# BactoReal® Kit
# *Borrelia burgdorferi* sensu lato

**Kit version 1.1**

# Instructions for Use

CE

IVD **For *in vitro* diagnostic use**

REF **DHUB05053**

Σ **50 reactions**

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# Explanation of symbols

| | | | |
|---|---|---|---|
| LOT | Batch code | | Use by date |
| REF | Catalogue number | | Manufacturer |
| Σ | Contains sufficient for <n> tests | | Temperature limit (Store at) |
| CE | This product fulfils the requirements of the European Directive 98/79 EC for *in vitro* diagnostic medical devices | IVD | In vitro diagnostic medical device |
| | Consult instructions for use | UDI | Unique device identifier |
| | Keep away from sunlight | QTY | Contents |

## 1. Intended purpose

BactoReal® Kit *Borrelia burgdorferi* sensu lato is a non-automated IVD real-time PCR test for the qualitative detection of DNA (ospA gene) of *Borrelia* species belonging to the *Borrelia burgdorferi* sensu lato (s.l.) complex (*Borrelia afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto, *B. valaisiana*, *B. lusitaniae, B. bavariensis, B. bissettii,* and *B. spielmanii* (*B. spielmanii* is detected with lower sensitivity, *B. japonica* is not detected).
Proper specimens are DNA extracts isolated from samples of synovial fluid, cerebrospinal fluid, and skin biopsies.
This test is suitable for patients of all ages with suspected infection with *Borrelia* species belonging to the *Borrelia burgdorferi* s.l. complex (causative agent of Lyme borreliosis) and is intended as an aid in the diagnosis of infection with these pathogens in combination with patient history and additional clinical information.

The test is intended for professional use and is limited to qualified personnel instructed in the procedures of real-time PCR and *in vitro* diagnostic procedures.

## 2. Product description

BactoReal® Kit *Borrelia burgdorferi* sensu lato is a real-time PCR test and detects the ospA (outer surface protein A) gene of *Borrelia burgdorferi* s.l. species, namely, *Borrelia afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto, *B. valaisiana*, *B. lusitaniae, B. bavariensis, B. bissettii,* and *B. spielmanii* (*B. spielmanii* is detected with lower sensitivity, *B. japonica* is not detected).

A probe-specific amplification-curve in the FAM channel indicates amplification of *Borrelia burgdorferi* s.l. specific DNA. The internal DNA positive control (IPC) is detected in the Cy5 channel and serves as a control for DNA extraction and possible real-time PCR inhibition. The target of the DNA IPC (artificial target DNA) is added during sample extraction.

This test has been validated with the ABI® 7500 Fast Real-Time PCR System (fast cycle parameters are not supported, Thermo Fisher Scientific) and tested with a LightCycler® 480 II (Roche Diagnostics), Mx3005P® (Agilent), CFX Opus Real-time PCR System (Bio-Rad), CFX Dx (Bio-Rad) and T-COR 8 (Tetracore).
It is also compatible with other real-time PCR instruments which detect and differentiate fluorescence in FAM and Cy5 channel (e.g., QuantStudio™ 5, QuantStudio™ 7 real-time PCR system (Thermo Fisher Scientific), qTOWER$^3$G (Analytik Jena), Mic instrument (bio molecular systems), cobas z 480 Analyzer (Roche)).
When using PCR-platforms not tested by ingenetix, an evaluation of the multiplex-PCR shall be done. Keep in mind that some PCR-platforms first must be calibrated with the corresponding dye before performing multiplex-PCR.

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

## 3. Pathogen information

*Borrelia* are gram-negative bacteria belonging to the family *Spirochaetaceae*. The genus *Borrelia* consists of numerous different species. BactoReal® Kit *Borrelia burgdorferi* sensu lato detects species belonging to the *B. burgdorferi* sensu lato-complex causing Lyme borreliosis (LB). These bacteria are transmitted by ticks, mainly by *Ixodes ricinus*. Lyme disease is a multisystemic illness, which can affect various organs, especially the nervous system, skin, heart, and joints (O'Connell, 2010).

In Europe, the main pathogenic and potentially pathogenic genospecies are *B. afzelii*, *B. garinii, B. spielmanii, B. bavariensis*, *B. valaisiana, B. bissettii and B. lusitaniae, while B. burgdorferi* sensu stricto is less common. Pathogenic species in North America are *Borrelia burgdorferi* senso stricto and *B. mayonii* so far found in the Upper Midwest of the USA. The *B. burgdorferi* sensu stricto pathogen probably migrated from Europe to North America some 60 000 years ago and dominates in this geographical area. Evidence is accumulating that different genospecies cause different clinical symptoms in humans.

Species detected by BactoReal® Kit *Borrelia burgdorferi* sensu lato include *Borrelia afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto, *B. valaisiana, B. lusitaniae, B. bavariensis, B. bissettii* and *B. spielmanii*. Other

human pathogenic *Borrelia* species such as *B. recurrentis, B. duttonii* and *B. hermsii,* which can cause relapsing fever are not detected by this kit.

## 4. Principle of real-time PCR

The test is based on multiplex real-time PCR by 5´-nuclease-assay technology. Specific DNA sequences are amplified and the generated PCR-products are detected by oligonucleotide-probes labelled with fluorescent dyes. This allows a sequence-specific detection of PCR amplificates.

During PCR, primers are extended by *Taq* polymerase and probes hybridized to the target are cleaved by the 5'-exonuclease activity of *Taq* polymerase. According to the accumulation of PCR product, the fluorescence of the probe increases with each PCR cycle. The change in fluorescence of the different dyes is recorded cycle by cycle in the real-time PCR instrument in the closed reaction tube at different fluorescence wavelengths.
The Cq value (Cq = Quantification cycle, Ct = Cycle threshold, Cp = Crossing point) describes the cycle at which the fluorescence first rises significantly above the background fluorescence.

## 5. Kit contents, stability and storage

**Table 1**

| **Labelling** | **Content** | **Amount** | **Storage** |
|---|---|---|---|
| Borrelia burgdorferi s.l. + IPC3 Assay Mix (green cap) | Primers and probes for detection of<br>- *Borrelia burgdorferi* s.l. (FAM)<br>- DNA IPC (Cy5) | 1 x 50 µl | -25 to -15 °C |
| IPC-Target (DNA) (orange cap) | Target for DNA IPC (internal DNA positive control system) | 1 x 200 µl | -25 to -15 °C |
| Borrelia burgdorferi s.l. Positive Control (red cap) | DNA positive control (approx. 1,000 target copies/µl) | 1 x 300 µl | -25 to -15 °C |
| DNA Reaction Mix (white cap) | PCR reaction mix for DNA amplification | 1 x 500 µl | **-25 to -15 °C, after first opening 2 to 8 °C** |
| Nuclease-free water (blue cap) | Nuclease-free water | 1 x 1000 µl | -25 to -15 °C |

**DNA Reaction Mix**
The Master Mix provided with the kit has been designed for reliable, high-sensitivity real-time PCR. The Master Mix contains a highly purified Taq Polymerase for rapid hot-start PCR, dNTPs with dUTP and Uracil-N glycosylase (UNG) to eliminate amplicon carryover, ROX™ dye (passive reference) and buffer components – additives optimized to handle RT-PCR inhibitors.

**Delivery and Storage**
Shipment is with dry ice or with coolpacks. When stored properly, the kit components are stable until the specified expiration date. This also applies after opening. Store kit protected from light.

**Quality Control Release Testing**
In accordance with the ISO 13485 certified Quality Management System of ingenetix, each lot is tested against predetermined specifications to ensure consistent product quality. Quality control is performed with a plasmid containing parts of the pathogen DNA.

## 6. Additionally required materials and devices

- Reagents and devices for DNA-extraction which are appropriate for the listed sample material (see 9. Preparation of the samples)
- Nuclease-free water
- Powder-free disposable gloves
- Pipettes (adjustable)
- Filter pipette tips
- Vortex-Mixer
- Benchtop centrifuge with rotor for 2 ml reaction tubes
- Real-time PCR instrument detecting and differentiating fluorescence in FAM and Cy5 channel (see 2. Product description)
- Appropriate 96 well optical reaction plates or reaction tubes with corresponding (optical) closing material
- Optional: Laminar flow box
- Optional: PCR workstation

## 7. Precautions and safety information

### 7.1. General information

- For *in vitro* diagnostic use. The use of this kit is limited to qualified personnel instructed in real-time PCR and *in vitro* diagnostic procedures.
- Transportation of clinical specimens must comply with local regulations for the transport of biological materials.
- Specimens should be handled as if infectious in accordance with safe laboratory procedures. Wear protective powder-free disposable gloves when handling kit reagents and specimens.
- Discard specimens, reagents, and waste according to your local safety regulations.
- Improper collection, transport or storage of specimens may hinder the ability of the assay to detect the target sequences.
- Quality of DNA has a profound impact on the test performance. Ensure that the used DNA extraction system is compatible with real-time PCR technology.
- The real-time PCR instrument should be calibrated, serviced, and cleaned regularly.
- Protect kit components from light.
- Avoid mixing reagents of different kits and lots and check expiry date of the kit.

### 7.2. Specific information

A workflow must be followed to avoid false positive results due to detection of contaminating DNA.

<u>Recommended measures to avoid DNA contamination:</u>

- Use separated workspaces for specimen preparation, set-up of real-time PCR and amplification. Supplies and equipment must be dedicated to each of these separate workspaces to ensure workflow in the laboratory from pre- to post-PCR.
- Laboratory benches and devices must be cleaned regularly.
- Sample preparation should be performed in a laminar flow box. Clean laminar flow box regularly in all areas.
- If possible, leave consumables and pipettes in the laminar flow sterile bench and PCR workstation.
- Preparation of real-time PCR should be done in a PCR workstation.
- The use of sterile aerosol-resistant pipette tips is required.
- Use only DNA-free consumables.
- Wear lab coat.
- Work only with powder-free disposable gloves, do not touch the outer surface of the palm and fingers of the gloves when putting them on. Change gloves frequently. To avoid skin contact, wear gloves over the sleeves of the lab coat. Use disposable sleeve protectors if possible.
- Do not touch the rim or threads of open vials.
- Caution is advised when handling specimens and positive control to avoid cross-contamination.
- Store positive and potentially positive material separately from all other reagents.

- For a valid interpretation of results, a negative control shall be included during DNA-extraction (e.g., extraction of water instead of sample material), in to exclude false-positive results due to contamination with DNA of pathogen during extraction.
- Optional: include a negative control of PCR per PCR-run (nuclease-free water instead of sample, NTC).

## 8. Limitations

- Reliable results with this test are only achieved by appropriate specimen collection, transport, and storage, as well as an appropriate DNA extraction procedure.
- BactoReal® Kit *Borrelia burgdorferi* sensu lato was validated with DNA extracted (QIAamp® DNA Mini Kit, Qiagen) from synovial fluid, cerebrospinal fluid, and skin biopsy. Test performance with other specimen types has not yet been assessed.
- A negative test result does not exclude the possibility of *Borrelia burgdorferi* s.l. infection, because test results may be affected by improper specimen collection, technical error, specimen mix-up or pathogen quantities below the assay sensitivity. PCR inhibitors may lead to invalid results.
- For this kit highly specific primers and probes have been selected. However, false-negative, or less sensitive results might be obtained due to sequence heterogeneity within the target region of not yet described clinical subtypes. *Borrelia spielmanii* is detected with lower relative fluorescence intensities (RFI).
- Results should be interpreted in context of other clinical and laboratory findings.

# 9. Preparation of samples

BactoReal® Kit *Borrelia burgdorferi* sensu lato is suitable for analysis of DNA extracts from human tissue biopsies, CSF or tissue fluid.

Sample preparation should be performed with the recommended measures to avoid DNA contamination (see precautions and safety information). Always include a DNA extraction negative control (e.g., extraction of water).

Purified DNA should be stored at -25 to -15 °C.

## 9.1. Sample collection and storage

- Synovial fluid and cerebrospinal fluid can be stored in microcentrifuge tubes. It is recommended to process samples immediately after collection. Store samples at 2-8 °C for no longer than 48 hours or freeze at -20/-80 °C.
- Skin punch biopsies can be stored in microcentrifuge tubes containing sterile physiological saline (approx. 100 µl) at 2-8 °C for up to 48 h. If it is not guaranteed that the sample will be processed within 48 h, the sample shall be immediately frozen dry without additives at -20/-80 °C.

## 9.2. Recommended extraction methods

Ensure that the extraction system used is not contaminated with DNA of pathogens detected with BactoReal® Kit *Borrelia burgdorferi* sensu lato. Extract the sample using a DNA extraction system compatible with real-time PCR technology and appropriate for the sample material.

For manual extraction recommended
- QIAamp DNA Mini Kit (Qiagen)

For automated extraction recommended
- innuPREP AniPath DNA RNA – KFFLX Kit (Analytik Jena) with the KingFisher FLEX instrument (Thermo Fisher Scientific)

When using extraction methods not recommended by ingenetix, an evaluation of the extraction method must be performed.

## 9.3. Quality control for DNA extraction and PCR inhibition with IPC

The DNA IPC system (internal DNA positive control) is used as a control for DNA extraction, identifies possible PCR inhibition and confirms the integrity of kit reagents.
For this, an artificial target DNA (IPC-Target (DNA), approx. $6x10^5$ copies/µl) is added during extraction.

**Note:** The Cq values of the IPC depend on the extraction method and on the type of sample material. Negative samples should show Cq values of the IPC between 27-30. The extraction method used must be validated accordingly with sample material. Use the IPC-Target (DNA) freshly diluted (1:10 with nuclease-free water) in the extraction, if Cq values < 27 are determined during validation with samples.

### 9.3.1. Application of IPC as control of extraction and real-time PCR

The IPC-Target (DNA) must be added during extraction.

→ Per sample spike 1 µl IPC-Target (DNA) (orange cap) directly into the appropriate volume of lysis buffer (or spike it to the sample after the lysis buffer has been added to the sample), then continue the extraction procedure.

**Caution:** The IPC-Target (DNA) shall not be added to sample material in the absence of lysis buffer, as degradation may occur. It must be added to the lysis buffer.

### 9.3.2. Application of IPC as quality control for PCR reaction

If the IPC-Target (DNA) has not been added during extraction, it can be added at a later stage to the PCR master mix.

→ Freshly dilute the IPC-Target (DNA) 1:100 with nuclease-free water and add 1 µl of the dilution/PCR reaction (approx. 6000 target copies).

**Caution:** The IPC-Target (DNA) shall not be added to the master mix undiluted.

## 10. Preparation of real-time PCR

- Include one positive control (red cap), one extraction negative control and optional one PCR negative control (NTC, e.g., nuclease-free water) per PCR run.
- It is generally recommended to analyse samples in duplicates, which increases the probability of pathogen detection and facilitates interpretation of results.
- Thaw DNA samples on ice.
- Thaw kit components completely at room temperature. When thawed, mix components carefully, centrifuge briefly with low speed.
- Mix the DNA Reaction Mix gently to ensure homogeneity of solution.
- **Positive Control**
  → Use 9 µl of Positive Control (red cap). Always, pipette positive control at last.

### 10.1. Pipetting scheme

| | | **Per sample** |
|---|---|---|
| **Preparation of Master Mix** (mix well) | DNA Reaction Mix | 10.0 µl |
| | Borrelia burgdorferi s.l. + IPC3 Assay Mix | 1.0 µl |
| | **Total volume Master Mix** | **11.0 µl** |
| **Preparation of PCR** | Master Mix | 11.0 µl |
| | DNA-Sample* | 9.0 µl |
| | **Total volume** | **20.0 µl** |

*1-9 µl of the sample can be used. For ≠ 9 µl sample, the volume must be adjusted with nuclease-free water.

→ **If IPC-Target (DNA) was not added during extraction:** Freshly dilute the IPC-Target (DNA) (orange cap) 1:100 with nuclease-free water and add 1 µl per sample directly to the master mix. In this case, the IPC is used for quality control of the PCR reaction. Only 8 µl of DNA sample can be analyzed.
**Caution:** The use of more than 1 µl diluted IPC-Target (DNA) per reaction might cause inhibition of the real-time PCR reaction.

- Prepare the Master Mix according to the number of samples, calculating an additional volume of approx. 10% to account for pipetting loss.
- Pipette 11 µl of the prepared Master Mix per sample into the well of the optical reaction plate.
- Then add 9 µl of the extracted sample or controls. Pipette the positive control at last.
- Seal the plate with a suitable optical sealing material.
- Vortex the sealed plate for 1-2 seconds and briefly centrifuge the plate.

### 10.2. Programming of temperature profile

Please find further information on programming of the real-time PCR instrument in the respective operator's manual. Keep in mind that some PCR-platforms must be calibrated with the corresponding dye before performing a multiplex-PCR.

**Sample Volume:** 20 µl
**Ramp speed:** "Standard" without "fast cycling" parameter for ABI® 7500 Fast, QuantStudio™ (Thermo Fisher Scientific)

| **Program 1**<br>Cycles: 1<br>Analysis: None | **Program 2**<br>Cycles: 1<br>Analysis: None | **Program 3**<br>Cycles: 45<br>Analysis: Quantification<br>Acquisition at 60 °C |
|---|---|---|
| 50 °C<br>2 min | 95 °C<br>2 min | 95 °C<br>5 sec<br>60 °C<br>1 min |

**Ad program 1:** If viral RNA should be also detected in the same PCR run, program 1 must be prolonged to 15 min at 50 °C. This temperature profile can be used for all ingenetix ViroReal®, BactoReal®, MycoReal®, PanReal® and ParoReal® kits for the detection of DNA or RNA.
**Ad program 2:** the previous temperature profile with 20 sec in program 2 can still be used.

**<u>Select detection channels</u>**

**FAM channel:** Detection of *Borrelia*
**Cy5 channel:** Detection of IPC

**For ABI® 7500 Instrument, QuantStudio™ 5/6/7 (Thermo Fisher Scientific), Mx3005P® (Agilent)**
FAM-NONE
Cy5-NONE
Passive refence dye: ROX

**For CFX Opus Real-time PCR System (Bio-Rad), CFX Dx (Bio-Rad) and T-COR 8 (Tetracore)**
FAM
Cy5
Passive refence dye: no ROX needed

**For MIC Instrument (bio molecular systems)**
FAM Green
Cy5 Red
Passive reference dye: no ROX needed

**For cobas z 480 Analyzer / LightCycler® 480 II (Roche)**
Detection format: 2 Color Hydrolysis Probe
FAM: Excitation at 465 nm, Emission at 510 nm
Cy5: Excitation at 610 / 618 nm, Emission at 670 / 660 nm
Passive reference dye: no ROX needed

# 11. Interpretation of data

For the analysis of the PCR results, select the fluorescence display options FAM channel for the pathogen target and Cy5 channel for the IPC.
Please note that some PCR platforms require Color Compensation when using a multiplex PCR.

**IMPORTANT:** Samples should be checked in both logarithmic (Roche instrument: Abs Quant/Fit Points) and linear view and compared to the negative controls. In addition to the Cq values, check the amplification curves and adjust the threshold (noise band) if necessary.
After you have saved the new settings, export the data. For the cobas z 480 Analyzer, export tables per dye.

Assessment of clinical specimen test results should be performed after the positive and negative controls have been examined and determined to be valid. If results of controls are not valid, no interpretation of results with clinical samples is possible.

**Negative samples:**
Samples without amplification curves (no Cq values, undetermined) are regarded as negative. No DNA was detected in these samples because there is no infection with the corresponding pathogen or the pathogen DNA concentration is below the detection limit of the test.

**Positive samples:**
Samples with Cq values < 45 in the fluorescence channel for the pathogen target are regarded as positive.

Table 2 shows the criteria for valid positive and negative controls. Table 3 shows interpretation of data with clinical samples.

## 11.1. Controls

**Table 2**: Criteria for valid controls, IPC-Target (DNA) was added during extraction

| | Cq FAM channel *Borrelia* target | Cq Cy5 channel IPC[1] | Interpretation | Action |
|---|---|---|---|---|
| **Positive control** | < 30 | Negative | Valid | - |
| **Positive control** | Negative | Negative | Invalid | See 12.1 |
| **Extraction negative control** | Negative | 27-30[2] | Valid | - |
| **Extraction negative control** | Negative | Negative | Invalid | See 12.1 |
| **Extraction negative control** | Positive | 27-30[2] | Invalid | See 12.3 |
| **NTC PCR (optional)** | Negative | Negative | Valid | - |
| **NTC PCR (optional)** | Positive | Negative | Invalid | See 12.2 |

[1] If the IPC-Target (DNA) was added directly to the master mix, all samples in the Cy5 channel must be positive.
[2] Cq values of the IPC are dependent on the extraction method and should show Cq values of 27-30 (see 9. preparation of samples).

### 11.2. Clinical samples

Samples with positive Cq-values are interpreted as positive, see Table 3.

**Table 3** Interpretation of data with clinical samples

| | Cq FAM channel *Borrelia* target | Cq Cy5 channel IPC | Interpretation | Action |
|---|---|---|---|---|
| **Clinical sample** | Negative | 27-30[1] | Negative | - |
| **Clinical sample** | Positive | Positive/Negative[2] | Positive | - |
| **Clinical sample** | Negative | Negative | Invalid | See 12.5 |

[1] A positive signal of the IPC excludes potential PCR inhibition. However, IPC Cq-values should show comparable results among samples. A shift of Cq- values can indicate a partial inhibition of PCR. Cq values of the IPC are dependent on the extraction method and should show Cq values of 27-30 (see 9. preparation of samples).
[2] High pathogen loads in the sample can lead to a reduced or absent fluorescence signal of the IPC.

In case of invalid data, analysis must be repeated with the remaining or newly extracted DNA sample (see 12. Troubleshooting).

# 12. Troubleshooting

## 12.1. No pathogen specific signal with positive control and with IPC

- Incorrect programming of the temperature profile or incorrect setting of detection channels on the real-time PCR instrument.
  → Compare temperature profile and setting of detection channels with details specified in the protocol
- Incorrect configuration of PCR reaction.
  → Check your pipetting steps with the pipetting scheme and repeat PCR, if necessary.
  → The DNA may have been degraded.
- The IPC-Target (DNA) was added undiluted directly to the master mix and not freshly diluted 1:100. The PCR reaction is therefore inhibited.
  → Freshly dilute IPC-Target (DNA) 1:100 and repeat PCR.
- No Positive Control was added.
  → Repeat PCR in case all clinical samples are negative.
- For control of real-time PCR only: 1 µl of freshly 1:100 diluted IPC-Target (DNA) must be added to the master mix. If the addition of IPC-Target has been forgotten:
  → Freshly dilute IPC-Target (DNA) and repeat PCR.
- For control of DNA extraction and PCR inhibition, the undiluted IPC-Target (DNA) must be added during extraction to the lysis buffer. If the addition of IPC-Target has been forgotten:
  → Repeat DNA extraction.

## 12.2. Pathogen signal with negative control

- If there are Cq values in the negative control below the cut-off specified in the Instructions for Use, a contamination occurred during the preparation of the PCR.
  → Repeat PCR with new reagents in replicates.
  → Strictly pipette positive control at last.
  → Make sure that workspace and instruments are cleaned at regular intervals.

## 12.3. Pathogen signal with extraction negative control

- If there are Cq values in the negative control of extraction below the cut-off specified in the Instructions for Use, a contamination occurred during the preparation of the PCR or the DNA extraction.
  → Repeat the PCR with unused reagents in replicates.
  → If repeatedly positive: Repeat DNA extraction and PCR using new reagents.
  → Strictly pipette positive control at last.
  → Make sure that workspace and instruments are cleaned at regular intervals and that workstations are separated from samples with high pathogen concentration.

## 12.4. IPC specific signal with PCR negative control and positive control

- The IPC-Target (DNA) has been added to lysis buffer during extraction, but there is IPC specific signal with PCR-NTC and positive control: Contamination with the IPC-Target (DNA).
  → Make sure that workspace and instruments are cleaned at regular intervals.

## 12.5. No signal with IPC and no pathogen specific signal with sample

- Incorrect assignment of detection channels in sample.
  → Please verify the correct assignment of detection channels.
- The DNA might be degraded.
- If the IPC-Target (DNA) was added during extraction:
  - Inhibition of PCR may have occurred.
  - DNA extraction was unsuccessful.
  - The IPC-Target (DNA) was not added to the lysis buffer of the sample.
  - The extracted sample was not added to the PCR-reaction.

  → No statement is possible. Verify you use a recommended method for DNA isolation and re-examine the single steps of the DNA extraction.

→ If no operating mistakes during DNA 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 volume of nuclease-free water).

# 13. Specifications and performance evaluation

## 13.1. Kit performance

Performance of BactoReal® Kit *Borrelia burgdorferi* sensu lato with an ABI® 7500 instrument (Thermo Fisher Scientific) is shown in Figure 1.

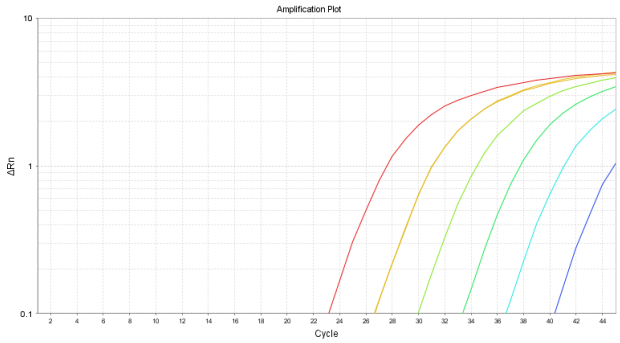
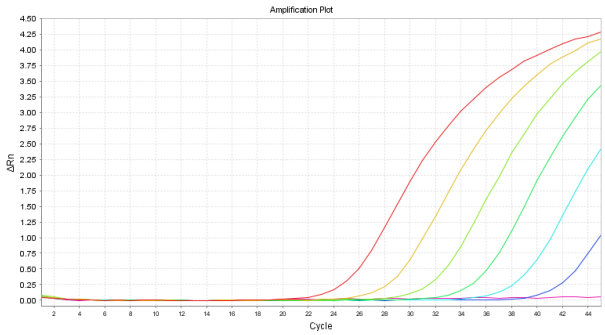
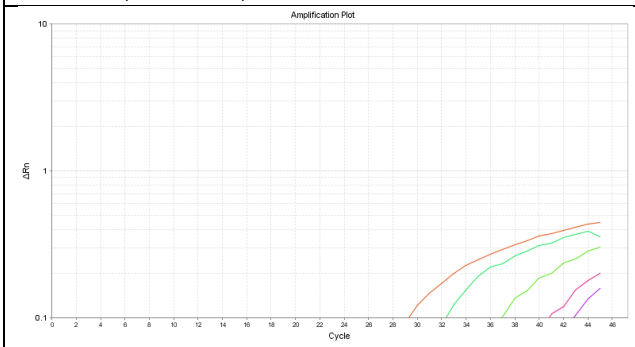
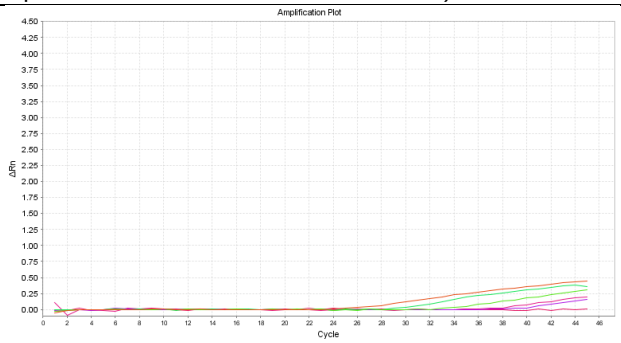
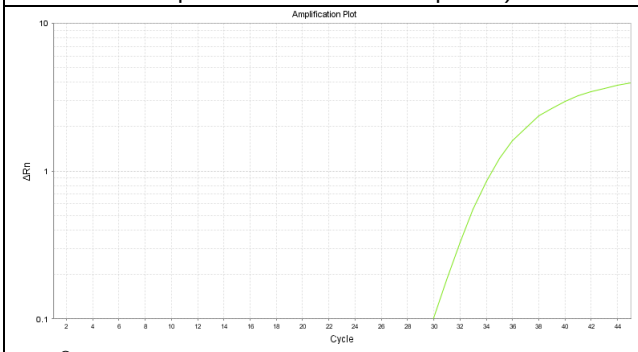
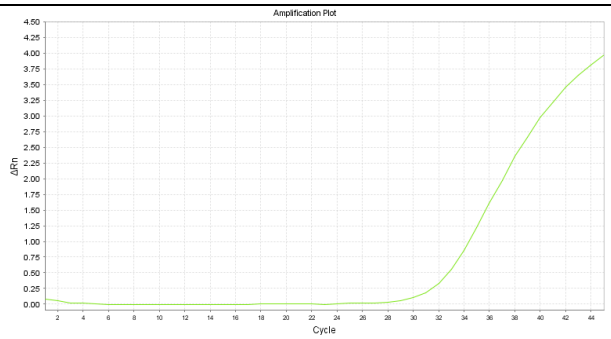
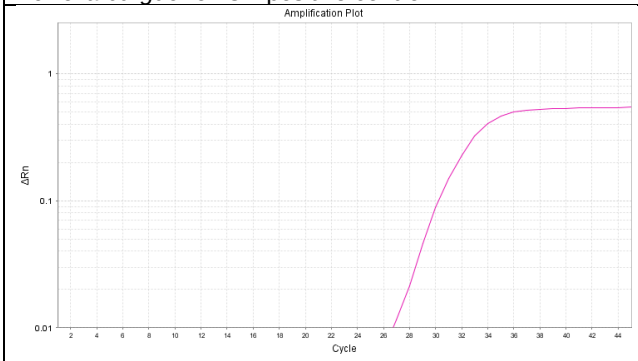
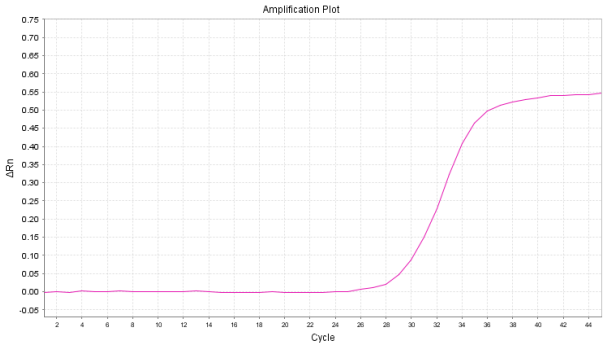
| Logarithmic view | Linear view |
|---|---|
|  |  |
| **ABI® 7500:** FAM channel<br>1:10 serial dilution of a *Borrelia afzelii* DNA target (amplification plots of *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, *B. valaisiana, B. bissetii, B. bavariensis* and *B. lusitaniae* show comparable relative fluorescence intensities) | |
|  |  |
| **ABI® 7500:** FAM channel<br>1:10 serial dilution of a *Borrelia spielmanii* DNA target (amplification plots of *B. spielmanii* show lower relative fluorescence intensities compared to other *Borrelia* species) | |
|  |  |
| **ABI® 7500:** FAM channel<br>*Borrelia burgdorferi* s.l. positive control | |
|  |  |
| **ABI® 7500:** Cy5 channel<br>Internal DNA positive control | |

**Figure 1** Performance of BactoReal® Kit *Borrelia burgdorferi* sensu lato

This test has been validated with the ABI® 7500 instrument (Thermo Fisher Scientific) and was also tested with a LightCycler® 480 II (Roche Diagnostics), Mx3005P® (Agilent), CFX Opus Real-time PCR System (Bio-Rad), CFX Dx (Bio-Rad) and T-COR 8 (Tetracore).

### 13.2. Limit of detection, LoD95%

**Method:** BactoReal® Kit *Borrelia burgdorferi* sensu lato was tested with a 10-fold dilution series of a plasmid containing a fragment of *Borrelia afzelii* DNA. The limit of detection (LoD95%: number of target copies detected in 95% of cases) was determined with plasmid dilutions around the analytical sensitivity using a non-linear curve fitting with the Graph Pad Prism Software.

**Result:** At least 10 target copies/reaction could be detected. The LoD95% is 42 target copies/reaction.

### 13.3. Linearity and dynamic measuring range

**Method:** Linearity was determined using a 10-fold serial dilution ($10^6$ – $10^1$ target copies/reaction) of the plasmid. The number of determinations (n) per dilution was nine.

**Result:** The assay shows linearity over the range of 100 to 1,000,000 target copies/reaction with a slope of -3.35 ± 0.1 and a $R^2$ of 0.99 as shown in Figure 2.

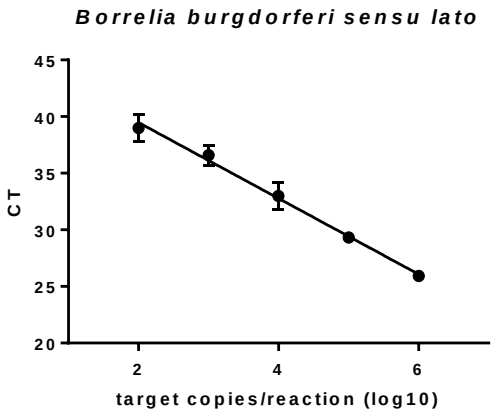



**Figure 2** Ten-fold dilution series of a *Borrelia afzelii* DNA standard plotted against CT

### 13.4. Precision

**Method:** Precision within a run (intra-assay), between multiple runs (inter-assay) and between two lots (inter-lot) was determined.

**Result:** The mean values of the coefficients of variation (CV%) are 0.93% for intra-assay precision, 1.21% for inter-assay precision and 0.35 for inter-lot precision.

### 13.5. Analytical specificity

**Method BLAST analysis:** The selection of highly specific primers and probes ensures analytical specificity. *In silico* validation of primers and probes was carried out with the Basic Local Alignment Search Tool (BLAST) against the NCBI database. Primers and probes have been checked for possible homologies to currently published sequences by sequence comparison analysis. This approach validates the detection of so far known *Borrelia burgdorferi* s.l. strains.

**Result:** No cross-reactions were observed in the BLAST analyses and no relevant sequence variability was observed in the primer and probe region of *Borrelia burgdorferi* sensu lato strains. An exception was *B. spielmanii* - this species shows sequence variability in the probe region. This species is therefore detected with lower sensitivity.

**Method testing exclusivity:** 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*).

**Result:** No cross-reactions have been observed. Other spirochetes, including *Leptospira* (n = 2) and *Treponema phagedenis (n = 1)* were not detected. *Borrelia* species causing relapsing-fever including *B. hermsii* (n = 1) and *B. miyamotoi* (n = 1) were not detected.

**Method testing inclusivity:** 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*).

**Result:** *Borrelia spielmanii* was detected with lower relative fluorescence intensity as visualized by lower amplification curves compared to the other isolates. Signal over background has to be evaluated using both, linear and log-scale view of the amplification plot as shown in Figure 1.
Serial 10-fold dilutions of ring trial samples indicated comparable sensitivity of *B. burgdorferi* sensu stricto (n = 2), *B. afzelii* (n = 2), *B. garinii* (n = 5), *B. bavariensis* (n = 1), *B. bisetti* (n = 1), *B. lusitaniae* (n = 1) *and B. valaisiana* (n = 1). *Borrelia japonica*, a non-human pathogenic *Borrelia* species was not detected.

### 13.6. Diagnostic evaluation

**Method:** Diagnostic evaluation of BactoReal® Kit *Borrelia burgdorferi* sensu lato was performed by an external service provider with 80 DNA isolates from clinical specimens with known positivity and negativity for *Borrelia*. Results were compared with those obtained with a DIN EN ISO 15189 accredited pathogen-specific quantitative real-time PCR reference method detecting the ospA gene using hybridization probes. The analytical sensitivity of the reference method with clinical specimens was 20 – 50 genome equivalents. DNA was isolated from relevant clinical specimens using the QIAamp® DNA Mini Kit, Qiagen. Real-time PCR was performed using a LightCycler® 480 II (Roche Diagnostics). Specimens included one tissue biopsy (skin), one stool sample, 46 cerebrospinal fluids, 27 synovial fluids and 5 EDTA-blood samples.

**Result:** A total of 44 known *Borrelia* spp. positive specimens (including *B. miyamotoi* (n=1), *B. hermsii* (n=1), which do not belong to the *B. burgdorferi* s.l. complex) and 36 known *Borrelia* negative nucleic acid preparations (including three *Leptospira* or *Treponema* positive specimens) were included in the study.

Using the reference method, 41 of the 42 *Borrelia burgdorferi* s.l. positive samples were positive (*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=5) and one sample was negative (*Borrelia japonica* n=1). The remaining 38 samples were negative (*B. hermsii* n=1, *B. miyamotoi* n=1, *Leptospira* sp. n=2, *Treponema* sp. n=1 and negative for *Borrelia burgdorferi* s.l. n=33).

In sum, 38 out of the 41 samples tested positive with the reference method were positive with BactoReal® Kit *Borrelia burgdorferi* sensu lato also. The two *B. spielmanii* positive samples showed lower relative fluorescence intensities and were considered negative.
Those 39 samples tested negative with the reference method, including the *Borrelia japonica* positive sample, were tested negative with the BactoReal® Kit *Borrelia burgdorferi* sensu lato also.

The diagnostic evaluation of BactoReal® Kit *Borrelia burgdorferi* sensu lato is shown in Table 3 and Table 4.

**Table 3:** Overall results obtained with 80 tested clinical isolates, 2x2 contingency table

| | **Reference** | | | |
|---|---|---|---|---|
| | | **pos** | **neg** | **Total** |
| **BactoReal® Kit *Borrelia burgdorferi* sensu lato** | **pos** | **39** | **0** | **39** |
| | **neg** | **2** | **39** | **41** |
| | **Total** | **41** | **39** | **80** |

**Table 4** Statistical evaluation of the diagnostic validation

| | Value | 95% CI |
|---|---|---|
| Sensitivity | 95.12% | 83.47% to 99.40% |
| Specificity | 100.00% | 90.97% to 100.00% |
| NPV | 95.12% | 83.46% to 98.69% |
| PPV | 100.00% | |
| Prevalence | 51.25% | 39.81% to 62.59% |
| Accuracy | 97.50% | 91.26% to 99.70% |

## 14. References

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

## 15. Revision history

| Revision | Date | Description |
|---|---|---|
| 1.1en | February 2020 | |
| 1.2en | 18 November 2021 | Added: Amendment of IFU with “Notice to purchaser: Limited license”.<br>**2. Product description**<br>“tissue” specified, validation of kit with extraction kit specified.<br>Definition of internal DNA positive control (DNA IPC) as a quality control added.<br>More examples of real-time PCR instruments added.<br>Recommendation of an evaluation of the multiplex PCR (plus dye calibration) added.<br>**3. Pathogen information**<br>update<br>**4. Contents of the kit, stability and storage**<br>Amendment of IFU with description of DNA Reaction Mix and Quality Control Release Testing.<br>**5. Additionally required materials and devices**<br>“appropriate for the biological sample material” added<br>**6. Precautions and safety information**<br>Added: Additional information on sample collection and storage, processing of samples after collection, storage conditions of samples, transportation and storage conditions of purified DNA and recommended tubes.<br>Amendment of filter pipette tips with “free from DNases, RNases”.<br>**7. Limitations**<br>Amendment of IFU with DNA extraction kit used for validation of BactoReal® Kit *Borrelia burgdorferi* sensu lato and specification of validated biological samples.<br>**8. Preparation of samples**<br>Recommendation of extraction negative control changed to “always include”.<br>Description of the DNA IPC (internal DNA positive control system).<br>Addition of a note with additional information on when the target should be added depending on the stage of extraction or PCR set-up.<br>**9. Preparation of real-time PCR**<br>Amendment of IFU with the requirement of the inclusion of “one extraction negative control per PCR run”.<br>“Always,” added to “pipette positive control at last”.<br>New ingenetix products PanReal and SeptiReal Kits added.<br>**10. Interpretation of PCR data**<br>Amendment of IFU with the definition of Cq, Ct and Cp values.<br>Update of tables for results with positive and negative controls and clinical samples. |

| Revision | Date | Description |
|---|---|---|
| | | Inclusion of a statement for assessment of clinical specimen test results with respect to controls.<br>**11. Troubleshooting**<br>Update of Troubleshooting with respect to references in new tables for results.<br>**12. Specifications and Performance evaluation**<br>**12.3 Analytical specificity**<br>Amendment of IFU with ring trial sample results and species not detected<br>**12.4 Diagnostic evaluation**<br>Amendment of IFU with specification of accredited reference method and its analytical sensitivity in genome equivalents. Extraction method used for clinical specimens and real-time PCR instrument added to document. More detailed description of obtained data with clinical specimens.<br>Amendment of IFU with a table depicting overall results from 80 clinical isolates tested with BactoReal® Kit *Borrelia burgdorferi* sensu lato.<br>**13. References**<br>New reference added: Kingry L. C. et al. 2018. |
| 1.3en | 01 May 2023 | **Change of the company address**<br><br>**Explanation of symbols**<br>Update<br>**1. Intended purpose**<br>Intended use specified in more detail<br>**5. Contents, stability and storage**<br>Kit version 1.1:<br>Filling volume of IPC-Target (DNA) and Positive Control was changed.<br>The DNA IPC Target is not stored in RNA/DNA Stabilizer anymore and was renamed to IPC-Target (DNA).<br>IPC-3 Assay Mix has been removed. The former DNA IPC-3 Assay Mix is now included in the Borrelia Assay Mix.<br>**7. Precautions and safety instructions**<br>Update of this section<br>Inclusion of additional information on specimen collection and storage, processing of specimens after collection, storage conditions of specimens, transport and storage conditions of purified DNA.<br>Recommended measures to avoid DNA contamination added.<br>**9. Preparation of samples**<br>innuPREP AniPath DNA RNA – KFFLX Kit is added<br>Note that for Cq values < 27, the IPC-Target (DNA) must be diluted prior to extraction.<br>**10. Preparation of real-time PCR**<br>Pipetting scheme has changed. |
| 1.4en | 02 June 2023 | CFX Dx (Bio-Rad) and T-COR 8 (Tetracore) were included as real-time PCR instruments which were validated with this kit. |

**Note:**
Any serious incident that has occurred in relation to the product shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.

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