ELISABETH PHARMACON Ltd.
Rokycanova 4437/5
615 00 Brno-Zidenice, Czech Republic
Phone: +420 542 213 851
E-mail: info@elisabeth.cz
Web: www.elisabeth.cz
VAT: CZ26258412

EliGene® Babesia/Bartonella RT

CE REF **90094-RT (for 50 samples)**

Kit components:

5 x 150 µl **Bab/Bar Mix**
2 x 50 µl **PC DNA Bab/Bar**
5 x 200 µl **IAC DNA**
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® Babesia/Bartonella RT kit is intended for DNA diagnostics of pathogenic species *Babesia spp.* and *Bartonella spp.* from isolated DNA. The kit detects all known species of these microorganisms.

Principle of the method

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

Channel	FAM	HEX	Cy5
DNA target	Babesia	IAC	Bartonella

Introduction

Babesiosis is an infection caused by parasitic protozoa of *Babesia spp.*, the most common is *Babesia microti*. This parasite is transmitted by ticks and primarily infects erythrocytes. If a tick carries a protozoon, sporozoite form enters the human bloodstream. Sporozoites enter erythrocytes, where they mature and divide by binary cleavage to form merozoites. These merozoites then leave the host erythrocyte, disrupt the cell and continue to infect other erythrocytes, repeating the above-mentioned cycle. The spleen is essential for the host's ability to control this infection. People, who have undergone a splenectomy are at high risk of a severe infection. Other high-risk populations include people with HIV, people over 50, newborns, and immunosuppressed patients. PCR diagnostics from blood is a more sensitive alternative to microscopic examination.

Bartonella spp. are slowly growing facultatively intracellular bacteria that can cause a variety of diseases in both, humans and animals. These zoonotic bacteria can be transmitted directly from animals to humans (eg. *Bartonella henselae*) or various blood-sucking vectors, including lice and flies. It is known more than 40 *Bartonella spp.* species, of which at least 14 are considered human pathogens. Most human infections are caused by *Bartonella quintana*, *B. henselae* and *Bartonella bacilliformis*, whereas *B. bacilliformis* is endemic in the Andes in South America.

Ticks are thought to transmit Bartonella, because *Bartonella henselae* and other species were proved in the blood of patients exposed to ticks. Various species of *Bartonella spp.* have been found in ticks of *Ixodes ricinus*. Human bartonellosis can manifest with a wide range of symptoms such as recurrent fever, headache, lymphadenopathy, myalgia, endocarditis, etc. PCR diagnostics from blood and tissues is an alternative to other diagnostic procedures.

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Primary sample collection, handling and storage

Clinical material:

Blood, CSF, tissue

Recommended NA isolation procedure:

Manual: Quick-DNA/RNA Miniprep Plus Kit (Zymo Research)

Automatic: **ZEPHYRUS Magneto** (ELISABETH PHARMACON)

DNA isolated from ticks

Blood:

It is recommended to take blood into fluid EDTA. 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 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® Babesia/Bartonella RT kit.

- For *Babesia* DNA detection the probe labeled with FAM is used (exc. 494 nm – em. 518 nm).
- For *Bartonella* DNA 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 (Babesia), VIC (IAC DNA), Cy5 (Bartonella) and NONE as a passive reference dye.

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 of cycling stage 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, 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" and "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 DNA 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 Bab/Bar Mix and after the thawing pipette 15 µl of the mix to amplification microtube or plate and add 5 µl of isolated DNA. During the pipetting of samples be careful to avoid cross-contamination of samples.
2. Positive control: Take one microtube with Bab/Bar Mix and after the thawing pipette 15 µl of mix to amplification microtube or plate and add 5 µl of PC DNA Bab/Bar. 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, 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 *Babesia*: 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 *Bartonella*: 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” and “Apply Fluorescence Drift Correction” option.

In Data Analysis window select a single fluorophore (FAM, HEX, 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 *Babesia*: 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 *Bartonella*: 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) 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 *Babesia*: 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 *Bartonella*: 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 or Cy5 channel does not appear before cycle number 40, the result of test should be interpreted as probably *Babesia* and *Bartonella* DNA negative or with concentration below the detection limit of this kit (10 genomic DNA/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.

Positive result:

Amplification signal in FAM channel will appear before cycle number 40. *Babesia* DNA was detected in the sample. The sample is *Babesia* DNA positive.

Amplification signal in Cy5 channel will appear before cycle number 40. *Bartonella* DNA was detected in the sample. The sample is *Bartonella* DNA 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 analyses.

Inhibited sample:

In the case that increasing of amplification signal in FAM or Cy5 channel (specific for 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 extraction process.

Control procedure

EliGene® Babesia/Bartonella RT Kit involves Internal Control (IAC DNA) and Positive Control. Internal isolation control follows the quality of DNA isolation and detects mistakes in the isolation process. It detects the occurrence of an inhibition of amplification process. In the case that the sample is *Babesia/Bartonella* DNA 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 DNA 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 samples.

Reference material:

To monitor all examination process covering DNA isolation and RealTime PCR detection is possible to use specimen positive for *Babesia* and *Bartonella* DNA. 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 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® Babesia/Bartonella RT has a very high sensitivity - detects 10 or more copies of genomic NA of *Babesia spp.* and *Bartonella spp.* added to the amplification mix.

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, *Borrelia burgdorferi sensu lato*, *Anaplasma phagocytophilum*, HBV, EBV, CMV, HSV1, HSV2, VZV.

Clinical specificity was tested on 45 DNA samples isolated from different materials. These were DNA samples 3x *Babesia microti* and 3x *Babesia divergens* from the reference workplace of the Academy of Sciences of the Czech Republic. 4 samples positive for *Bartonella spp.* were obtained from Ixodes ricinus ticks analyzed at a reference university site. Further there were 35 samples of Ixodes ricinus ticks from different regions of the Czech Republic. Of all the samples listed, there were 10 samples positive for *Babesia spp.* and 4 samples were positive for *Bartonella spp.* Compared to reference methods, it was achieved 100% agreement in results.

Diagnostic performance characteristics:

The clinical sensitivity and specificity of EliGene® Babesia/Bartonella RT kit is 100%.

Measuring interval

The kit enables the detection of 10 or more copies of genomic NA of microorganisms *Babesia spp.* and *Bartonella spp.* in analyzed sample.

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 specimen (isolation of DNA). It is strictly recommended to use isolation kits and procedures mentioned above.

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.

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Warnings and precautions for molecular biology

- Molecular biology procedures, such as extraction, 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 and must be treated accordingly, e.g., due to possible contamination, degradation of nucleic acids, etc. 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 (not in 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 (Bab/Bar Mix) are disposable and therefore must be used once only in the preparation of the reaction mixture.
- The tubes containing IC 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.

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\EC for *in vitro* diagnostic medical device.
	Contains sufficient for "N" tests
	Attention, consult instructions for use
	Manufacturer

Manufacturer

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