

Instruction for Use

diarellaBorrelia/Rickettsia real time PCR Kit

For qualitative in vitro detection of Borrelia burgdorferi sensu lato DNA and Rickettsia species DNA, extracted from biological specimens incl. ticks.

REF

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1 Intended Use

The diarellaBorrelia/Rickettsia real time PCR is an assay for the detection of *Borrelia burgdorferi* sensu lato DNA and Rickettsia species DNA, extracted from biological specimens incl. ticks.

2 Pathogen Information

Borrelia are gram-negative bacteria of the spirochaete family. Members of the genus *Borrelia* are the causative agents of two important tick-borne diseases: relapsing fever and Lyme disease.

In Europe, Lyme borreliosis is the most common vector-borne disease. The highest incidence is reported from Austria, Switzerland, the Czech Republic, Germany, Slovenia, as well as from the northern countries bordering the Baltic Sea.

Lyme borreliosis is a multi-system disorder, which can lead to severe complications of the neurological system, the heart and the joints. At an early stage of its manifestation borreliosis is treatable with antibiotics, however, clinical diagnosis is complicated. Antibodies are not detectable in the blood until weeks after infection and symptoms are highly variable.

Rickettsia is a genus of bacteria of the tribe Rickettsiae, made up of small, gram-negative, rod-shaped to coccoid, often pleomorphic microorganisms, which multiply only in host cells. Organisms occur in the cytoplasm of tissue cells or free in the gut lumen of lice, fleas, ticks, and mites and are transmitted by their bites.

R. conorii is the etiologic agent of Boutonneuse Fever (a tickborne disease endemic in the Mediterranean area, Crimea, Africa, and India with chills, fever, primary skin lesion (tache noire), and rash appearing on the second to fourth day).

R. prowazekii is transmitted between humans by the human body louse and from flying squirrels to humans by fleas and lice. *R. prowazekii* is the agent of epidemic typhus and Brill-Zinsser disease. Epidemic typhus is a form of typhus so named because the disease often causes epidemics following wars and natural disasters. The Brill-Zinsser disease is characterized by a delayed relapse of epidemic typhus. After a patient contracts epidemic typhus from the fecal matter of an infected louse (*Pediculus humanus*), the rickettsia can remain latent and reactivate months or years later, with symptoms similar to or even identical to the original attack of typhus, including a maculopapular rash.

R. typhi is the cause of murine typhus, which is transmitted to humans chiefly by rat fleas. Murine typhus is a mild, acute, endemic form of typhus characterized by fever, headache, and muscular pain. Rickettsial diseases are not common in communities with good sanitary standards, since prevention depends on controlling the rodent and insect populations. Major epidemics have occurred, especially in times of war when standards of sanitation drop.

3 Principle of the Test

The diarellaBorrelia/Rickettsia real time PCR Kit contains specific primers and dual-labeled probes for the amplification and detection of *Borrelia burgdorferi* sensu lato DNA and Rickettsia species DNA.

The presence of nucleic acid is detected by an increase in fluorescence due to hydrolysis of the probes during amplification. The fluorescence of the Borrelia-specific probes is measured in the FAM channel. The fluorescence of the Rickettsia-specific probes is measured in the Cy5 channel.

Furthermore, diarellaBorrelia/Rickettsia real time PCR Kit contains a Control DNA, which is added during DNA extraction and detected in the same reaction by a differently labeled probe. The Control DNA allows the detection of PCR inhibition and acts as control, that the nucleic acid was isolated from the clinical specimen. The fluorescence of the Control DNA is measured in the HEX channel.

4 Package Contents

The reagents supplied are sufficient for 32 or 96 reactions respectively.

Table 1: Components of the diarellaBorrelia/Rickettsia real time PCR Kit.

Label	Lid Color	Content	
		32	96
Reaction Mix	yellow	1 x 512 µl	1 x 1536 µl
Positive Control	red	1 x 50 µl	1 x 100 µl
Negative Control	green	1 x 50 µl	1 x 100 µl
Control DNA	colorless	1 x 160 µl	1 x 480 µl

5 Equipment and Reagents to be Supplied by User

- DNA purification kit (e.g. NukEx Pure RNA/DNA, gerbion Cat. No. G05004 or NukEx Mag RNA/DNA, gerbion Cat. No. G05012)
- PCR grade Water
- Sterile microtubes
- Pipets (adjustable volume)
- DNase/RNase-free disposable pipette tips with aerosol barriers
- Table centrifuge
- Vortexer
- Real time PCR instrument
- Optical PCR reaction tubes or optical PCR reaction plates with optical foil
- Optional: Liquid handling system for automation

6 Transport, Storage and Stability

The diarellaBorrelia/Rickettsia real time PCR Kit is shipped on dry ice or cool packs All components must be stored at maximum -18°C in the dark immediately after receipt. Do not use reagents after the date of expiry printed on the package. Up to 20 freeze and thaw cycles are possible. For convenience, opened reagents can be stored at +2-8°C for up to 6 months. Protect kit components from direct sunlight during the complete test run.

7 Warnings and Precautions

Read the Instructions for Use carefully before using the product.

Before first use check the product and its components for:

- Use of this product is limited to personnel specially instructed and trained in the techniques of real-time PCR procedures.
- Specimens should always be treated as infectious and/or biohazardous in accordance with safe laboratory procedures.
- Avoid microbial and nuclease (DNase/RNase) contamination of the eluates and the components of the kit.
- Always use DNase/RNase-free disposable pipette tips with aerosol barriers.
- Always wear protective disposable powder-free gloves when handling kit components.
- Use separated and segregated working areas for (1) sample preparation, (2) reaction setup and (3) amplification/detection activities. The workflow in the laboratory should proceed in unidirectional manner. Always wear disposable gloves in each area and change them before entering a different area.
- Dedicate supplies and equipment to the separate working areas and do not move them from one area to another.
- Store positive and/or potentially positive material separated from all other components of the kit.
- Do not open the reaction tubes/plates post amplification, to avoid contamination with amplicons.
- Additional controls may be tested according to guidelines or requirements of local, state and/or federal regulations or accrediting organizations.
- Do not autoclave reaction tubes after the PCR, since this will not degrade the amplified nucleic acid and will bear the risk to contaminate the laboratory area.
- Discard sample and assay waste according to your local safety regulations.

8 Sample Preparation

Purified DNA is suitable for downstream processing in real time PCR. For the extraction and purification of DNA from various biological materials, commercial kits are available. The operator needs to evaluate the suitability of respective DNA extraction kit.

Commercial kits for RNA isolation such as the following are recommended:

- NukEx Pure RNA/DNA, gerbion
- NukEx Mag RNA/DNA, gerbion

Important: In addition to the samples always run a ,water control' in your extraction. Treat this water control analogous to a sample.

Comparing the amplification of the Control DNA in the samples to the amplification of the internal control in the water control will give insights on possible inhibitions of the real time PCR. Furthermore, possible contaminations during DNA extraction will be detectable.

Please note the chapter ,Control DNA'.

If the real time PCR is not performed immediately, store extracted DNA according to the instructions given by the manufacturer.

9 Control DNA

A Control DNA is supplied and can be used as extraction control or only as inhibition control. This allows the user to control the DNA isolation procedure and to check for possible real time PCR inhibition.

a) Control DNA used as Extraction Control:

diarellaBorreliar/Rickettsia real time PCR Control DNA is added to the DNA extraction. Add 5 µl Control DNA per extraction (5 µl x (N+1)). Mix well. Perform the DNA isolation according to the manufacturer's instructions. Please follow protocol A.

The Control DNA must be added to the Lysis Buffer of the extraction kit.

b) Control DNA used as Internal Control of the real time PCR:

If only inhibition will be checked please follow protocol B.

10 Real time PCR

10.1 Important Points Before Starting:

- Please pay attention to the chapter ,Warnings and Precautions‘.
- Before setting up the real time PCR familiarise yourself with the real time PCR instrument and read the user manual supplied with the instrument.
- The programming of the thermal profile should take place before the PCR set up.
- In every PCR run one Positive Control and one Negative Control should be included.
- Before each use, all reagents should be thawed completely at room temperature, thoroughly mixed and centrifuged very briefly.

10.2 Procedure

If the Control DNA is used to control both, the real time PCR and the DNA isolation procedure, please follow protocol A. If the Control DNA is solely used to detect possible inhibition of the real time PCR, please follow protocol B.

Protocol A

The Control DNA was added during DNA extraction (see chapter ,Control DNA‘). In this case, prepare the Master Mix according to Table 2.

The Master Mix contains all of the components needed for PCR except the sample. Prepare a volume of Master Mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 2:Preparation of the Master Mix (Control DNA was added during DNA extraction)

Volume per Reaction	Volume Master Mix
16.0 µl Reaction Mix	16.0 µl x (N+1)

Protocol B

The Control DNA is used for the control of the real time PCR only (see chapter ,Control DNA‘). In this case, prepare the Master Mix according to Table 3.

The Master Mix contains all of the components needed for real time PCR except the sample. Prepare a volume of Master Mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 3: Preparation of the Master Mix (Control DNA) is added directly to the Master Mix)

Volume per Reaction	Volume Master Mix
16.0 µl Reaction Mix	16.0 µl x (N+1)
0.5 µl Control DNA*	0.5 µl x (N+1)*

*The increase in volume caused by adding the Control DNA is not taken into account when preparing the PCR assay.

Protocol A and B: real time PCR set-up

- Place the number of optical PCR reaction tubes needed into the respective tray of the real time PCR instrument or use an optical PCR reaction plate.
- Pipet **16 µl** of the Master Mix into each optical PCR reaction tube / optical PCR reaction plate.
- Add **4 µl** of the eluates from the DNA isolation (including the eluate of the water control), the Positive Control and the Negative Control to the corresponding optical PCR reaction tube / optical PCR reaction plate (Table 4).
- Close the optical PCR reaction tubes/ optical PCR reaction plate immediately after filling in order to reduce the risk of contamination.

Table 4: Preparation of the real time PCR

Component	Volume
Master Mix	16.0 µl
Sample	4.0 µl
Total Volume	20.0 µl

10.3 Instrument Settings

For the real time PCR use the thermal profile shown below.

Table 5: real time PCR thermal profile

Description	Time	Temperature	Number of Cycles
<i>Initial Denaturation</i>	10 min	95°C	1
<i>Amplification of DNA</i>			
Denaturation	10 sec	95°C	
Annealing	20 sec	60°C	45
		Acquisition at the end of this step	
Extension	10 sec	72°C	

If in the same run samples should be tested for pathogens with RNA genome, e.g. with the virellaTBE real time RT-PCR Kit, use the thermal profile shown in Table 6.

Table 6: real time RT-PCR thermal profile

Description	Time	Temperature	Number of Cycles
<i>Reverse Transcription</i>	20 min	45°C	1
<i>Initial Denaturation</i>	5 min	95°C	1
<i>Amplification of DNA</i>			
Denaturation	10 sec	95°C	
Annealing	20 sec	60°C	45
		Acquisition at the end of this step	
Extension	10 sec	72°C	

Dependent on the real time instrument used, further instrument settings have to be adjusted according to Table 7.

Table 7: Overview of the instrument settings required for the diarellaBorrelia/Rickettsia real time PCR.

Real time PCR Instrument	Parameter	Detection Channel	Notes
LightCycler 480II	Borrelia	FAM (465-510)	no color compensation required
	Rickettsia	CY5 (618-660)	
	Control DNA	HEX (533-580)	
Stratagene Mx3000P / Mx3005P	Borrelia	FAM	Gain 8
	Rickettsia	CY5	Gain 4
	Control DNA	HEX	Gain 1
ABI 7500	Borrelia	FAM	Option Reference Dye ROX: NO
	Rickettsia	CY5	
	Control DNA	JOE	
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	Borrelia	Green	Gain 5
	Rickettsia	Red	Gain 5
	Control DNA	Yellow	Gain 5
Mic qPCR Cyclor	Borrelia	Green	Gain 8
	Rickettsia	Red	Gain 10
	Control DNA	Yellow	Gain 10

11 Data Analysis

The *Borrelia* specific amplification is measured in the FAM channel. The *Rickettsia* specific amplification is measured in the CY5 channel. The amplification of the Control DNA is measured in the HEX channel.

Table 8: Interpretation of results

Signal/Ct Values			Interpretation
FAM Borrelia	CY5 Rickettsia	HEX Control DNA	
positive	negative	positive or negative*	Positive result, the eluate contains <i>Borrelia</i> DNA.
negative	positive	positive or negative*	Positive result, the eluate contains <i>Rickettsia</i> DNA.
negative	negative	≤ 34**	Negative result, the eluate contains neither <i>Borrelia</i> DNA nor <i>Rickettsia</i> DNA.
negative	negative	negative or > 34**	No diagnostic statement can be made. The real time PCR is either inhibited or errors occurred while DNA extraction.

* A strong positive signal in the FAM and/or CY5 can inhibit the amplification of the Control DNA. In such cases the result for the Control DNA can be neglected.

** Depending on the PCR instrument and/or the chosen extraction method, the Ct values might be shifted. The water control can be used as reference. If the HEX Ct value of a sample differs a lot from the water control, partial inhibition has occurred, leading to false negative results in case of weak positive samples.

Figure 1 and **Figure 2** show examples for positive and negative real time PCR results.

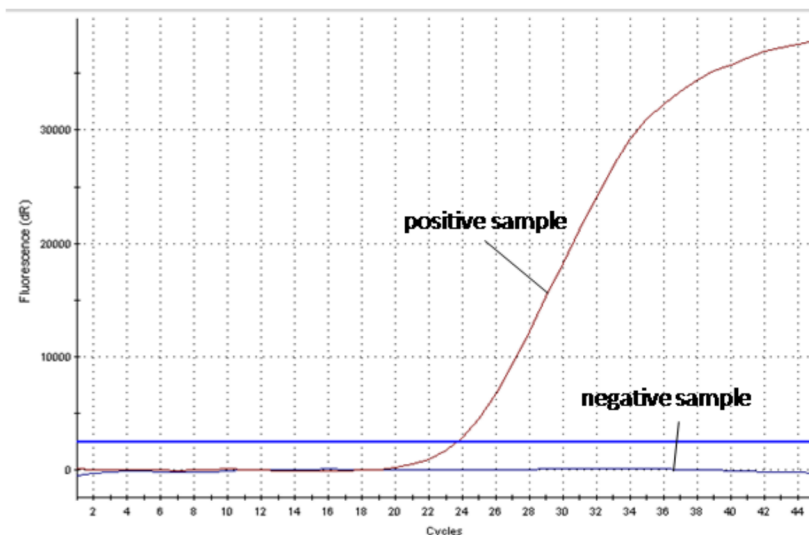


Figure 1: The positive sample shows bacteria specific amplification in the FAM or CY5 channel, whereas no fluorescence signal is detected in the negative sample.

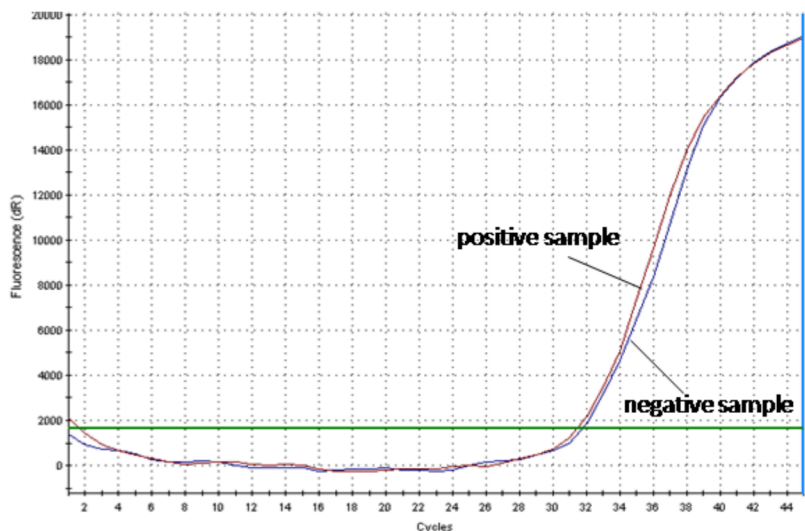


Figure 2: The positive sample as well as the negative sample show a signal in the Control DNA specific HEX channel. The amplification signal of the Control DNA in the negative sample shows, that the missing signal in the bacteria specific FAM or CY5 channel is not due to PCR inhibition or failure of DNA isolation, but that the sample is a true negative.

12 Assay Validation

Set a threshold as follows:

Negative Controls

All negative controls should be below the threshold. If there is a potential contamination (appearance of a curve in the negative control or a cluster of curves in specimens at high C_T – for example above 36), results obtained are not interpretable and the whole run (including extraction) has to be repeated.

Positive Controls

All the positive controls must show a positive (i.e. exponential) amplification curve. The positive controls must fall below a C_T of 30.

Internal Controls

All internal controls must show a positive (i. e. exponential) amplification curve. The internal control must fall below a C_T of ≤ 34 . If the internal control is above C_T 34, this points to a purification problem or a strong positive sample that can inhibit the IC. In the latter case, the assay is valid. If a water control run is performed, the IC must fall below a C_T of ≤ 34

13 Limitations of the Method

- Strict compliance with the Instructions for Use is required for optimal results.
- Use of this product is limited to personnel specially instructed and trained in the techniques of real-time PCR and in vitro diagnostic procedures.
- Good laboratory practice is essential for proper performance of this assay.
- All reagents should be closely monitored for impurity and contamination. Any suspicious reagents should be discarded.
- This assay must not be used on a biological specimen directly. Appropriate nucleic acid extraction methods have to be conducted prior to using this assay.
- The presence of PCR inhibitors may cause false negative or invalid results.
- Potential mutations within the target regions of the *Borrelia* or *Rickettsia* genome covered by the primers and/or probes used in the kit may result in failure to detect the respective DNA.
- As with any diagnostic test, results of the *diarellaBorrelia/Rickettsia* real time PCR kit need to be interpreted in consideration of all clinical and laboratory findings.

14 Troubleshooting

The following troubleshooting guide is included to help you with possible problems that may arise when performing a real time PCR. If you have further questions, please do not hesitate to contact our scientists on info@gerbion.com.

No fluorescence signal in the FAM channel and/or Cy5 channel of the Positive Control.

The selected channel for analysis does not comply with the protocol	Select the FAM channel for analysis of the Borrelia DNA specific amplification, the Cy5 channel for Rickettsia DNA specific and the HEX channel for the amplification of the Control DNA.
Incorrect configuration of the real time PCR	Check your work steps and compare with ,Procedure' on page 7.
The programming of the thermal profile is incorrect	Compare the thermal profile with the protocol (Table 5, page 9).
Incorrect storage conditions for one or more kit components or kit expired	Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in ,Transport, Storage and Stability', page 5.

Weak or no signal of the Control DNA and simultaneous absence of a signal in the bacteria specific FAM channel and/or Cy5 channel.

real time PCR conditions do not comply with the protocol	Check the real time PCR conditions (page 9).
real time PCR inhibited	Make sure that you use an appropriate isolation method and follow the manufacturer's instructions. Make sure that the ethanol-containing washing buffers have been completely removed. An additional centrifugation step at high speed is recommended before elution of the DNA.
DNA loss during isolation process	In case the Control DNA was added before extraction, the lack of an amplification signal can indicate that the DNA isolation was not successful. Make sure that you use an appropriate isolation method (commercial kits are recommended) and stick to the manufacturer's protocol.
Incorrect storage conditions for one or more components or kit expired	Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in ,Transport, Storage and Stability'.

Detection of a fluorescence signal in the FAM channel and/or Cy 5 channel of the Negative Control.

Contamination during preparation of the PCR	Repeat the real time PCR in replicates. If the result is negative in the repetition, the contamination occurred when the samples were pipetted into the optical PCR reaction tubes. Make sure to pipet the Positive Control last and close the optical PCR reaction tube immediately after adding the sample. If the same result occurs, one or more of the kit components might be contaminated. Make sure that work space and instruments are decontaminated regularly. Use a new kit and repeat the real time PCR.
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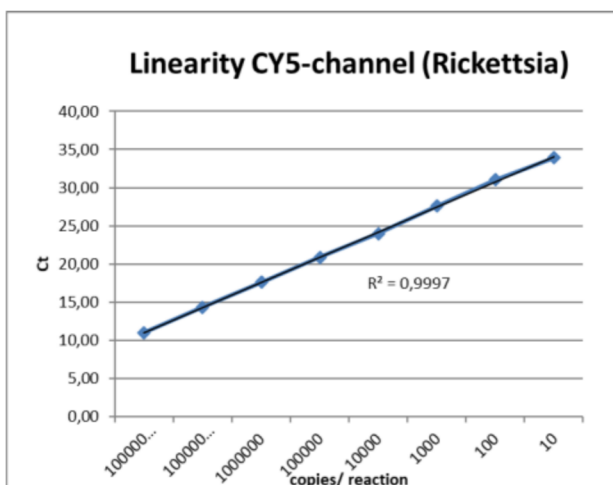
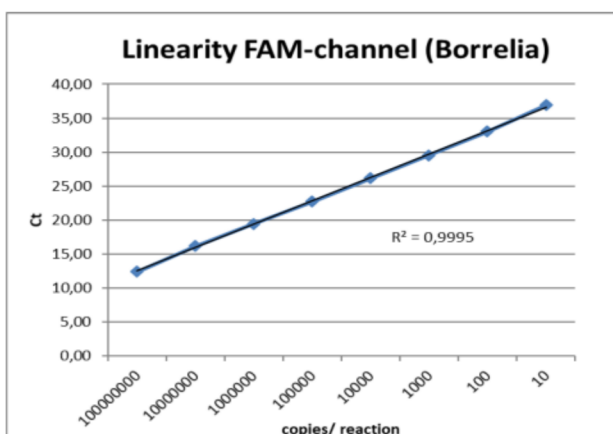
15 Kit Performance

15.1 Analytical Sensitivity

The limit of detection (LoD) of diarellaBorrelia/Rickettsia real time PCR was determined using serial dilutions of synthetic target DNA-sequences in a Stratagene Mx3005P real time PCR instrument. The LoD of diarellaBorrelia/Rickettsia real time PCR is ≤ 10 target copies per reaction.

15.2 Linear Range

The linear range of the diarellaBorrelia/Rickettsia real time PCR was evaluated by logarithmic dilution series of synthetic DNA fragments.



15.3 Analytical Specificity

The specificity of the diarellaBorrelia/Rickettsia real time PCR was evaluated by testing a panel of DNA extracted from bacteria. The diarellaBorrelia/Rickettsia real time PCR kit did not cross-react with the DNA and RNA of the following bacteria and viruses.

Table 9: Determination of the analytical specificity of diarellaBorrelia/Rickettsia real time PCR.

Culture Samples	Expected Result FAM-channel	Result diarellaBorrelia/Rickettsia
<i>Borrelia miyamotoi</i>	negative	negative
<i>Borrelia spielmanii</i> PSigII	positive	positive
<i>Borrelia afzelii</i>	positive	positive
<i>Borrelia valaisiana</i>	positive	positive
<i>Babesia microti</i>	negative	negative
<i>Babesia divergens</i>	negative	negative
<i>Babesia canis canis</i>	negative	negative
<i>Babesia gibsoni</i>	negative	negative
<i>Anaplasma phagocytophilum</i>	negative	negative
<i>Ehrlichia canis ebony</i>	negative	negative
<i>Coxiella burnetii</i>	negative	negative
Leptospiren	negative	negative
TBE Virus K617	negative	negative
Adenovirus 2	negative	negative
CMV	negative	negative
EBV	negative	negative
<i>Mycobacterium tuberculosis</i> (MDR-TB, EAS)	negative	negative
<i>Streptococcus agalactiae</i>	negative	negative

Culture Samples	Expected Result Cy5-channel	Result diarellaBorrelia/Rickettsia
<i>Borrelia miyamotoi</i>	negative	negative
<i>Borrelia spielmanii</i> PSigII	negative	negative
<i>Borrelia afzelii</i>	negative	negative
<i>Borrelia valaisiana</i>	negative	negative
<i>Babesia microti</i>	negative	negative
<i>Babesia divergens</i>	negative	negative
<i>Babesia canis canis</i>	negative	negative
<i>Babesia gibsoni</i>	negative	negative
<i>Anaplasma phagocytophilum</i>	negative	negative
<i>Ehrlichia canis ebony</i>	negative	negative
<i>Coxiella burnetii</i>	negative	negative
Leptospiren	negative	negative
TBE Virus K617	negative	negative
Adenovirus 2	negative	negative
CMV	negative	negative
EBV	negative	negative
<i>Mycobacterium tuberculosis</i> (MDR-TB, EAS)	negative	negative
<i>Streptococcus agalactiae</i>	negative	negative

Field Samples*	Expected Result FAM-channel	Result diarellaBorreliia/Rickettsia FAM channel
BT-N 49	negative	negative
BT-N 51	negative	negative
BT-N 54	negative	negative
BT-N 55	negative	negative
BT-N 56	negative	negative
BT-N 58	negative	negative
BT-N 64	negative	negative
BT-N 65	negative	negative
BT-N 67	negative	negative
BT-N 71	negative	negative
BT-N 72	negative	negative
BT-N 75	negative	negative
<i>Rickettsia conorii</i>	negative	negative
<i>Rickettsia massiliae</i>	negative	negative

Field Samples*	Expected Result Cy5-channel	Result diarellaBorreliia/Rickettsia Cy5 channel
BT-N 49	positive	positive
BT-N 51	positive	positive
BT-N 54	positive	positive
BT-N 55	positive	positive
BT-N 56	positive	positive
BT-N 58	positive	positive
BT-N 64	positive	positive
BT-N 65	positive	positive
BT-N 67	positive	positive
BT-N 71	positive	positive
BT-N 72	positive	positive
BT-N 75	positive	positive
<i>Rickettsia conorii</i>	positive	positive
<i>Rickettsia massiliae</i>	positive	positive

*Samples were qualified for Rickettsia by an accredited governmental lab.

15.4 Precision

The precision of the diarellaBorreliia/Rickettsia real time PCR was determined as intra-assay variability, inter-assay variability and inter-lot variability.

Variability data are expressed by standard deviation and coefficient of variation. The data are based on quantification analyses of defined concentrations of Borrelia and Rickettsia specific DNA and on the threshold cycle of the Control DNA.









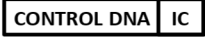





Table 10: Precision of diarellaBorrelia/Rickettsia real time PCR.

Borrelia	copies/μl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	25	0.23	0.70
Inter-Assay-Variability	25	0.34	1.03
Inter-Lot Variability	25	0.09	0.26

Rickettsia	copies/μl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	25	0.21	0.67
Inter-Assay-Variability	25	0.11	0.35
Inter-Lot Variability	25	0.00	0.02

Control DNA	copies/μl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	25	0.17	0.56
Inter-Assay-Variability	25	0.23	0.75
Inter-Lot Variability	25	0.08	0.27

16 Abbreviations and Symbols

DNA	Deoxyribonucleic Acid		Catalog number
PCR	Polymerase Chain Reaction		Content sufficient for <n> test
	Reaction Mix		Upper limit of temperature
	Positive Control		Manufacturer
	Negative Control		Use by YYYY-MM-DD
	Control DNA		Batch code
	Content		<i>In vitro</i> diagnostic medical device
	Consult instructions for use		European Conformity

17 Literature

- [1] Wilking, H. et al. Antibodies against *Borrelia burgdorferi* sensu lato among adults, Germany 2008 – 2011. CDC Emerging Infectious Diseases 21, 1, 2015.
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