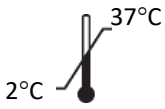
savyonDIAGNOSTICS
Member of the Gamida Diagnostics Division

savvy•gen Tick Borne

REF **624-01L / 624-01H**

Test kit for 24 determinations



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Intended Use

The Savvygen Tick Borne is designed for the specific identification and differentiation of viral RNA or genomic DNA specific for Tick Borne Encephalitis Virus (TBEV), *Rickettsia spp., Babesia microti, Babesia divergens, Ehrlichia chafeensis, Ehrlichia muris, Borrelia burgdorferi sensu lato (s.l.), Borrelia miyamotoi and/or Borrelia hermsii, Anaplasma phagocitophylum* and/or *Coxiella burnetii* in blood, serum, tissue samples and microbiological culture from ticks, biopsy skin, cerebrospinal fluid (CSF) and synovial fluid from patients with signs and symptoms of Tick Borne diseases. This test is intended for use as an aid in the diagnosis of Tick Borne diseases in combination with clinical and epidemiological risk factors.

For *in-vitro* professional diagnostic use.

Background

Tick Borne diseases comprise a group of infections transmitted to humans by the bite of ticks infected with bacteria, viruses, or parasites. Tick Borne diseases affecting humans include: Lyme disease, relapsing fever, babesiosis, anaplasmosis, ehrlichiosis, Q fever, Tick Borne encephalitis and among others.

Lyme disease (or Lyme borreliosis) is the most common Tick Borne disease worldwide. It is caused by the spirochaete *Borrelia burgdorferi* and it is transmitted to humans through the bite of infected blacklegged ticks of the genus Ixodes. Typical symptoms of Lyme disease include fever, headache, fatigue and a characteristic skin rash called erythema migrans. If left untreated, infection can spread to joints, the heart, and the nervous system.

Borrelia miyamotoi and *B. hermsii* are species of spiral-shaped bacteria that is closely related to the bacteria that cause tick-borne relapsing fever (TBRF). First identified in 1995 in ticks from Japan, *B. miyamotoi* has also been detected in different Ixodes ticks species, whereas spirochete *Borrelia hermsii* is transmitted by its argasid tick vector, *Ornithodoros hermsi*. Patients with this infection were most likely to have fever, chills, and headache. Other common symptoms included body and joint pain and fatigue.

Anaplasmosis is caused by the bacterium *Anaplasma phagocytophilium* and is transmitted to humans through ticks of the genus Ixodes. Anaplasmosis shows high clinical variability, with symptoms like headache, fever, chills, malaise, muscle pain, nausea, cough, confusion and rash. If not treated correctly, anaplasmosis can evolve to severe clinical manifestations and even death in <1% of cases.

Q fever is a zoonosis caused by the bacteria *Coxiella burnetii*. Cattle, sheep, and goats are the primary reservoirs of *Coxiella burnetii,* and transmission to humans occurs primarily through inhalation of aerosols from contaminated soil or animal waste, though it can be transmitted through tick bites. Only about 50% of the infected people show clinical symptoms, which are flu-like symptoms such as headache, fever, chills, fatigue, muscle aches, nausea, cough, chest pain and weight loss. In severe cases people may develop pneumonia or hepatitis.

Babesiosis is produced by many species of protozoa of the genus Babesia, mainly *Babesia microti* and *Babesia divergens*. Babesiosis is also transmitted by ticks of the genus Ixodes, and it is frequently found as a coinfection with Lyme disease. Babesiosis usually shows none or flu-like mild symptoms, such as headache, fever, chills, body aches, loss of appetite, nausea or fatigue. However, since Babesia parasites infect and destroy red blood cells, babesiosis can lead to hemolytic anemia.

Ehrlichiosis is due to different species of bacteria of the genus Ehrlichia. Causative agents in humans are *Ehrlichia chaffeensis* and *Ehrlichia muris*. Ehrlichiosis is transmitted by the lone star tick (Amblyomma americanum). Typical symptoms of ehrlichiosis include headache, fever, chills, malaise, muscle pain,

nausea, conjunctival infection, confusion and rash. Severe disease may present lethargy, myalgia, reduction of sodium levels and platelets and elevated liver enzymes, with fatal outcome in 3% of cases.

Tick Borne encephalitis is produced by the Tick Borne encephalitis virus (TBEV) of the family *Flaviviridae*. It is transmitted to humans through ticks of the genus *Ixodes*. The onset of the disease includes nonspecific symptoms such as fever, malaise, anorexia, muscle aches, headache, nausea and vomiting. In 20-30% of patients, a second phase is observed that involves the central nervous system with symptoms of meningitis, encephalitis, or meningoencephalitis, leaving neuropsychiatric sequelae in 10-20% of patients.

Spotted fever is caused by bacteria of the genus Rickettsia and is widely distributed by different geographical areas, being able to transmit depending on it by different ticks such as: wood tick, Dermacentor Andersoni (in the Rocky Mountain states, USA), dog tick, Dermacentor variabilis (other areas of the USA), ticks Amblyomma cajennense (in South America) and Rhipicephalus sanguineus (in Mexico). Spotted fever is characterized by darks scabs at the site of the tick bite (eschar), accompanied by nonspecific symptoms such as headache, fever, rash and muscle ache.

Since most Tick Borne diseases show similar symptoms, diagnosis can be problematic. Real-time PCR assays have been shown to be a sensitive and specific diagnostic tool for the detection of the causative agent.

Principles of the Procedure

The Savvygen Tick Borne test is a Real-Time PCR ready-to used test which contains in each well all the necessary reagents for real time PCR assay in a stabilized format. The test is designed for the diagnosis of TBEV, *Rickettsia spp., Babesia microti, Babesia divergens, Ehrlichia chafeensis, Ehrlichia muris, Borrelia burgdorferi s.l., Anaplasma phagocitophylum and Coxiella burnetii* in clinical samples.

Savvygen Tick Borne detection of TBEV is done in one step real RT-PCR format where the reverse transcription and the subsequent amplification of specific target sequence occur in the same reaction well. The isolated RNA target is transcribed generating complementary DNA by reverse transcriptase which is followed by the amplification of a conserved region of the 3'UTR sequence of TBEV using specific primers and a fluorescent–labelled probe. After DNA isolation, the identification of rest of pathogens is performed by the amplification of highly conserved fragments in the 23S rRNA gene (*Rickettsia spp*.), CCT-eta gene (*Babesia microti*), hsp70 gene (*Babesia divergens*),GroEl gene (*Ehrlichia chafeensis and Ehrlichia muris*), 23S rRNA gene (*Borrelia burgdorferi s.l., Borrelia miyamotoi and/or Borrelia hermsii*), msp2 gene (*Anaplasma phagocitophylum*) and IS1111 gene (*Coxiella burnetii*). Following extraction of viral RNA, the conserved fragments are reversely transcribed into cDNA in a primer-specific manner (Figure 1a). Reverse transcription is followed in a "one-pot reaction" by Taq Polymerase Chain Reaction (PCR). The assay is based on the 5'→3' exonuclease activity of Taq DNA Polymerase (Figure 1b). A fluorophore/quencher dual-labeled probe is annealing to an internal specific sequence. Upon primer elongation, Taq DNA Polymerase displaces and hydrolyzes the probe, thus releasing and activating the fluorophore. The presence of Tick Borne pathogens is detected by an increase in observed fluorescence during the reaction. The resulting increase in fluorescence signal is proportional to the amount of amplified product in the sample and detected by the real-time PCR instrument.

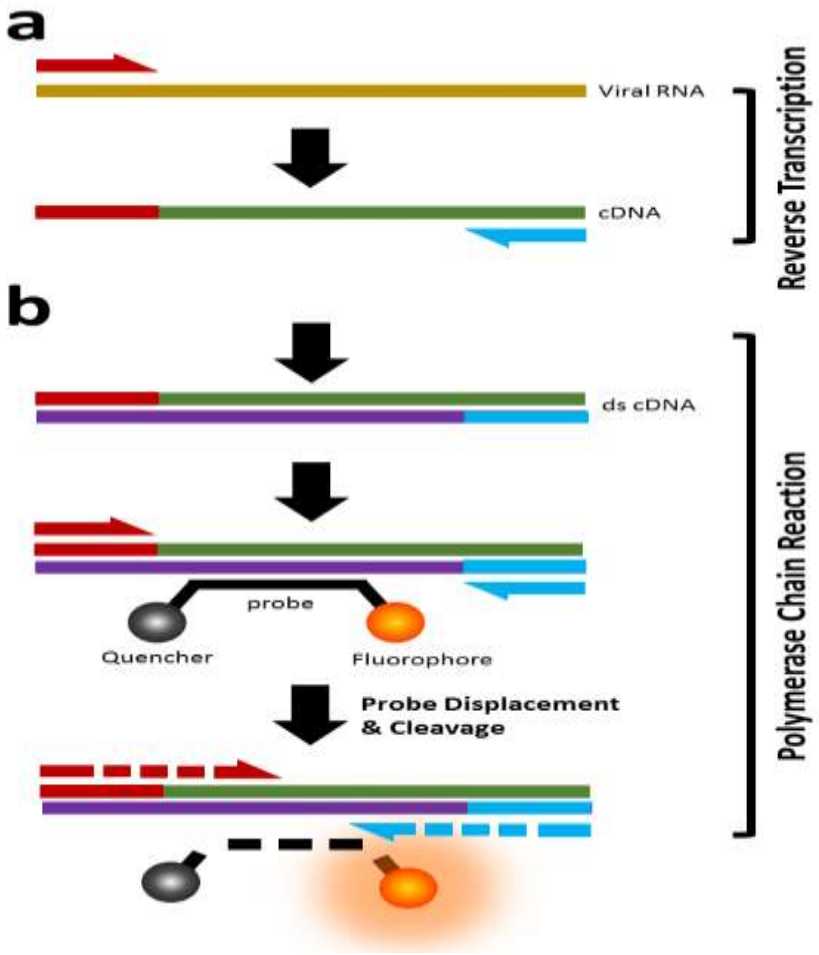


Figure 1. *Principle of the Savvygen assay*

The Savvygen Tick Borne Real Time PCR test is a ready-to used test which contains in each well all the necessary reagents for real time PCR assay in a stabilized format. In addition, an internal control allows the detection of a possible reaction inhibition. The Real Time PCR Detection kits contain into each kit 3 different strips, each one corresponds to one different assay. The optical channels used for multiplexed detection of the amplified fragments are outlined in table 1A,1B and 1C below:

Table 1A. Pathogens target and optical channel detection; Strip 1 (*Borrelia, Anaplasma, Coxiella)*

Target	**Optical channel**
Borrelia burgdorferi s.l. / Borrelia miyamotoi / Borrelia hermsii	FAM
Anaplasma phagocitophylum	ROX
Coxiella burnetii	Cy5
Internal Control	*HEX, VIC or JOE

Table 1B. Pathogens target and optical channel detection; Strip 2 (*Rickettsia, Babesia, Ehrlichia*)

Target	**Optical channel**
E. chafeensis / E. muris	FAM
B. microti & B. divergens	ROX
Rickettsia spp.	*HEX, VIC or JOE

Table 1C. Pathogen target and optical channel detection; Strip 3 (*TBEV*)

Target	Optical channel
TBEV (Tick Borne encephalitis virus)	FAM

(*) Depending on the equipment used, the proper detection channel should be selected (see table 4)

Fluorogenic data should be collected during the extension step (*) through the FAM (TBEV, *Borrelia burgdorferi s.l./Borrelia miyamotoi/Borrelia hermsii* and *Ehrlichia chafeensis/Ehrlichia muris*), HEX, JOE or VIC (*Rickettsia spp* and Internal Control (IC)), ROX (*Babesia microti / Babesia divergens* and *Anaplasma phagocitophylum*), and Cy5 channels (*Coxiella burnetii*).

Materials/ Reagents Provided

Product Description	Contents
Savvygen™ Tick Born; ***REF:*** *624-01L / 624-01H* *24 reactions.*	3 x *Borrelia, Anaplasma, Coxiella* - strip 1 3 x *Rickettsia, Babesia, Ehrlichia* - strip 2 3 x *TBEV* – strip3
	1x Tick Borne Positive Control
	1x Water RNAse/DNAse free 1mL
	1x Rehydration Buffer 1.8 mL
	1x Negative Control 1 mL
	Optical caps

Additional Equipment and Material Required

- RNA extraction kit.
- Centrifuge for 1.5 mL tube.
- Vortex.
- Micropipettes (0.5-20 µL, 20-200 µL).
- Powder-free disposal gloves
- Real Time PCR instrument (see table 2A+2B for compatible RT-PCRs).

Table 2A. Compatible *Real Time PCR instrument (Low-Profile)*

Bio-Rad	***Applied Biosystems***
CFX96 Touch™ Real-Time PCR Detection System	7500 Fast Real-Time PCR System (1)
Roche	7500 Fast Dx Real-Time PCR System (1)
LightCycler ®480 Real-Time PCR System (4)	QuantStudio™ 12K Flex 96-well Fast
LightCycler ®96 Real-Time PCR System (4)	QuantStudio™ 6 Flex 96-well Fast
Cobas z480 Analyzer (4)	QuantStudio™ 7 Flex 96-well Fast
Agilent Technologies	QuantStudio™ 5 Real-Time PCR System
AriaMx Real-Time PCR System	ViiA™ 7 Fast Real-Time PCR System
Qiagen	***Cepheid***
Rotor-Gene® Q (3)	SmartCycler® (3)

Table 2B. Compatible *Real Time PCR instrument (High-Profile)*

Bio-Rad	***Applied Biosystems***
CFX96 Touch Deep Well Real-Time PCR Detection System	*7500 Real-Time PCR System*
iCycler iQ Real-Time PCR Detection System	*QuantStudio™ 12K Flex 96-well*
iCycler iQ 5 Real-Time PCR Detection System	*QuantStudio™ 6 Flex 96-well*
DNA-Technology	*QuantStudio™ 7 Flex 96-well*
DTlite Real-Time PCR System (2)	*QuantStudio™ 5 Real-Time PCR System*
DT prime Real-Time Detection Thermal Cycler (2)	*ViiA™ 7 Real-Time PCR System*
Stratagene /Agilent Technologies	***Qiagen***
Mx3000P™ Real Time PCR System	*Rotor-Gen® Q (3)*
Mx3005P™ Real Time PCR System	***Cepheid***
Analytik Jena Biometra	*SmartCycler® (3)*
TOptical	***Abbot***
qTOWER 2.0	*Abbot m2000 RealTime System*

*(1)Select Ramp Speed "**Standard**".*
(2)See Annex 3 to check optical measurement exposure setting.
(3)The product should be reconstituted following the appropriate procedure (see Test Procedure, section 8.3) and transferred into the specific tubes designed to perform on Rotor-Gene® Q or SmartCycler® instruments.
(4)Shell Frame grid plate which fits in these Roche qPCR System is necessary.

Note: Tick Borne Diseases test has been validated on the following equipments: Applied Biosystems 7500 Fast Real-Time PCR System, Bio-Rad CFX96™ Real-Time PCR Detection System, Agilent Technologies AriaMx Real-Time PCR System, DNA-Technology DTprime Real-time Detection Thermal Cycler, DNA-Technology DTlite Real-Time PCR System, Rotor-Gene® Q (Qiagen), Roche Molecular Diagnostics Cobas z480 Analyzer,. When using the Applied Biosystems 7500 Fast with strips it is recommend to place a plate holder to reduce the risk of crushed tube (Ref. PN 4388506).

Precautions

Amplification technologies can amplify target nucleic acid sequences over a billion-fold and provide a means of detecting very low concentrations of target. Care must be taken to avoid contamination of samples with target molecules from other samples, or amplicons from previous amplifications. Follow these recommendations to help control contamination.

1. Separate pre-amplification steps from post-amplification steps. Use separate locations for pre- and post-amplification. Use dedicated lab equipment for each stage. Prepare samples in a laminar flow hood using dedicated equipment to minimize contamination. Set up the post-amplification area in a low-traffic area with dedicated equipment.
2. The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.
3. Be careful not to mix the BAC, REB and TBEV strips during the process.
4. Use disposable containers, disposable barrier pipette tips, disposable bench pads, and disposable gloves. Avoid washable lab wear.
5. Use a diluted bleach solution (0.2% sodium hypochlorite) to treat waste from the post-amplification and detection areas, as the waste contains amplicon. Use the bleach solution to wipe down equipment and bench areas, and to treat drains used to dispose of liquid waste.

6. Use negative controls to monitor for possible contamination during reaction setup. If reagent contamination is detected, dispose of the suspect reagents.
7. Make sure to use a well for determining TBEV, another well for determining *Rickettsia spp., Babesia microti/Babesia divergens* and *Ehrlichia chafeensis/Ehrlichia muris* and another well for determining *Borrelia burgdorferi s.l., Borrelia miyamotoi and/or Borrelia hermsii, Anaplasma phagocitophylum* and/or *Coxiella burnetii.* Be careful not to mix them throughout the process.
8. Do not use after expiration date.
9. Regular decontamination of commonly used equipment is recommended, especially micropipettes and work surfaces.
10. Specimens must be treated as potentially infectious as well as all reagents and materials that have been exposed to the samples and handled in the same manner as an infectious agent. Take necessary precautions during the collection, storage, treatment and disposal of samples.

Transport and Kit Storage

The Savvygen kits can be shipped and stored at 2-37ºC until expiration date stated in the label.

After resuspension of the Positive Control, store at -20ºC. Avoid repeated freeze/thaw cycles.

It is recommended to make aliquots of the positive control and stored at -20ºC once resuspended in order to avoid freeze & thaw cycles.

Test Procedure

Specimen Collection and processing

The proper collection and transport of clinical specimens is critical for the isolation, identification, and characterization of pathogens. Specimens (ticks, blood, serum, tissue samples, biopsy skin, cerebrospinal fluid (CSF) and synovial fluid) should be collected properly in a clean area and processed as soon as possible to avoid loss of viability of the etiological agents for suitable microbiological culture and/or to prevent nucleic acid degradation, as well as, to guarantee the quality of the test. We recommend to use fresh samples or immediately frozen. For longer storage, the samples should be frozen at -20ºC. In this case, the sample will be totally thawed and brought to room temperature before testing. Homogenize sample as thoroughly as possible prior to preparation. Freezing and thawing cycles before isolating nucleic acids should be avoided.

Given that the number of *B. burgdorferi* spirochetes in infected tissues or body fluids of patients is very low, appropriate procedures for sample collection and transport and preparation of DNA from clinical samples are critical for yielding reliable and consistent PCR results.

Blood samples might be collected using vacutainer system (tubes stabilized with EDTA) and be stored at 4ºC for up to one week. Blood components (serum and plasma samples) can be obtained after blood centrifugation and should be stored at 4 ºC for up to one week or at -20ºC indefinitely. Nucleic acids isolation from whole blood, serum or plasma could be performed using 100µL elution and/or following the recommendations of the manufacturer.

Tissue samples should be collected in a clean container and stored immediately at -20ºC or -80ºC until use.

Ticks could be collected directly from patients using a mechanical method (forceps). For bacterial growth, tissues from each tick could be pooled and cultivated in primary cell line.

For biopsy skin, the peripheral border of the cutaneous lesion should be identified and under sterile conditions 4-mm-diameter punch biopsy specimen could be obtained from the peripheral aspect of the lesion. The specimens should be stored immediately at -20ºC or -80ºC. For microorganism isolation, each skin biopsy specimen could be placed in a polystyrene tube containing 6 ml of isolation/broth medium without antibiotics and incubated at corresponding temperature during the corresponding time. If the tissue is kept in BSK medium for over 24h, some spirochetes will

have migrated from the skin biopsy to the culture medium. In this case, DNA should be prepared from both the skin biopsy and the medium.

Cerebrospinal fluid specimen (CSF) should be processed in a microbiology laboratory within 1 hour after collection or inoculated into Trans-Isolate (T-I) medium or similar for transport to the laboratory if processing within 1 hour is not feasible. These samples should be maintained at room temperature prior testing. Refrigeration is not recommended. If samples can be processed within 1 hour, centrifuge the CSF for 15 min at 1000 x g. Take the sediment and seed it in primary media. If not, incubate the T-I medium over-night.

Synovial fluids (SF) samples from patients with Lyme arthritis could be collected without additives and stored overnight initially at 4°C and then transferred to -20ºC or -80ºC until use.

After specimen microbiology cultivation (ticks, biopsy skin, cerebrospinal fluid (CSF) and synovial fluid), acid nucleic could be extracted using 1 mL of the culture after being pelleted by centrifugation (2 min 8000 x g). To perform an extraction of bacterial DNA from gram positive Bacteria addition of lysozyme is needed.

This section summarized a brief description of sample preparation. To perform more suitable sample collection, transport and storage; we suggest following the manufacturer's recommendations appearing in the instructions for use of the extraction kit used.

RNA/DNA extraction

For RNA/DNA extraction from serum, blood, tissue and microbiological culture samples you can use your manual or automatic routine optimized system. Also, you can use any commercially available RNA/DNA extraction kit and follow the manufacturer´s instructions.

• ZP02006 MagPurix Bacterial DNA Extraction Kit, using the MagPurix 12A instrument (Zinexts Life Science Corp.).

Positive Control Preparation

Note: *The Positive Control vial contains high copies number template of the assay targets with a contamination risk. Therefore, it is recommend resuspend the vial in a separate laboratory area or a special cabinet.*

Open the Positive control pouch to resuspend the lyophilized *Tick Borne* Positive Control (tube of red cap) with 100 µL of Water RNAse/DNAse free (transparent cap vial) supplied. To ensure a complete resuspension, vortex the tube thoroughly. After first use, dispense into aliquots in order to avoid multiple freeze-thaw cycles, and store them at -20ºC.

PCR protocol program

Set your thermocycler following the conditions below:

Table 3. Real time RT-PCR profile

Step	Temperature	Time	Cycles
Reverse transcription	45ºC	15 min	1
Initial denaturation	95ºC	2 min	1
Denaturation	95ºC	10 sec.	45
Annealing/Extension*	60ºC	50 sec.	

Note: Fluorogenic data should be collected during the extension step (*) through the FAM (TBEV, *Borrelia burgdorferi s.l./Borrelia miyamotoi/Borrelia hermsii* and *Ehrlichia chafeensis/Ehrlichia muris*), HEX, JOE or VIC (*Rickettsia spp* and Internal Control (IC)), ROX (*Babesia microti/Babesia divergens* and *Anaplasma phagocitophylum*), and Cy5 channels (*Coxiella burnetii*). Depending on the equipment used select the proper detection channel (see Annex 4). In Applied Biosystems 7500 Fast Real-Time PCR System and Stratagene Mx3005P™ Real Time PCR System check that passive reference option ROX is none. In the Applied Biosystems 7500 Fast Real-Time PCR System select Ramp Speed Standard in Select New Experiment/Advanced Setup/Experiment Properties.

Preparing reaction wells

A. Reconstitute the required reaction wells.

Calculate the number of required reactions including samples and controls. It is highly recommended to run at least one positive and one negative control per run.

1. Peel off protective aluminum seal from the strips/plate and
2. Pipette 15 µL of Rehydration Buffer (Blue cap vial) into each well.

B. Add samples and controls according to real-time PCR experimental plate set up.

1. Pipette 5 µL of RNA / DNA sample into each sample well (strip1+strip 2+strip 3).
2. Pipette 5 µL of resuspended Tick Borne Positive Control (tube of red cap) into each positive control well.
3. Pipette 5 µL of Negative Control (tube of orange cap) into each negative control well.
4. Cover the wells with the caps provided. Spin down briefly if needed.

C. Performing PCR.

1. Place the strips in the Real Time PCR instrument.
2. Start the run.

The fluorescence detection channels of common Real Time PCR Thermocyclers are specified in Table 4.

Table 4: Detection fluorescence channels of different Real Time PCR systems

RT- PCR THERMOCYCLER	*System Detection channels*	*Savvygen probes channels*	*Remarks*
Roche LightCycler® 96 or LightCycler®480II	465/510	FAM	Color Compensation is required only for LC480 system
	533/580	HEX	
	533/610	ROX	
	618/660	Cy5	
Applied Biosystems ABI 7500 fast	FAM	FAM	Passive reference option ROX is not mark
	VIC	HEX	
	ROX	ROX	
	Cy5	Cy5	
Bio-Rad CFX96 ™	FAM	FAM	
	HEX	HEX	
	ROX	ROX	
	Cy5	Cy5	
Agilent AriaMx	FAM	FAM	
	HEX	HEX	
	ROX	ROX	
	Cy5	Cy5	
DNA-Technology DTlite / DTprime	FAM	FAM	
	HEX	HEX	
	ROX	ROX	
	Cy5	Cy5	
Smartcycler® Cepheid	Channel 1	FAM	
	Channel 2	HEX	
	Channel 3	ROX	
	Channel 4	Cy5	
Abbott m2000rt	FAM	FAM	
	HEX	HEX	
	ROX	ROX	
	Cy5	Cy5	
Rotor-Gene®Q Qiagen	Green	FAM	
	Yellow	HEX	
	Orange	ROX	
	Red	Cy5	

Interpretation of results

Interpretation of results can be automatically performed if programed by the user using the RT-qPCR instrument software following manufacturer´s instructions. It is required to run assay controls (positive and negative controls) in each run to validate the reaction.

Note: *The positive controls used in each run, must show an amplification curve for Tick Borne targets which validates the reaction while the negative control well should demonstrate an absence of signal (except internal control target).*

Strip 1: results of strip 1 containing the multiplex reaction mixture for the detection of ***Borrelia, Anaplasma & Coxiella*** are interpreted as follows:

- A sample is considered positive for ***Borrelia burgdorferi s.l./Borrelia miyamotoi/ Borrelia hermsii*** if there is an amplification signal in FAM channel, the Ct value obtained is less than 40 and the internal control shows or not an amplification signal. Sometimes, the detection of internal control is not necessary because a high copy number of target can cause preferential amplification of target-specific nucleic acids (see figure 2).

- A sample is considered positive for ***Anaplasma phagocitophylum*** if there is an amplification signal in ROX channel, the Ct value obtained is less than 40 and the internal control shows or not an amplification signal. Sometimes, the detection of internal control is not necessary because a high copy number of target can cause preferential amplification of target-specific nucleic acids (see figure 2).

- A sample is considered positive for ***Coxiella burnetii*** if there is an amplification signal in Cy5 channel, the Ct value obtained is less than 40 and the internal control shows or not an amplification signal. Sometimes, the detection of internal control is not necessary because a high copy number of target can cause preferential amplification of target-specific nucleic acids (see figure 2).

- A sample is considered negative, if the sample shows no amplification signal in the detection system but the internal control is positive (*HEX, VIC or JOE channel) (see figure 2).

Strip 2: results of the first strip containing the multiplex reaction mixture for the detection of ***Rickettsia, Babesia & Ehrlichia*** are interpreted as follows:

- A sample is considered positive for Rickettsia spp. if there is an amplification signal in HEX/VIC/JOE channel and the Ct value obtained is less than 40 (see figure 3).

- A sample is considered positive for Babesia microti/Babesia divergens if there is an amplification signal in ROX channel and the Ct value obtained is less than 40 (see figure 3).

- A sample is considered positive for Ehrlichia chafeensis/Ehrlichia muris if there is an amplification signal in FAM channel and the Ct value obtained is less than 40 (see figure 3).

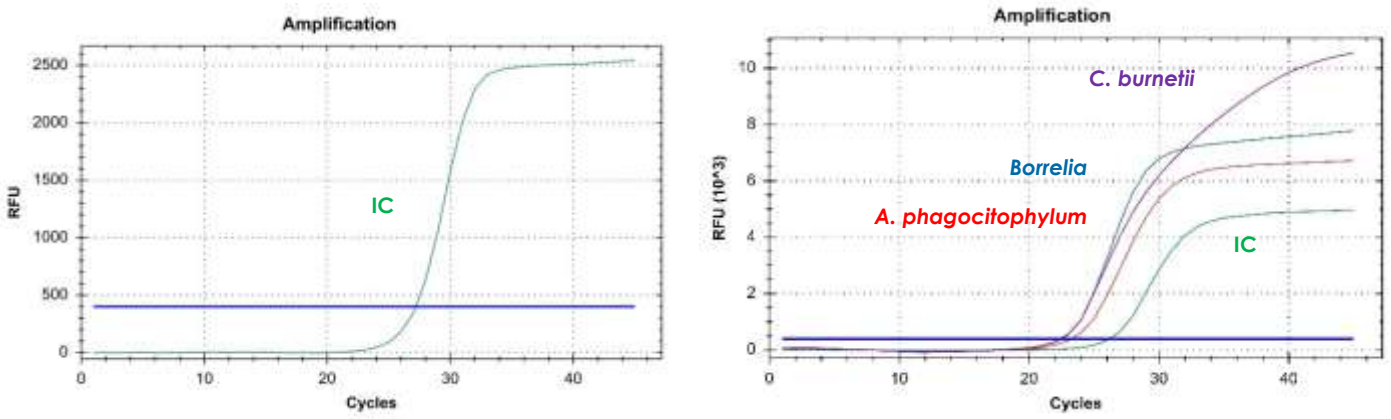
- A sample is considered negative, if the sample shows no amplification signal in the detection system, but the internal control of ***Borrelia, Anaplasma & Coxiella*** assay is positive (see figure 3).

- The experiment is considered failed if there is an amplification signal in the Negative Control well and/or there is not amplification signal in the Positive Control well. We recommend to repeat the assay again.

Strip 3: results of the first strip containing the multiplex reaction mixture for the detection of ***TBEV*** are interpreted as follows:

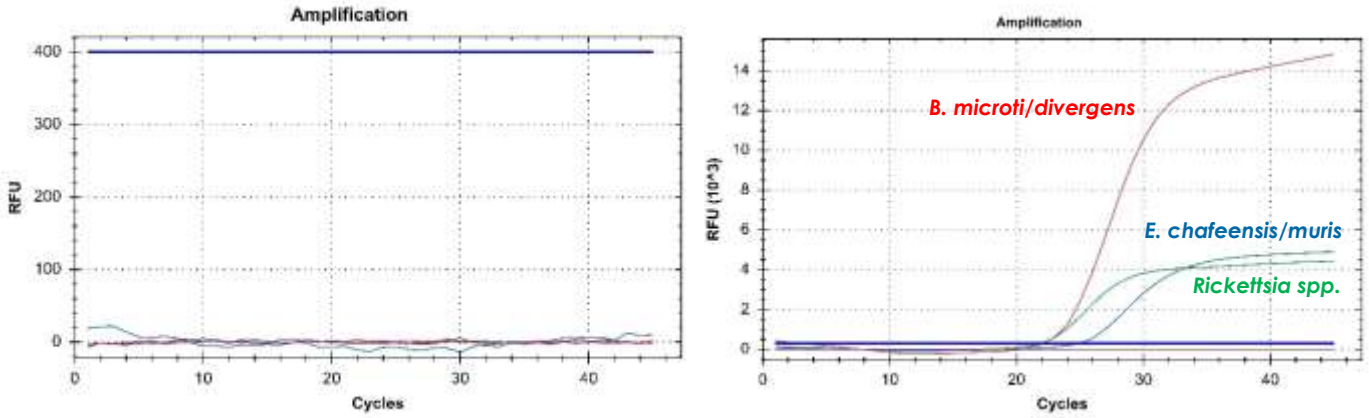
- A sample is considered positive for TBEV if there is an amplification signal in FAM channel and the Ct value obtained is less than 40 (see figure 4).
- A sample is considered negative, if the sample shows no amplification signal in the detection system, but the internal control of ***Borrelia, Anaplasma & Coxiella*** assay is positive (see figure 4).
- **Invalid run-** The assay should be considered as invalid and a new run should be performed if there is signal of amplification for one of the pathogens in the negative control well or absence of signal in the positive control well.

Note: If an amplification curve for the internal control is not shown, the sample should be retested by dilution of the original sample 1:10. Alternatively it is recommended to repeat the nucleic acid extraction due to possible problems caused by PCR inhibitors.



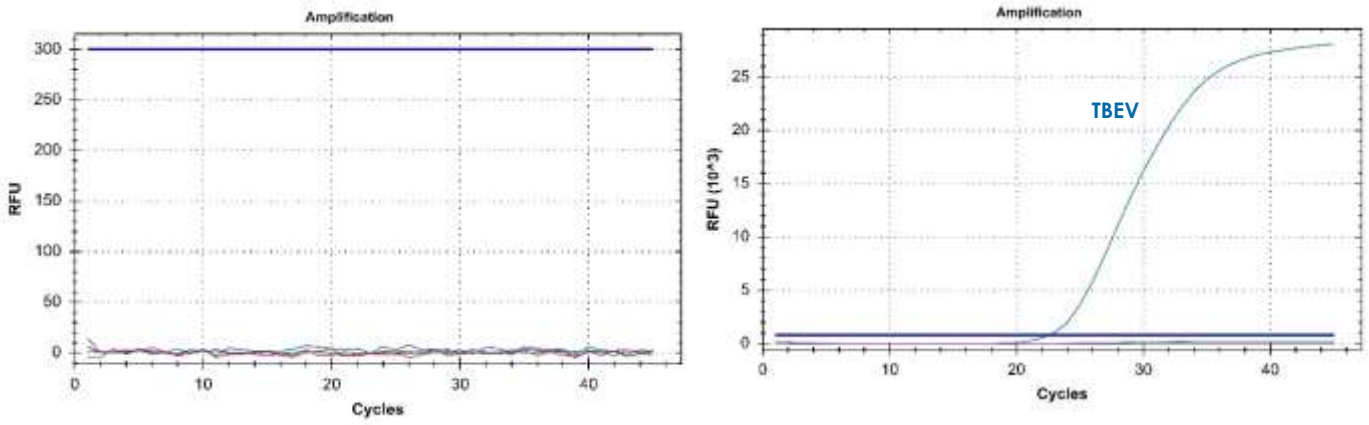
Negative control **Positive control**

Figure 2. Correct run of negative and positive control run on the Bio-Rad CFX96™ Real-Time PCR Detection System (Multiplex reaction mix *Borrelia, Anaplasma & Coxiella*).



Negative control **Positive control**

Figure 3. Correct run of negative and positive control run on the Bio-Rad CFX96™ Real-Time PCR Detection System (Multiplex reaction mix *Rickettsia, Babesia & Ehrlichia*).



Negative control **Positive control**

Figure 4. Correct run of negative and positive control run on the Bio-Rad CFX96™ Real-Time PCR Detection System (Monoplex reaction mix TBEV).

Limitations of the test

- The results of the test should be evaluated by a health care professional in the context of medical history, clinical symptoms and other diagnostic tests.
- Although this assay can be used with other types of samples it has been validated only with RNA/DNA extracted from blood, serum, tissue samples and microbiological culture from ticks, biopsy skin, cerebrospinal fluid (CSF) and synovial fluid.
- The quality of the test depends on the quality of the sample; proper extracted nucleic acid from clinical samples must be extracted. Unsuitable collection, storage and/or transport of specimens may give false negative results.
- Extremely low levels of target below the limit of detection might be detected, but results may not be reproducible.
- There is a possibility of false positive results due to cross-contamination by contamination by Tick Borne diseases, either samples containing high concentrations of target RNA/DNA or contamination due to PCR products from previous reactions.

Quality Control

Savvygen Tick Borne Detection Kit contains a positive and a negative control that must be included in each run to correctly interpret the results. Also, the internal control (IC) in each well included in the strip 1 (Borrelia, Anaplasma & Coxiella 8-well strips) confirms the correct performance of the technique.

Performance Characteristics

Clinical sensitivity and specificity

The clinical performance of Savvygen Tick Borne assay for Borrelia, Anaplasma & Coxiella 8-well strip was tested using 95 DNA samples extracted from microbiological culture from ticks, biopsy skin, cerebrospinal fluid (CSF) and synovial fluid. A total of 17 well characterized Borrelia strains comprising 9 different *Borrelia burgdorferi* sensu lato genospecies, notably *B. japonica* (n=1), *B. burgdorferi* sensu stricto (n=2; B31 and PBre strains*), B. bavariensis* (n=1; PBi strain), *B. garinii* (n=5; PBr, PHei, PWudII, PRef and PLa strains), *B. bissettii* (n=1; PGeb strain), *B. afzelii* (n=2; PKo and PVPM strains), *B. lusitaniae* (n=1; Poti B2strain), *B. spielmanii* (n=1; PSigII strain), *B. valaisiana* (n=1; VS116 strain)) were included. Additionally, two relapsing fever control strains *B. hermsii* (n=1) and *B. miyamotoi* (n=1) were included as well as the potentially cross-reactive spirochaetes *Leishmania spp.* (n=2) and *Treponema spp.* (n=1) were tested. The Savvygen Tick Borne successfully detected all tested Borrelia sensu lato genospecies and the relapsing fever group strains *B. hermsii* and *B. miyamotoi.* No cross reactivity was observed with DNA from Leptospira and Treponema species for this assay.

In addition, the Savvygen Tick Borne assay for Borrelia, Anaplasma & Coxiella 8-well strip was evaluated using 3 INSTAND Coxiella burnetii & Bacillus anthracis panels from 2017 and 2018, as well as, 17 additional tissue samples. The results were compared with the final EQA program reports or with those obtained by a commercial qPCR assay (EXOone Coxiella burnetii (EXOPOL)). All *Coxiella burnetii* positive samples (6/12) from 3 INSTAND programs were detected and 15/17 tissue samples showed a positive result in the identification of *Coxiella burnetii*.

The clinical performance of Savvygen Tick Borne Kit for Rickettsia, Babesia & Ehrlichia 8-well strips and for TBEV 8-well strips was evaluated using 90 samples from 16 different QCMD panels (Tropical diseases and borreliosis panels) and 2 clinical specimens (serum and blood). Savvygen Tick Borne assay for TBEV 8-well strips found 8/90 positive samples for Tick Borne Encephalitis Virus.

In conclusion, the results show a high sensitivity and specificity to detect *TBEV, Rickettsia spp., Borrelia burgdorferi s.l., Borrelia miyamoto* and/or *Borrelia hermsii, Anaplasma phagocitophylum* and *Coxiella burnetii* using Savvygen Tick Borne assay.

Analytical sensitivity

In series of experiments to establish the limit of detection for each pathogen- *Borrelia, Anaplasma & Coxiella* (strip 1). *Rickettsia, Babesia &* Ehrlichia (strip 2) and *TBEV (strip 3),* a 10-fold dilution of 10^7 to 10^1 copies/reaction was conducted for each target. According to the results, this assay has a detection limit of ≥10 viral RNA/DNA copies per reaction (Figure 5, 6, 7, 8, 9, 10, 11).

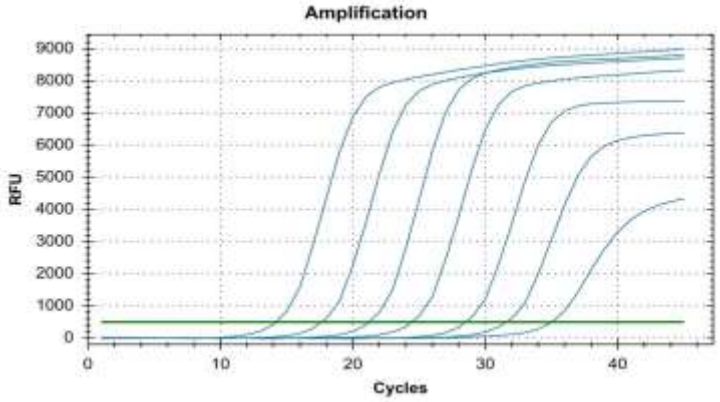


Figure 5. Dilution series of Borrelia burgdorferi/Borrelia miyamotoi/ B. hermsii (10^7-10^1 copies/rxn) template run on the Bio-Rad CFX96™ RealTime PCR Detection System (Multiplex reaction mix Borrelia, Anaplasma & Coxiella, channel FAM)

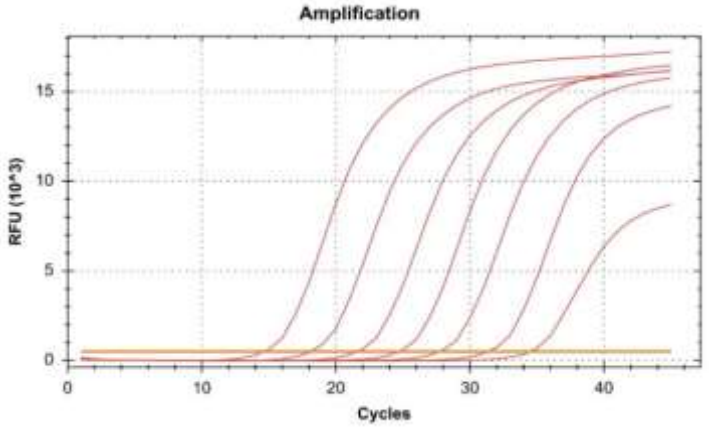


Figure 6. Amplification plot for 10-fold dilution series of *Anaplasma phagocitophylum* template ranging from 10^7 to 10^1 copies/rxn (Multiplex reaction mix Borrelia, Anaplasma & Coxiella, ROX channel).

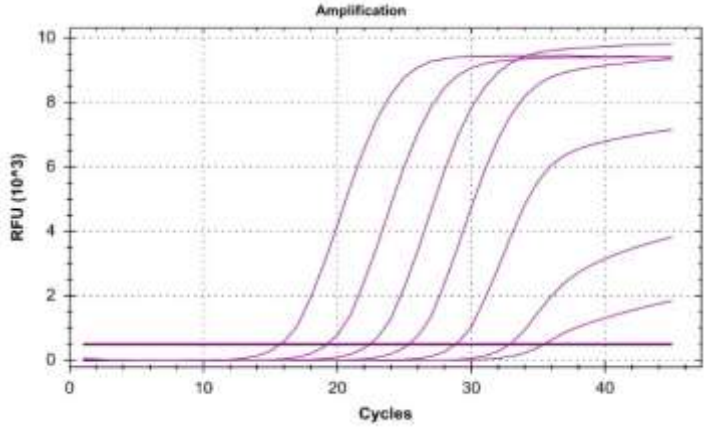


Figure 7. Amplification plot for 10-fold dilution series of *Coxiella burnetii* template ranging from 10^7 to 10^1 copies/rxn (Multiplex reaction mix Borrelia, Anaplasma & Coxiella, Cy5 channel).

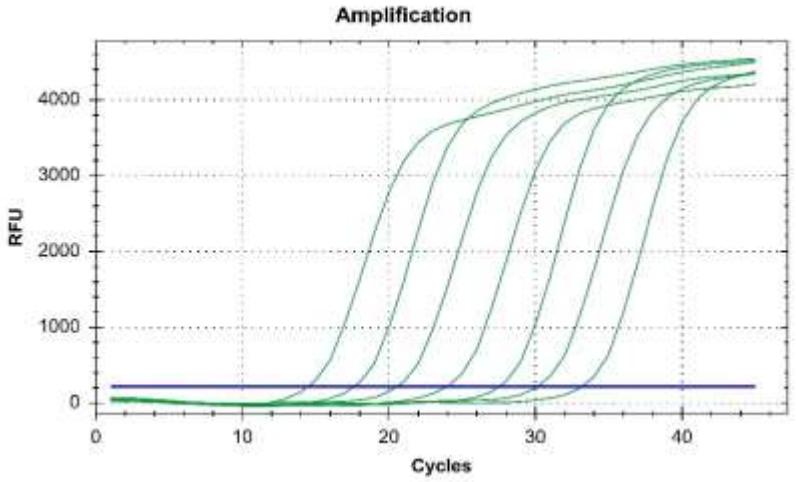


Figure 8. Amplification plot for 10-fold dilution series of ***Rickettsia spp***. template ranging from 10^7 to 10^1 copies/rxn (Multiplex reaction mix, Rickettsia, Babesia & Ehrlichia, HEX channel).

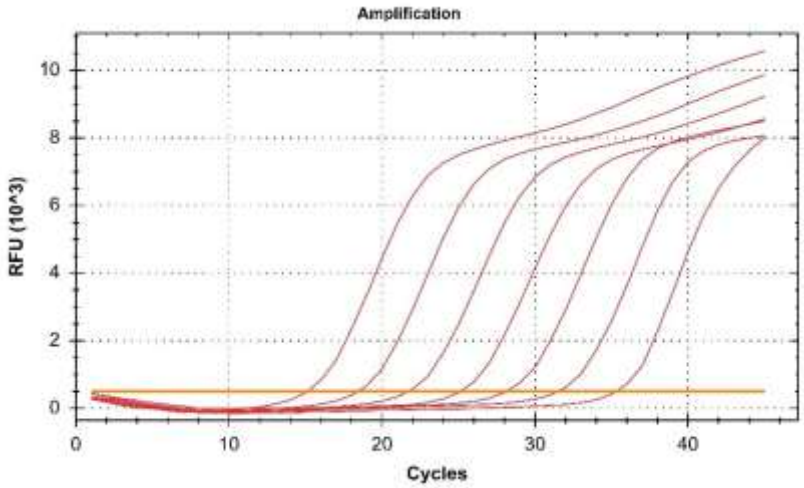


Figure 9. Amplification plot for 10-fold dilution series of *Babesia microti/Babesia divergens* template ranging from 107 to 101 copies/rxn (Multiplex reaction mix TBEV, Rickettsia, Babesia & Ehrlichia, ROX channel).

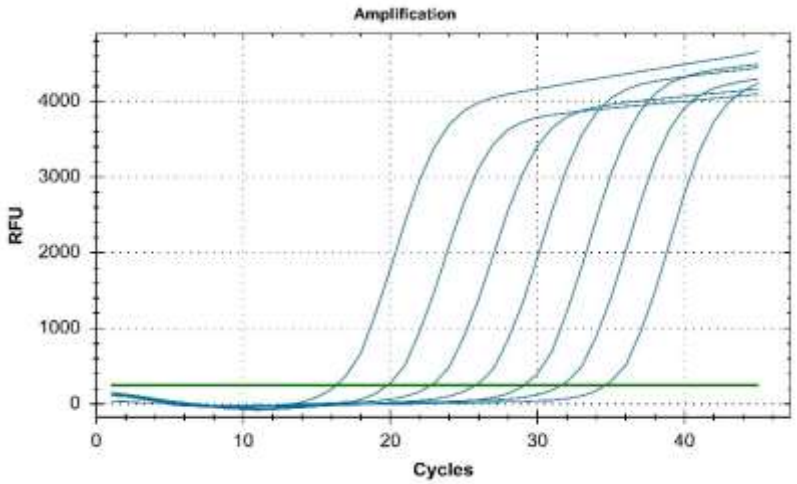


Figure 10. Amplification plot for 10-fold dilution series of *Ehrlichia chafeensis/Ehrlichia muris* template ranging from 10^7 to 10^1 copies/rxn (Multiplex reaction mix TBEV, Rickettsia, Babesia & Ehrlichia, Cy5 channel).

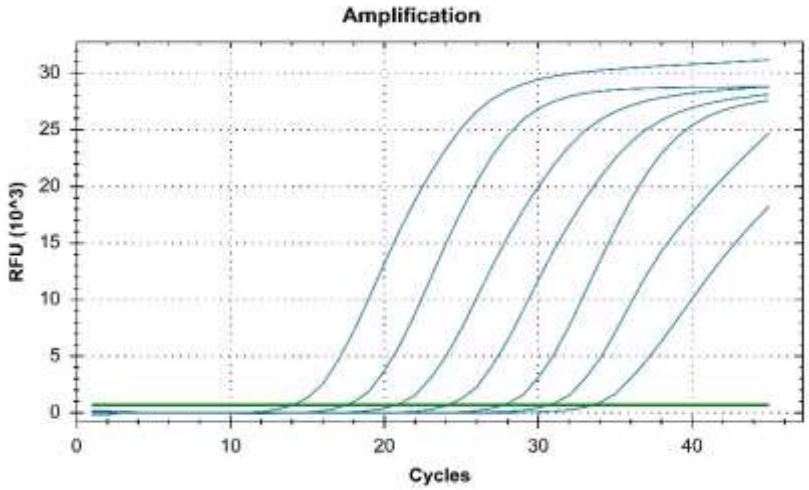


Figure 11. Dilution series of TBEV (10^7-10^1 copies/rxn) template run on the Bio-Rad CFX96™ Real-Time PCR Detection System (Monoplex reaction mix TBEV, channel FAM).

Analytical specificity

The specificity for the Savvygen Tick Borne assay was confirmed by testing a panel consisting of different microorganisms representing the most common Tick Borne pathogens. No cross-reactivity was observed for any of the listed species (table 5).

Table 5. Cross-reactivity testing.

Pathogen	Cross-Reactivity Test		
	Savvygen™ Tick Borne		
	Strip 1 Borrelia, Anaplasma & Coxiella	**Strip 2** Rickettsia, Babesia & Ehrlichia	**Strip 3** TBEV
Anaplasma marginale	+	-	-
Bartonella henselae strain Houston-1	-	-	-
Borrelia hermsii	+	-	-
Borrelia lusitianae	+	-	-
Borrelia valaisiana	+	-	-
Borrelia azfelii strain P-Ko/1984	+	-	-
Borrelia bavariensis	+	-	-
Borrelia bisetti	+	-	-
Borrelia burgdorferi sensu stricto strain IRS	+	-	-
Borrelia burgdorferi sensu stricto strain B31	+	-	-
Borrelia garinii	+	-	-
Borrelia japonica	+	-	-
Borrelia miyamotoi	+	-	-
Borrelia spielmanii	+	-	-
Coxiella burnetii strain Nine Mile Q	+	-	-
Theileria annulata	-	-	-
Rickettsia conorii. strain Moroccan	-	+	-
Leptospira	-	-	-
Treponema phagedenis	-	-	-
Tick Borne Encephalitis Virus (TBEV) strain Neudorfl	-	-	+

Analytical reactivity

The reactivity of Savvygen Tick Borne Real Time PCR Detection Kit was evaluated against TBEV strain NeordofI, Ricketsia coronii strain Moroccan, synthetic sequence of Babesia microti, synthetic sequence of Babesia divergens, synthetic sequence of Ehrlichia chafeensis, synthetic sequence of Ehrlichia muris, Borrelia azfelii (PKo/1984 and PVPM strains), Borrelia bavariensis (PBi strain), Borrelia bissettii (PGeb strain), Borrelia bisettiae, Borrelia burgdorferi sensu stricto (B31, IRS and PBre strains), Borrelia garinii (PHei, PWudII, PRef and PLa strains) and Borrelia garinii OspA Typ3 strains, B. japonica, B. lusitaniae (Poti B2 strain), B. spielmanii (PSigII strain), Borrelia valaisiana (VS116 strain), B. hermsii and B. miyamotoi, Anaplasma phagocitophylum and Coxiella burnetii strain Nine Mile Q showing positive results.

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Symbols for IVD Components and Reagents

Manufacturer	Use by	For *in vitro* diagnostic use only
Lot number	Temperature limitation	Consult instructions for use
Catalogue number	Contains sufficient for <n> test	Buffer (sample diluent)
Keep Dry		