BactoReal® Kit
Borrelia burgdorferi sensu lato

Manual

For use with the

- **ABI PRISM® 7500 (Fast)**
- **Mx3005P®**
- **LightCycler® 480**

For veterinary use only

REF **DVEB05011, DVEB05013** Σ **100**

REF **DVEB05051, DVEB05053** Σ **50**

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Symbols

LOT	Batch code		Use by
REF	Catalogue number		Manufactured by
Σ	Contains sufficient for <n> tests		Store at

1. Product description

BactoReal® Kit *Borrelia burgdorferi* sensu lato is a real-time PCR assay for detection of DNA of *Borrelia* species belonging to the *Borrelia burgdorferi* sensu lato complex, namely *Borrelia burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, *B. valaisiana*, *B. lusitaniae, B. bavariensis, B. bissettii* and *B. spielmanii. Borrelia spielmanii* is not well detected with this assay. *Borrelia* causing relapsing fever are not detected.
This test was developed and validated for the ABI PRISM® 7500 (Fast) instrument (Life Technologies), LightCycler® 480 (Roche) and Mx3005P® (Agilent), but is also suitable for other real-time PCR instruments. This test allows the rapid and sensitive detection of *Borrelia burgdorferi* sensu lato strains from samples purified from tissue, blood or cerebrospinal fluid (e.g. with the QIAamp DNA Mini Kit).

BactoReal® Kit *Borrelia burgdorferi* sensu lato detects the ospA gene of species of the *Borrelia burgdorferi* sensu lato complex. A probe-specific amplification-curve at 530 nm (FAM channel) indicates the amplification of *Borrelia* specific DNA.
An internal positive control system for detection in VIC/HEX channel, (554 nm, order no. DVEB05011 or DVEB05051) or Cy5 channel (667 nm; order no. DVEB05013 or DVEB05053) excludes false-negative interpretation of results due to inhibition of real-time PCR (see 8. Interpretation of PCR-data).
When using PCR-platforms not validated by ingenetix, an evaluation of the multiplex-PCR is recommended. Please be aware that some PCR-platforms have to be calibrated with the corresponding dye before performing multiplex-PCR.

BactoReal®, MycoReal, ParoReal and ViroReal® Kits are optimized to run under the same thermal cycling conditions. RNA and DNA material can be analysed in one run.

2. Pathogen information

Borrelia are Gram-negative bacteria belonging to the S*pirochaetaceae*. *Borrelia burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, *B. valaisiana*, *B. lusitaniae* and *B. spielmanii* belong to the *B. burgdorferi* sensu lato-complex and cause the Lyme disease (Lyme borreliosis). These species are associated with different genospecies specific symptoms. They are transmissed by ticks, mainly by *Ixodes ricinus*. The Lyme disease is a multisystemic illness, which can affect various organs, especially the nervous system, skin, heart and joints.

References:
O'Connell S. 2010. Lyme borreliosis: current issues in diagnosis and management. Curr. Opin. Infect. Dis. 23:231-5.

3. Principle of real-time PCR

A specific DNA sequence of the pathogen genome is amplified and the generated PCR-product is detected by an oligonucleotide-probe labelled with a fluorescent dye. This technology allows for a sequence-specific detection of PCR amplificates.

4. General Precautions

The user should always pay attention to the following:
- Always include a negative control per PCR-run (water instead of sample).
- Optional: for valid interpretation of results, a negative control should be included during DNA-extraction (for example extraction of water instead of sample material), in order to exclude false-positive results due to contamination with *Borrelia* DNA during extraction.
- Be careful when handling the positive control.
- Store and extract positive material (specimens, controls and amplicons) separately from all other reagents and add it to the reaction mix in a spatially separated workspace.
- Periodically decontaminate benches and devices.
- Use sterile pipette tips with filters.
- Thaw all components thoroughly at room temperature before starting an assay. When thawed, mix the components and centrifuge briefly.
- For MSDS, see www.ingenetix.com.

5. Contents of the Kit

5.1. BactoReal® Kit *Borrelia burgdorferi* sensu lato order no. DVEB05011 or DVEB05051

Labelling	Content	Amount		Storage
		DVEB05011	DVEB05051	
Borrelia burgdorferi (ospA) Assay Mix (green cap)	Primer and probe (FAM) for detection of *Borrelia*	2 x 50 µl	1 x 50 µl	-20°C
CR-1 Assay Mix (yellow cap)	Primer, probe (VIC/HEX) and target for detection of IPC	2 x 50 µl	1 x 50 µl	-20°C
Borrelia burgdorferi Positive Control (red cap)	Control-DNA (approx. 10,000 target copies/µl)	1 x 25 µl	1 x 25 µl	-20°C
DNA Reaction Mix (white cap)#	Reaction Mix	2 x 500 µl	1 x 500 µl	**-20°C until first use, then at +4°C**
Water (blue cap)	Water	1 x 1000 µl	1 x 1000 µl	-20°C to +4°C

#DNA Reaction Mix contains uracil-N glycosylase (UNG)

5.2. BactoReal® Kit *Borrelia burgdorferi* sensu lato order no. DVEB05013 or DVEB05053

Labelling	Content	Amount		Storage
		DVEB05013	DVEB05053	
Borrelia burgdorferi (ospA) Assay Mix (green cap)	Primer and probe (FAM) for detection of *Borrelia*	2 x 50 µl	1 x 50 µl	-20°C
CR-3 Assay Mix (yellow cap)	Primer, probe (Cy5) and target for detection of IPC	2 x 50 µl	1 x 50 µl	-20°C
Borrelia burgdorferi Positive Control (red cap)	Control-DNA (approx. 10,000 target copies/µl)	1 x 25 µl	1 x 25 µl	-20°C
DNA Reaction Mix (white cap)#	Reaction Mix	2 x 500 µl	1 x 500 µl	**-20°C until first use, then at +4°C**
Water (blue cap)	Water	1 x 1000 µl	1 x 1000 µl	-20°C to +4°C

#DNA Reaction Mix contains uracil-N glycosylase (UNG)

The components of BactoReal® Kit *Borrelia burgdorferi* sensu lato are stable until the expiry date stated on the label. Repeated thawing and freezing should be avoided. Please protect kit components from light.

6. Additionally required materials and devices

- Reagents and devices for DNA-extraction
- PCR-grade water
- Disposable powder-free gloves
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Vortex mixer
- Desktop centrifuge with rotor for 2 ml reaction tubes
- Real-time PCR instrument which is able to detect and differentiate fluorescence in FAM and VIC/HEX or Cy5 channel
- Appropriate 96 well reaction plates or reaction tubes with corresponding (optical) closing material

7. Preparation of real-time PCR

Please make sure that at least one negative control (water, blue cap), as well as one positive control (red cap) and one extraction negative control (optional, recommended) are included per PCR run.
Ingenetix highly recommends performing PCR analyses in duplicates, which increases the probability of detection of the pathogen and facilitates interpretation of results.

7.1. Pipetting scheme

		Per sample
Preparation of Master Mix (mix well)	Water*	3.0 µl
	DNA Reaction Mix (2x)	10.0 µl
	Borrelia burgdorferi (ospA) Assay Mix	1.0 µl
	CR Assay Mix	1.0 µl
	Total volume Master Mix	**15.0 µl**
Preparation of PCR	Master Mix	15.0 µl
	Sample*	5.0 µl
	Total volume	**20.0 µl**

*1-8 µl of the sample can be used. When using an amount other than 5 µl of the sample, the amount of H_2O has to be changed accordingly.

Positive Control: As positive control use 1 µl of the *Borrelia burgdorferi* Positive Control + 4 µl H_2O.
Optional: a 1:10 dilution of the positive control can be used and defined as second standard value (approx. 1000 target copies/µl).

7.2. Programming of the temperature profile

Please find further information on programming the real-time PCR instrument in the respective operator's manual. Please be aware that some PCR-platforms have to be calibrated with the corresponding dye before performing multiplex-PCR.

Select dyes: FAM-TAMRA for detection of *Borrelia burgdorferi* s.l.
Cy5-NONE (CR-3 Assay Mix) **or** VIC-TAMRA (CR-1 Assay Mix) for detection of IPC
Select reference dye (passive reference): ROX
Sample Volume: 20 µl
Temperature Profile:

Program 1	**Program 2**	**Program 3**
Cycles: 1 Analysis: None	Cycles: 1 Analysis: None	Cycles: 45 Analysis: Quantification Acquisition at 60°
50°C 2 min*	95°C 20 sec	95°C 5 sec 60°C 1 min

For ABI PRISM® 7500:
Ramp speed: Without "fast cycling" parameter

For LightCycler® 480 instrument:
Detection format: 2 Color Hydrolysis Probe
(dyes see above)

***Note:** If viral RNA should be also detected in the same PCR run, program 1 has to be prolonged to 15 min at 50°C. This temperature profile can be used for all BactoReal®, MycoReal, ParoReal and ViroReal® kits for the detection of DNA or RNA.

8. Interpretation of PCR-data

Examples for interpretation of positive reactions are shown in the amplification plots below.

For a valid interpretation, the following criteria must be fulfilled:

	Ct/Cp (FAM channel) *Borrelia* target	Ct/Cp IPC target	Interpretation
Negative control	Negative	36.0 ± 2	Valid
Positive control (undiluted, 1 µl/PCR)	28.0-31.0	36.0 ± 2	Valid
Or: positive control (1:10 diluted, 1 µl/PCR)	31.0-34.0	36.0 ± 2	Valid
Extraction negative control (optional)	Negative	36.0 ± 2	Valid
Negative sample	Negative	36.0 ± 2	Valid
Positive sample	Positive	Positive/Negative	Valid

For analysis of PCR data please proceed as follows:
For analysis of PCR results gained with BactoReal® Kit *Borrelia burgdorferi* sensu lato please select fluorescence display options FAM channel for the *Borrelia* target and VIC/HEX channel (order no. DVEB05011, DVEB05051) or Cy5 channel (order no. DVEB05013, DVEB05053) for the internal positive control target. Samples with a positive Cp or Ct-value are considered positive. Please also check the presence of amplification-curves manually.
With ABI PRISM® 7500 (Fast) instrument:
The threshold should be set manually above the background signal of the negative control.

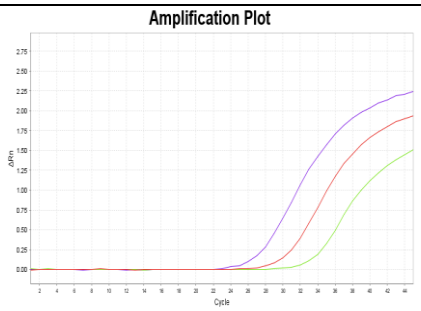
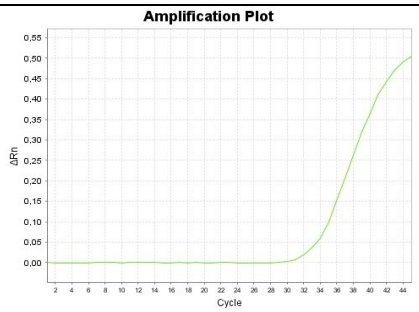
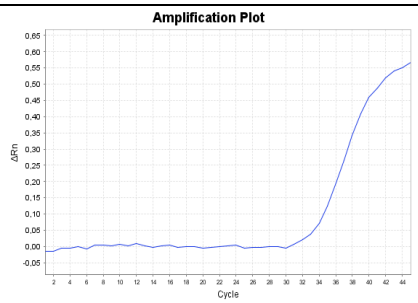
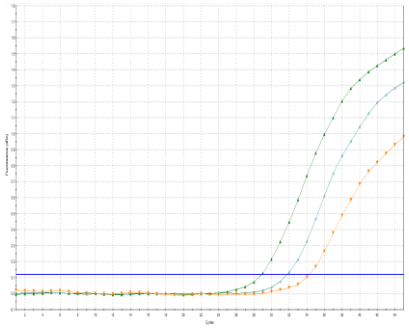
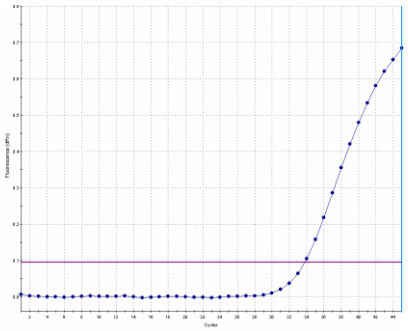
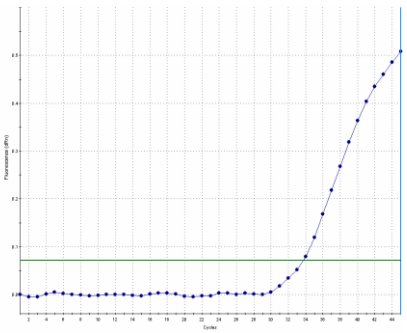
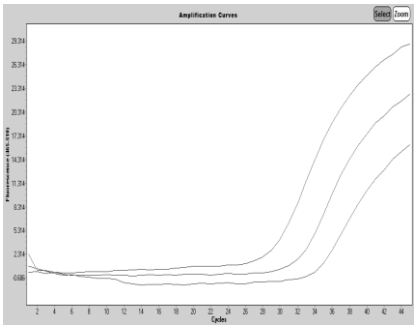
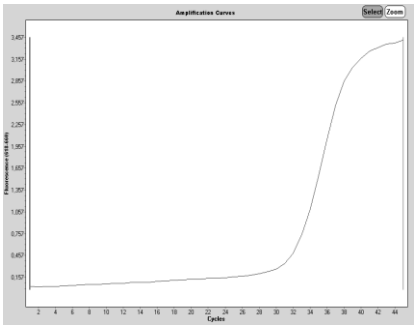
Once the analysis is completed, the following results are possible:

1. Signal in FAM channel:
→ DNA of *Borrelia burgdorferi* sensu lato was amplified. The sample has to be interpreted as positive.
Borrelia DNA can lead to a reduced or absent fluorescence signal of the internal positive control (competition of PCR).

2. No signal in FAM channel:
→ No *Borrelia burgdorferi* sensu lato DNA is detectable in the sample. The sample has to be interpreted as negative.
An inhibition of PCR cannot be excluded.

2a. No signal in FAM channel but signal of the internal positive control:
→ No *Borrelia burgdorferi* sensu lato DNA is detectable in the sample. The sample has to be interpreted as negative.
The positive signal of the internal positive control assay excludes a putative PCR inhibition.

2b. No signals in FAM channel and no signal with internal positive control:
→ No interpretation statement can be made.
Information about possible sources of error and their solution can be found in 9. Troubleshooting.

Detection of ***Borrelia burgdorferi***	Detection of internal positive control **CR-3**	Detection of internal positive control **CR-1**
		
ABI Prism® 7500: FAM channel, 530 nm 1:10 serial dilution of *Borrelia* DNA	**ABI Prism® 7500:** Cy5 channel, 667 nm Internal positive control	**ABI Prism® 7500:** VIC channel, 554 nm Internal positive control
		
Mx3005P®: FAM channel 1:10 serial dilution of *Borrelia* DNA	**Mx3005P®:** CY5 channel Internal positive control	**Mx3005P®:** HEX channel Internal positive control
		
LightCycler® 480: FAM channel 1:10 serial dilution of *Borrelia* DNA	**LightCycler® 480:** Cy5 channel Internal positive control	

9. Troubleshooting

1. No *Borrelia* specific signal with positive control:

- Incorrect programming of the temperature profile of the real-time PCR instrument.
  → Compare the temperature profile with the protocol (see 7. Preparation of real-time PCR).
- Incorrect configuration of the PCR reaction.
  → Check your work steps (see 7. Preparation of real-time PCR) and repeat the PCR, if necessary.

2. No signal with the internal positive control and no *Borrelia* specific signal with the sample:

- The PCR reaction was inhibited. No interpretation can be made.
  → Make sure that you use a recommended method for DNA isolation and stick closely to the manufacturer's instructions.
  → If no operating mistakes during extractions can be retraced, it is recommended to repeat the PCR with lower amounts of DNA-eluate (1/5 or 1/10 of sample volume + the adequate amount of H_2O).
- Incorrect PCR conditions.
  → Check the PCR conditions and repeat the PCR, if necessary.

3. *Borrelia* specific signal with the negative control:

- A contamination occurred during preparation of the PCR.
  → Repeat the PCR with new reagents in replicates.
  → Strictly pipette the positive controls at last.
  → Make sure that work space and instruments are decontaminated at regular intervals.

4. *Borrelia* specific signal with the negative control of DNA-extraction:

- A contamination occurred during extraction.
  → Repeat the extraction and PCR using new reagents.
  → Make sure that work space and instruments are decontaminated at regular intervals.

10. Specifications

BactoReal® Kit *Borrelia burgdorferi* sensu lato was evaluated with the ABI PRISM® 7500 (Fast) instrument, with the LightCycler® 480 and the Mx3005P®. For further validation data please contact ingenetix.

10.1. Analytical sensitivity

BactoReal® Kit *Borrelia burgdorferi* sensu lato has an analytical sensitivity of 10 target copies/PCR.
The limit of detection (LoD95 = smallest number of copies of target DNA which can be detected in 95% of cases) is 42 target copies/reaction.

10.2. Analytical specificity

The selection of highly specific primers and probes ensures analytical specificity. Primers and probes have been checked for possible homologies to currently published sequences by sequence comparison analysis. This validates the detection of so far known *Borrelia burgdorferi* s.l. strains.
Analytical specificity has been further evaluated by testing genomic DNA of adenovirus and of bacteria not belonging to the *Borrelia burgdorferi* sensu lato complex (*Borrelia hermsii, Borrelia miyamotoi, Bordetella pertussis, Bordetella parapertussis, Chlamydophila pneumoniae, Haemophilus influenzae, Legionella pneumophila, Leptospira sp., Listeria monocytogenes, Neisseria meningitidis, Mycoplasma pneumoniae* and *Treponema phagedenis*). No cross-reactions have been observed.
Furthermore, genomic DNA of bacteria belonging to the *Borrelia burgdorferi* sensu lato complex was tested (*B. afzelii, B. burgdorferi* sensu stricto, *B. bavariensis, B. bissettii, B. coriaceae, B. garinii*, *B. lusitaniae, B. spielmanii*, *B. turdi*, *B. valaisiana* and *B. japonica*). *B. spielmanii* was detected with lower relative fluorescence intensities compared to other *Borrelia* species. *Borrelia japonica*, a non-humanpathogenic *Borrelia* species was not detected. All other *Borrelia* isolates were detected and showed comparable relative fluorescence values.
Furthermore, 80 DNA isolates from clinical specimens tested with accredited reference methods were analyzed. Specimens included one tissue biopsy, one stool sample, 46 cerebrospinal fluids, 27 synovial fluids and 5 EDTA-blood samples. With the reference methods 42 samples were positive for *B. burgdorferi* s.l. (*B. afzelii* n=6, *B. burgdorferi* sensu stricto n=6, *B. bavariensis* n=3, *B. bissettii* n=3, *B. garinii* n=16, *B. spielmanii* n=2, *B. valaisiana* n=4, *B. japonica* n=1) and 38 were negative (*B. hermsii* n=1, *B. miyamotoi* n=1, *Leptospira* sp. n=2,*Treponema* sp. n=1 and samples negative for *B. burgdorferi* s.l. n=33).
With BactoReal® Kit *Borrelia burgdorferi* sensu lato 41 out of the 42 positive samples were also positive, albeit the two *B. spielmanii* positive samples showed a lower relative fluorescence intensity and were assessed questionable positive. The *B. japonica* positive sample was tested negatively (non-humanpathogenic species). The 38 samples negative with the reference methods were negative with the kit.