degradation of the nucleic acids contained in the samples or due to sample contamination by amplification products.
- It is necessary to have separate areas for the extraction/preparation of amplification reactions and for the amplification/detection of amplification products. Never introduce an amplification product in the area designed for extraction/preparation of amplification reactions.
- It is necessary to have lab coats, gloves and tools which are exclusively employed in the extraction/preparation of amplification reactions and for the amplification/detection of amplification products. Never transfer lab coats, gloves or tools from the area designed for the amplification/detection of amplification products to the area designed for the extraction/preparation of the amplification reactions.
- The samples must be exclusively employed for this type of analysis. Samples must be handled under a laminar safety box. Tubes containing different samples must be never opened at the same time. Pipettes used to handle samples must be exclusively employed for this specific purpose. The pipettes must be of the positive displacement type or be used with aerosol filter tips. The tips employed must be sterile, free from DNases and RNases, free from DNA and RNA.
- Reagents must be handled under PCR box or laminar flow box. The reagents required for amplification must be prepared in such a way that they can be used in a single session. The pipettes employed to handle the

reagents must be used exclusively for this purpose. The pipettes must be of the positive displacement type or be used with aerosol filter tips. The tips employed must be sterile, free from DNases and RNases, free from DNA and RNA.

- Amplification products must be handled in such way as to reduce dispersion into the environment as much as possible, in order to avoid the possibility of contamination. Pipettes used to handle amplification products must be employed exclusively for this specific purpose.

Warnings and precautions specific to components of the kit

- The tubes containing mix (TBD Mix) are disposable and therefore must be used once only in the preparation of the reaction mixture.
- The tubes containing IAC DNA are disposable and therefore must be used once only in the preparation of the reaction mixture.
- These mixes carry the following safety warnings (P):

P280 Wear protective gloves/protective clothing/eye protection/face protection.

P281 Use personal protective equipment as required.

In the case of any problems, contact the customer center of ELISABEHT PHARMACON, spol. s r.o.

Literature

Bannister BA, Begg NT, Gillespie SH. 2000. Infectious Disease. Blackwell Science, 2th Ed.

Barbara A. Bannister, Norman T. Begg and Stephen H. Gillespie: Infectious Disease. Blackwell Science, 2th Ed., 2000

Bonczek O, Žákovská A, Vargová L, Šerý O. Identification of Borrelia burgdorferi genospecies isolated from Ixodes ricinus ticks in the South Moravian region of the Czech Republic. Ann Agric Environ Med. 2015;22(4):637-41.

Gary P. Wormser et al. 2006. The Clinical Assessment, Treatment, and Prevention of Lyme Disease, Human Granulocytic Anaplasmosis, and Babesiosis: Clinical Practice Guidelines by the Infectious Diseases Society of America. Clin Infect Dis. 43 (9): 1089-1134.

Hytönen J, Hartiala P, Oksi J, Viljanen MK. 2008. Borreliosis: recent research, diagnosis, and management. Scand J Rheumatol. 37(3):161-172

Johan S. Bakken, MD, Stephen Dumler. 2008. Human Granulocytic Anaplasmosis. Clin Infect Dis. 22 (3): 433–448

Josko D. Molecular virology in the clinical laboratory. Clin Lab Sci. 2010 Fall;23(4):231-6.

Priem S, Rittig MG, Kamradt T, Burmester GR, Krause A. 1997. An optimized PCR leads to rapid and highly sensitive detection of Borrelia burgdorferi in patients with Lyme borreliosis. J Clin Microbiol. 35(3): 685–690

Symbols

	Catalog number
	Upper limit of temperature
	Batch code
	Use by (last day of month)
	*in vitro* diagnostic medical device
	Fulfilling the requirements of European Directive 98\79\EEC for *in vitro* diagnostic medical device.
	Contains sufficient for "N" tests
	Attention, consult instructions for use
	Manufacturer

Manufacturer

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