MIKROGEN
DIAGNOSTIK

Instruction for Use

*alpha*Cube Borrelia/Rickettsia

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

REF	**830577**	**830576**
Σ	32	96

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1 Intended Use

The *alpha*Cube Borrelia/Rickettsia 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.

Analysis of ticks offers the possibility to identify the risk of infection very quickly, and therefore minimizing the delay of an antibiotic treatment.

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

*alpha*Cube Borrelia/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, *alpha*Cube Borrelia/Rickettsia 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 *alpha*Cube Borrelia/Rickettsia.

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 isolation kit (e.g. *alpha*Clean Pure RNA/DNA or *alpha*Clean Mag RNA/DNA, *alpha*Clean TS (tissue shred))
- Sterile microtubes
- Pipets (adjustable volume)
- Sterile pipet tips with filter
- Table centrifuge
- Vortexer
- Real time PCR instrument
- Optical PCR reaction tubes with lid or optical microtiter plates with optical foil
- Optional: Liquid handling system for automation

6 Transport, Storage and Stability

*alpha*Cube Borrelia/Rickettsia is shipped on dry ice or cool packs. All components must be stored at ≤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 at any time.

7 Important Notes

- *alphaCube* Borrelia/Rickettsia must be performed by qualified personnel only.
- Good Laboratory Practice (GLP) has to be applied.
- Clinical samples must always be regarded as potentially infectious material and all equipment used has to be treated as potentially contaminated.

8 General Precautions

- Stick to the protocol described in the Instruction for use.
- Set up different laboratory areas for the preparation of samples and for the set up of the PCR in order to avoid contaminations.
- Pipettes, tubes and other materials must not circulate between those different laboratory areas.
- Always use filter tips.
- Regulary decontaminate equipment and benches with ethanol-free decontaminant.
- Do not combine *alpha*Cube Borrelia/Rickettsia components of different lot numbers.

9 Sample Material

Starting material for the *alpha*Cube Borrelia/Rickettsia real time PCR is DNA, extracted from biological specimens (e.g. EDTA-blood, plasma, serum, cerebrospinal fluid and tissue samples) or from ticks.

10 Sample Preparation

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

- *alpha*Clean Pure RNA/DNA
- *alpha*Clean Mag RNA/DNA

It is recommended to use mechanical disruption of ticks before DNA extraction. Please follow the instructions for use of the respective extraction kits.

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 11 ‚Control DNA'.

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

11 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.

DNA isolation from EDTA-blood, plasma, serum, cerebrospinal fluid, tissue samples and ticks

a) Control DNA used as Extraction Control:
*alpha*Cube Borrelia/Rickettsia 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.

12 Real time PCR

12.1 Important Points Before Starting:

- Please pay attention to the chapter 7 ‚Important Notes'.
- 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 (do NOT vortex the Reaction Mix but mix by pipetting up and down repeatedly), and centrifuged very briefly.

12.2 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 11 ‚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 11, Control DNA'). In this case, prepare the Master Mix according to table 3.

The Master Mix contains all of the components needed for real 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.
- Pipet **16 µl** of the Master Mix into each optical PCR reaction tube.
- 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 (Table 4).
- Close the optical PCR reaction tubes 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

12.3 Instrument Settings

For the real time PCR use the thermal profile shown in Table 5.

Table 5: real time PCR thermal profile

Description	Time	Temperature	Number of Cycles
Initial Denaturation	10 min	95°C	1
Amplification			
Denaturation	10 sec	95°C	45
Annealing	20 sec Aquisition at the end of this step	60°C	
Extension	10 sec	72°C	

If in the same run samples should be tested for pathogens with RNA genome, e.g. with *alpha*Cube TBE, use the thermal profile shown in Table 6.

Table 6: real time RT-PCR thermal profile

Description	Time	Temperature	Number of Cycles
Reverse Transcription	20 min	45°C	1
Initial Denaturation	5 min	95°C	1
Amplification			
Denaturation	10 sec	95°C	45
Annealing	20 sec Aquisition at the end of this step	60°C	
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 *alpha*Cube Borrelia.

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	Reference Dye: None
	Rickettsia	CY5	Gain 4	
	Control DNA	HEX	Gain 1	
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	Borrelia	Green	Gain 5	
	Rickettsia	Red	Gain 5	
	Control DNA	Yellow	Gain 5	
Mic qPCR Cycler	Borrelia	Green	Gain 8	
	Rickettsia	Red	Gain 10	
	Control DNA	Yellow	Gain 10	

13 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 Borrelia DNA.
negative	**positive**	positive or negative*	Positive result, the eluate contains Rickettsia DNA.
negative	negative	≤ 34**	Negative result, the eluate contains neither Borrelia DNA nor Rickettsia 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.

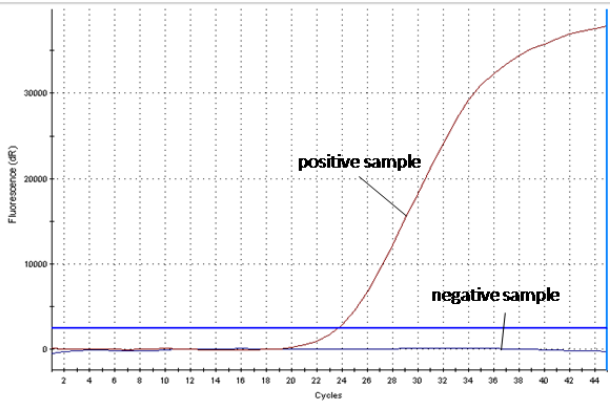


Figure 1: The positive sample shows bacteria specific amplification in the FAM or Cy5 channel, whereas no fluorescence signal is detected in the negative sample.

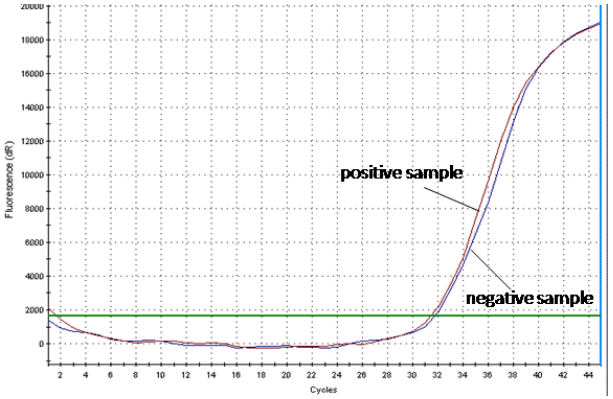


Figure 2: The positive sample as well as the negative sample show a signal in the Control DNA specific HEX channel. The amplification signal of the Control DNA in the negative sample shows, that the missing signal in the bacteria specific FAM or Cy5 channel is not due to PCR inhibition or failure of DNA isolation, but that the sample is a true negative.

14 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.

15 Limitations of the Method

*alpha*Cube Borrelia/Rickettsia is designated for the detection of Borrelia burgdorferi sensu lato DNA and Rickettsia species DNA in watery solutions (eluates) from biological samples. The extraction of the DNA from biological samples is not part of *alpha*Cube Borrelia/Rickettsia. The Control DNA added during extraction does not reflect the quality of the sample but gives a hint for the quality of the extraction and/or remaining inhibitors in the eluate. The LoD of the reaction is $\leq$10 Borrelia burgdorferi sensu lato DNA target copies and $\leq$10 Rickettsia DNA target copies per reaction, respectively. Therefore, a negative result in *alpha*Cube Borrelia/Rickettsia does not exclude the complete absence of Borrelia burgdorferi sensu lato or Rickettsia DNA in an eluate.

16 Troubleshooting

The following troubleshooting guide is included to help you with possible problems that may arise when performing a real time PCR.

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 6.
The programming of the thermal profile is incorrect	Compare the thermal profile with the protocol (Table 5, page 8).

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 4.
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 6).
real time PCR inhibited	Make sure that you use an appropriate isolation method (see ‚Sample Preparation') 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', page 4.
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.

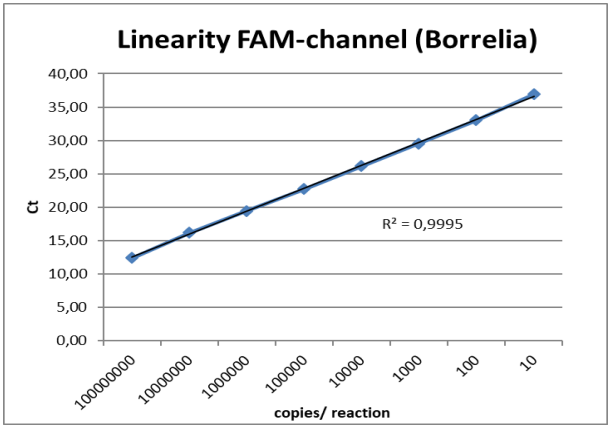
17 Kit Performance

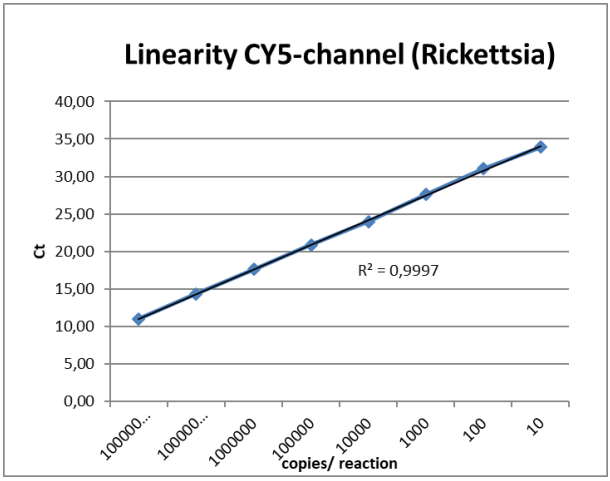
17.1 Diagnostic Sensitivity and Specificity

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

17.2 Linear Range

The linear range of *alpha*Cube Borrelia/Rickettsia was evaluated by logarithmic dilution series of synthetic DNA fragments.





17.3 Analytical Specificity

The specificity of *alpha*Cube Borrelia/Rickettsia was evaluated by testing a panel of DNA extracted from bacteria.

*alpha*Cube Borrelia/Rickettsia did not cross-react with the DNA and RNA of the following bacteria and viruses.

Table 9: Determination of the analytical specificity of *alpha*Cube Borrelia/Rickettsia.

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

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

Field Samples*	**Expected Result FAM-channel**	**Result *alpha*Cube Borrelia/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
Rickettsia conorii	negative	negative
Rickettsia massiliae	negative	negative

Field Samples*	**Expected Result Cy5-channel**	**Result *alpha*Cube Borrelia/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
Rickettsia conorii	positive	positive
Rickettsia massiliae	positive	positive

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

17.4 Precision

The precision of *alpha*Cube Borrelia/Rickettsia 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 *alpha*Cube Borrelia/Rickettsia.

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

18 Abbreviations and Symbols

DNA	Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction
REACTION MIX	Reaction Mix
CONTROL +	Positive Control
CONTROL −	Negative Control
CONTROL DNA IC	Control DNA

REF	Catalog number
Σ	Content sufficient for <n> test
-18°C	Upper limit of temperature
	Manufacturer
	Use by YYYY-MM-DD
LOT	Batch code
CONT	Content
	Consult instructions for use
IVD	*In vitro* diagnostic medical device
CE	European Conformity

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19 Literature

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