Instruction for Use

diarellaBorrelia

real time PCR Kit TM

For qualitive *in vitro* detection of *Borrelia burgdorferi* sensu lato DNA, extracted from clinical specimens and ticks.

REF	G01062-32	G01062-96
Σ	32	96

- 18°C

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1 Intended Use

The diarellaBorrelia real time PCR TM is an assay for the detection of *Borrelia burgdorferi* sensu lato DNA, extracted from clinical specimens and 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, Slowenia, 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 minimising the delay of an antibiotics treatment.

3 Principle of the Test

The diarellaBorrelia real time PCR Kit TM contains specific primers and dual-labeled probes for the amplification and detection of *Borrelia burgdorferi* sensu lato DNA, extracted from clinical specimens and ticks.

The presence of nucleic acid is detected by an increase in fluorescence due to hydrolysis of the probes during amplification.

The fluorescence of the pathogen-specific probes is measured in the FAM channel.

Furthermore, diarellaBorrelia real time PCR Kit TM 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 VIC®/HEX/JOE/TET channel.

4 Package Contents

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

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

Label	Lid Colour	Content	
		32	96
Reaction Mix	yellow	1 x 512 µl	2 x 768 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	colourless	1 x 160 µl	2 x 240 µl

5 Equipment and Reagents to be Supplied by User

- DNA isolation kit (e.g. NukEx Pure RNA/DNA, gerbion Cat. No. G05004 or NukEx Mag RNA/DNA, gerbion Cat. No. G05012), NukEx TS (tissue shred, gerbion Cat. No. G06007).
- PCR grade Water
- Sterile microtubes
- Pipets (adjustable volume)
- Sterile pipet tips with filter
- Table centrifuge
- Vortexer
- Real time PCR instrument
- Optical PCR reaction tubes with lid
- Optional: Liquid handling system for automation

6 Transport, Storage and Stability

The diarellaBorrelia real time PCR Kit TM 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 test run.

7 Important Notes

- The diarellaBorrelia real time PCR TM 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 diarellaBorrelia real time PCR Kit TM components of different lot numbers.

9 Sample Material

Starting material for the diarellaBorrelia real time PCR is DNA, extracted from clinical specimens (e.g. EDTA-blood, plasma, serum, cerebrospinal fluid and tissue samples) or from ticks.

10 Sample Preparation

The diarellaBorrelia real time PCR TM is suitable for the detection of *Borrelia burgdorferi* sensu lato DNA, isolated from clinical specimens or ticks with appropriate isolation methods.
Commercial kits for DNA isolation such as the following are recommended:

- NukEx Pure RNA/DNA, gerbion Cat. No. G05004
- NukEx Mag RNA/DNA, gerbion Cat. No. G05012

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

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.

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:
diarellaBorrelia real time PCR TM 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, 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 (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 of DNA			
Denaturation	10 sec	95°C	
Annealing	20 sec	60°C	45
	Aquisition 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
Reverse Transcription	20 min	45°C	1
Initial Denaturation	5 min	95°C	1
Amplification of DNA			
Denaturation	10 sec	95°C	
Annealing	20 sec	60°C	45
	Aquisition 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 real time PCR TM.

Real time PCR Instrument	Parameter	Detection Channel	Notes
LightCycler 480I	Borrelia	483-533	pre-installed universal Color Compenation FAM (510) - VIC (580)
	Control DNA	523-568	
LightCycler 480II	Borrelia	FAM (465-510)	
	Control DNA	HEX (533-580)	
Stratagene Mx3000P / Mx3005P	Borrelia	FAM	Gain 8 Reference Dye: None
	Control DNA	HEX	Gain 1
ABI 7500	Borrelia	FAM	Option Reference Dye ROX: NO
	Control DNA	JOE	
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	Borrelia	Green	Gain 5
	Control DNA	Yellow	Gain 5
Mic qPCR Cycler	Borrelia	Green	Gain 8
	Control DNA	Yellow	Gain 10

13 Data Analysis

The *Borrelia* specific amplification is measured in the FAM channel. The amplification of the Control DNA is measured in the VIC®/HEX/JOE™/TET channel.

Following results can occur:

- A signal in the FAM channel is detected:
  The result is positive, the sample contains *Borrelia* DNA.
  In this case, detection of a signal of the Control DNA in the VIC®/HEX/JOE™/TET channel is inessential, as high concentrations of bacterial DNA may reduce or completely inhibit amplification of the Control DNA.

- No signal in the FAM channel, but a signal in the VIC®/HEX/JOE™/TET channel is detected:
  The result is negative, the sample does not contain *Borrelia* DNA.
  The signal of the Control DNA excludes the possibilities of DNA isolation failure (in case the Control DNA is being used as an Extraction Control) and/or real time PCR inhibition. If the C_T value of a sample differs significantly from the C_T value of the water control, a partial inhibition occured, which can lead to negative results in weak positive samples (see chapter „Troubleshooting“).

- Neither in the FAM nor in the VIC®/HEX/JOE™/TET channel a signal is detected:
  A diagnostic statement cannot be made.
  The DNA isolation was not successful or an inhibition of the PCR has occurred. In case the Control DNA was added during DNA isolation and not directly to the PCR Master Mix, the Negative Control is negative in both channels.

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

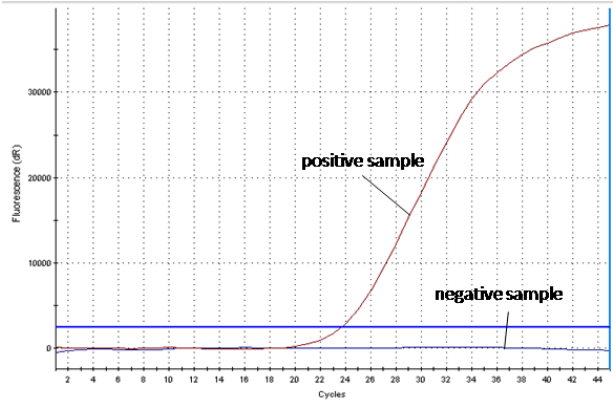

Figure 1: The positive sample shows bacteria specific amplification in the FAM channel, whereas no fluorescence signal is detected in the negative sample.

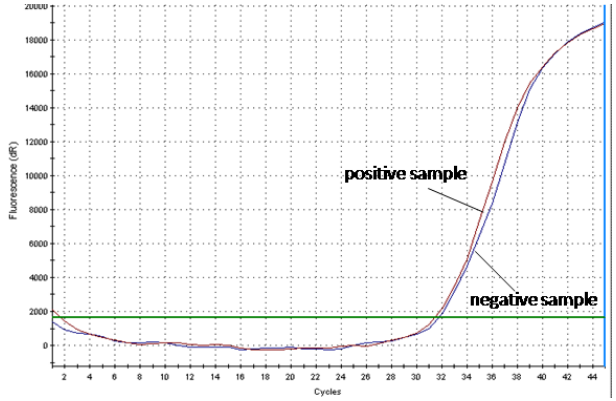

Figure 2: The positive sample as well as the negative sample show a signal in the Control DNA specific VIC®/HEX/JOE™/TET channel. The amplification signal of the Control DNA in the negative sample shows, that the missing signal in the bacteria specific FAM channel is not due to PCR inhibition or failure 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 33. 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 33.

15 Limitations of the Method

The results must always be considered in relation to the clinical symptoms. Therapeutical consequences should be made in consideration of clinical data.
A negative test result does not exclude a *Borrelia burgdorferi* infection.

16 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 of the Positive Control	
The selected channel for analysis does not comply with the protocol	Select the FAM channel for analysis of the *Borrelia* specific amplification and the VIC®/HEX/JOETM/TET 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 4.
Weak or no signal of the Control DNA and simultaneous absence of a signal in the bacteria specific FAM channel.	
real time PCR conditions do not comply with the protocol	Check the real time PCR conditions (page 7).
real time PCR inhibited	Make sure that you use an appropriate isolation method (see chapter 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'.
Detection of a fluorescence signal in the FAM 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 occured 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

During the validation study of the diarellaBorrelia real time PCR TM 48 positive and 120 negative samples were tested. The diagnostic sensitivity was found to be 100 % and the diagnostic specificity 100 %
The positive predictive value was found to be 100 %, the negative predictive value showed to be 100 %.

Table 8: Overview of the amount of samples tested and the resulting positive and negative predictive values

	positive samples	negative samples
diarellaBorrelia positive	48	0
diarellaBorrelia negative	0	120

Sensitivity	100 %
Specificity	100 %

17.2 Analytical Sensitivity

The limit of detection (LoD) of the diarellaBorrelia real time PCR Kit TM was determined using serial dilutions of Borrelia burgdorferi in culture medium in a Stratagene Mx3000 real time PCR instrument. Total nucleic acids were extracted using NukEx Pure RNA/DNA (gerbion) according to the manufacturer´s instructions. Each sample was supplemented with 5 µl Control-DNA prior to extraction. Total nucleic acids were eluted with 50 µl. and 4 µl of the eluates were applied to the subsequent real time PCR.
The LoD of the diarellaBorrelia real time PCR Kit TM for *Borrelia burgdorferi sensu lato* is $\geq$10 genome copies per reaction each.

The sensitivity of the diarellaBorrelia real time PCR Kit TM was also analysed by testing round robin samples of known status.
All samples of the QCMD Borrelia panels were detected correctly. Likewise the samples of the Borrelia ring trial (INSTAND e.V.). Results are shown in table 9.

Table 9: Samples tested for the validation of the sensitivity of the diarellaBorrelia real time PCR Kit TM.

Sample	Sample Content	Expected Result	Result diarellaBorrelia	Sample Type
BbDNA14-01	Borrelia garinii	positive	positive	core
BbDNA14-07	Borrelia garinii	positive	positive	core
BbDNA14-08	Borrelia garinii	positive	positive	educational
BbDNA14-09	Borrelia burgdorferi s.s.	positive	positive	core
BbDNA14-03	Borrelia burgdorferi s.s.	positive	positive	core
BbDNA14-04	Borrelia burgdorferi s.s.	positive	positive	educational
BbDNA14-10	Borrelia afzelii	positive	positive	core
BbDNA14-05	Borrelia afzelii	positive	positive	core
BbDNA14-02	Treponema phagedenis	negative	negative	core
BbDNA14-06	Borrelia negative	negative	negative	core
1515351	Borrelia miyamotoi	negative	negative	-
1515352	Borrelia bavariensis	positive	positive	-
1515353	Borrelia garinii Ospa Typ 8	positive	positive	-
1515354	Borrelia kurtenbachii	positive	positive	-

17.3 Analytical Specificity

The specificity of the diarellaBorrelia real time PCR TM was evaluated additionally with different other relevant viruses and bacteria found in clinical samples.

Results:

The diarellaBorrelia real time PCR Kit TM showed a positiv result for the sample containing *Borrelia burgdorferi,* whereas samples containing other pathogens were reliably tested negative. The results are shown in Table 10.

Table 10: Bacterial and viral pathogens tested for the determination of the analytical specificity of the diarellaBorrelia real time PCR Kit TM.

Strain	Expected Result	Result
Enterovirus 68	negative	negative
Coxsackievirus B3	negative	negative
Coxsackievirus A16	negative	negative
Coxsackievirus B5	negative	negative
Influenza Virus A A/ Brisbane H1N1 59/2007 E40/08	negative	negative
Influenza Virus A Indonesia H5N1 05/2005	negative	negative
Influenza Virus A Panama H3N2 2007/99	negative	negative
Influenza Virus B B/ Brisbane 60/2008 E09/09	negative	negative
TBE-Virus	negative	negative
Ehrlichia chaffeensis	negative	negative
Ehrlichia ewingii	negative	negative
Ehrlichia canis	negative	negative
Ehrlichia phagocytophilum	negative	negative
Anaplasma platy	negative	negative
Babesia divergens	negative	negative
Babesia microti	negative	negative
Babesia sp. EU1	negative	negative
Borrelia burgdorferi Strain 4681	positive	positive
Borrelia burgdorferi sensu stricto	positive	positive
Borrelia afzelii	positive	positive
Borrelia garinii	positive	positive
Borrelia spielmanii	positive	positive
Borrelia bavariensis	positive	positive
Borrelia bisettii	positive	positive
Borrelia lustianae	positive	positive
Borrelia valaisiana	positive	positive
Borrelia kurtenbachii	positive	positive
Borrelia japonica	negative	negative
Borrelia miyamotoi	negative	negative
T. phagedenis	negative	negative
Leptospira	negative	negative

18 Abbreviations and Symbols

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

REF	Catalog number
Σ	Contains 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

19 Literature

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

[2] Wilking H, Stark K. Trends in surveillance data of human Lyme borreliosis from six federal states in eastern Germany, 2009–2012. Ticks Tick Borne Dis. 2014; 5:219–24