Instructions for Use

RealLine Borrelia burgdorferi s.l.
Str-Format

ASSAY KIT FOR THE QUALITATIVE DETECTION OF DNA OF BORRELIA BURGDORFERI SENSU LATO COMPLEX USING REAL-TIME PCR METHOD

In vitro Diagnostics

RealLine Borrelia burgdorferi s.l. (Str-Format)	VBD1498	48 Tests
valid from	September 2019	

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Explanation of symbols used in labeling

IVD	*In vitro* diagnostic medical device
LOT	Batch code
REF	Catalogue number
Σ	Contains sufficient for <n> tests
	Use-by-date
	Temperature limit
	Consult instructions for use
	Keep away from sunlight
	Manufacturer

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1. INTENDED USE

Clinical Information:

Lyme disease or borreliosis is considered to be one of the fastest spreading illnesses in the world. Gram negative spirochetal bacteria from the genus Borrelia, which are collectively known as Borrelia burgdorferi sensu lato group cause Lyme disease and are transmitted by infected ticks. Early symptoms may include fever, headache, fatigue, depression, and a characteristic circular skin rash called erythema migrans (EM). Left untreated, later symptoms may involve the joints, heart, and central nervous system. In most cases, the infection and its symptoms are eliminated by antibiotics, especially if the illness is treated early. Delayed or inadequate treatment can lead to the more serious symptoms, which can be disabling and difficult to treat.

RealLine Borrelia burgdorferi s.l. (Str-format) assay kit is intendet for the detectionof DNA of pathogenic to human Borrelia species of *Borrelia burgdorferi* sensu lato complex:

- B. afzelii,
- B. garinii,
- B. burgdorferi sensu stricto

using the method for real-time polymerase chain reaction (PCR) with fluorescence detection of amplified products.

RealLine Borrelia burgdorferi s.l. kit can be used in clinical praxis for testing clinical material: cerebrospinal fluid, whole blood, blood serum, plasma, leukocyte blood fraction, biopsy material and tick suspension samples. The results of PCR analysis are taken into account in complex diagnostics of disease.

The extraction of DNA can be performed using following extraction kits from BIORON Diagnostics GmbH:

RealLine DNA-Extraction 2 (REF VBC8897)
RealLine DNA-Extraction 3 (REF VBC8889)
RealLine Extraction 100 (REF VBC8896)
RealLine Extraction 1000 (REF VBC8895)
For tick suspension samples please use the RealLine Extraction 100 or 1000 kit for the NA isolation (see Annex I).

When using NA extraction kits of other manufacturers it is highly recommended to use Internal Control sample (IC) from BIORON Diagnostics GmbH.

The **Str-Format Kit** contains 48 tubes (0.2 ml) in strips with lyophilized Mastermix. 50 µl of extracted DNA have to be pipetted into the tube and the ready mastermix is diluted. The kit contains reagents required for 48 tests, including control samples and the positive control sample.

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The kit is validated for use with: iQ5 iCycler, CFX96, iQ iCycler (Bio-Rad, USA) and RealLine Cycler (BIORON Diagnostics GmbH) and DT-96 (DNA-Technology, Russia).

The use of:

- **Extraction Kits for nucleic acids from clinical specimen from other supplier**
- **other real-time PCR devices**
- **appropriate reaction volumes, other than 50 µl**

has to be validated in the lab by the user. The special notes regarding the internal control IC have to be strongly followed.

2. KIT CONTENTS

Positive Control Sample (PC)	1 tube, 1 ml
Ready Master Mix (RMM), lyophilized	48 test-tubes (6 strips x 8 tubes)
Solution for Sample Preparation SSP	4 vials, 4 ml each
The kit also includes PCR optical film	1 sheet

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3. PRINCIPLE OF THE METHOD

The Real time PCR is based on the detection of the fluorescence, produced by a reporter molecule, which increases as the reaction proceeds. Reporter molecule is dual-labeled DNA-probe, which specifically binds to the target region of pathogen DNA. Fluorescent signal increases due to the fluorescent dye and quencher separating by Taq DNA-polymerase exonuclease activity during amplification. PCR process consists of repeated cycles: temperature denaturation of DNA, primer annealing and complementary chain synthesis.

Threshold cycle value – Ct – is the cycle number at which the fluorescence generated within a reaction crosses the fluorescence threshold, a fluorescent signal rises significantly above the background fluorescence. Ct depends on initial quantity of pathogen DNA template.
The use of **Internal Control (IC)** prevents generation of false negative results associated with possible loss of DNA template during specimen preparation. IC indicates if PCR inhibitors occur in the reaction mixture. IC template should be added in each single sample (including control samples) prior to DNA extraction procedure. The amplification and detection of IC does not influence the sensitivity or specificity of the target DNA PCR.

Note: IC is a component of the NA extraction kits of RealLine series. Internal Control is added to the sample during NA isolation step and is used throughout the whole process of NA extraction, amplification, detection.

4. SPECIFICATIONS

4.1 Sensitivity

Sensitivity control was performed on 5 samples containing 100 *Borrelia burgdorferi s.l.* DNA copies per sample, prepared from SRS (Standard Reference Sample containing *Borrelia burgdorferi s.l.* DNA).
The sensitivity equals 100%.

4.2 Specificity

Specificity of *Borrelia burgdorferi s.l.* DNA detection was determined using 4 negative samples, prepared from Standard Reference Panel (SRP). Specificity of *Borrelia burgdorferi s.l.* DNA detection equals 100%.

4.3 Diagnostic sensitivity

Diagnostic sensitivity determination was performed on 138 positive samples. Sensitivity equals 100% (within the range 97.9 – 100 %, with a confidence level of 90 %).

4.4 Diagnostic specificity

Diagnostic specificity determination was performed on 132 negative samples. Specificity equals 100 % (within the range 97.8 – 100 %, with a confidence level 90 %).

Analysis by the CE-marked reference kit showed full match of results.

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5. PRODUCT USE LIMITATIONS

- This assay must not be used on the clinical specimen directly. Appropriate nucleic acids extraction methods have to be conducted prior to using this assay.
- The presence of PCR inhibitors (e.g. heparin) may cause false negative or invalid results.
- When monitoring a patient the same extraction method must be used in all determinations. Otherwise, results may not be comparable.
- The kit is designed for use in patients with a clinical history and/or symptoms consistent with *Borrelia burgdorferi s.l.* infections. The kit may be used for screening purposes.
- Diagnostic sensitivity of the kit may vary depending on the pathogen prevalence and characteristics of the enrolled cohort.
- Reliable results depend on adequate specimen sampling.
- Positive results indicate active or asymptomatic infection; clinical history and symptoms should be taken into account.
- Negative results indicate lack of detectable DNA but do not exclude the infection or disease.
- Potential mutations within the target regions of the *Borrelia burgdorferi s.l.* genome covered by the primers and/or probes used in the kit may result in failure to detect the presence of the pathogens.
- The kit is not intended to replace culture and other methods (e.g., cervical exam) for diagnosis of infections.

6. WARNING AND PRECAUTIONS

- For in vitro use only.
- The kits must be used by skilled personnel only.
- When handling the kit, follow the national safety requirements for working with pathogens.
- To prevent contamination, the stages of DNA isolation and PCR test run must be spatially separated.
- Avoid microbial and nuclease contamination of reagents when removing aliquots from reagent vials.
- Wear protective disposable gloves, laboratory coats and eye protection when handling specimens and kit reagents.
- Every workplace must be provided with its own set of variable-volume pipettes, necessary auxiliary materials and equipment. It is prohibited to relocate them to other workplaces.
- The use of sterile disposable pipette tips is recommended.
- Never use the same tips for different Samples.
- Do not pool reagents from different lots or from different vials of the same lot.
- Dispose unused reagents and waste in accordance with country, federal, state and local regulations.
- Do not use the kit after the expiration date at the side label of the box.

7. ADDITIONAL MATERIALS AND DEVICES REQUIRED BUT NOT SUPPLIED

- real time PCR system, like described in p.1
- DNA-Extraction Kit: RealLine DNA-Extraction 3, RealLine DNA-Extraction-2, RealLine Extraction 100 or 1000.
- Internal Control reagent (VBC8881) and Negative Control Sample, if the kit is used with the extraction kits of other supplier;
- laminar safety box;
- refrigerator;
- vortex;
- half-automatic variable-volume single-channel pipettes;
- disposable medical non-sterile powder-free gloves;
- disposable pipette tips with aerosol barrier;
- biohazard waste container
- razor or scalpel.

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8. PREPARATION OF THE ANALYSED SAMPLES

*Each group of samples undergoing the procedure of DNA isolation must include a **Positive Control sample (PC)** from this kit and a **Negative Control sample (NC)** which is a component of the DNA extraction kit.*

We strongly recommend the implementation of the Internal Control IC, the Negative Control NC and Positive Control PC samples to the extraction procedure.

When using a kit of another supplier for the extraction of nucleic acids as recommended in chapter 1, add **20 µl** of **IC (VBC8881)** to each tube.

- For the NC use **100 µl** of the Negative Control Sample or H_2O (molecular biology grade).
- For the PC use **70 µl** of Negative Control Sample or H_2O (molecular biology grade) and **30 µl** of Positive Control to the tube marked PC.

The assay is performed on extracted DNA specimens obtained from the clinical material using one of the DNA extraction kits listed in p.1, according to their Instruction Manuals. To prepare tick suspension, SSP is used (see Annex I).
After initial opening, store SSP at (2 – 8) °C for no more than 3 months.
Store extracted DNA at (2 – 8) °C for no more than 24 hours.

9. PROCEDURE

9.1. Preparation of the reagents.

Prior to the test take the kit out of the refrigerator and keep the **Ready Master Mix (RMM)** closed in the package at (18 – 25) °C for at least 30 minutes. Then open the package and cut the necessary number of tubes in strips with RMM (*including prepared specimens and controls: 1 NC and 1 PC*) with a razor or a scalpel. Cut the tubes together with the covering film Put the remaining strips immediately back into the foil pouch, squeeze the air out and tightly close with the clip.

After initial opening, store RMM at (2 – 8) °C for no more than 3 months.

After initial opening, store PC at (2 – 8) °C for no more than 1 month, or in 50 µl aliquots at minus (18 – 60) °C for no more than 3 months.

9.2. Label the tubes with RMM for each specimen and control.
Attention! *Labels should be placed on the lateral side of the tubes.*

9.3. Add **50 µl** of corresponding isolated DNA solution to each tube using a separate pipette tip with filter. Tightly close the tubes with caps or seal with the PCR transparent film.

9.4. Place the tubes into the real-time PCR system.

9.5. Program real time PCR system as follows:

Step 1:	50°C	2 min	
Step 2:	95°C	2 min	
Step 3:	94°C	10 sec	50 Cycles
	60°C*	20 sec	
* measurement of fluorescent at 60 °C in FAM and ROX			

9.6. Select the amplification detection channels:

- Collect real-time PCR data through the **FAM** channel for detection of amplification of **IC** DNA.
- Collect real-time PCR data through the **ROX** channel for detection of amplification of ***Borrelia burgdorferi s.l.*** **DNA**.

9.7. Program the positions of test tubes with Samples, positive and negative controls according to the instruction manual for the real time PCR system in use.

9.8. Run the program.

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10. DATA ANALYSIS AND INTERPRETATION

10.1 For **PC** the program should detect:

- increase of the IC DNA amplification signal (channel **FAM**) and determine the threshold cycle, IC **Ct**;
- increase of the Borrelia burgdorferi s.l. DNA amplification signal (channel **ROX**) and determine the **Ct** value;

10.2 For **NC** the program should detect the increase of the amplification signal of IC DNA (channel **FAM**) and determine the threshold cycle, IC **Ct**. No **ROX** fluorescent increase should appear (*no Borrelia burgdorferi s.l. DNA amplification*).

10.3 If Ct value for NC through ROX channel is less than or equal to 40, this indicates the presence of contamination (see paragraph 9.7.).

10.4 For each sample the program should detect the increase of the amplification signal of IC DNA (channel FAM) and determine IC Ct.

10.5 Calculate (IC Ct)av as an average IC Ct of all analyzed samples (including PC and NC). IC Ct values that differ by more than 2 from the (IC Ct)av should be ignored. Recalculate the (IC Ct)av for the remaining values after the screening.

10.6 The sample is considered negative (not containing *Borrelia burgdorferi s.l.* DNA), if Ct value via ROX channel for this sample is above 40 or is not determined.

10.7 If IC Ct value for such sample differs from the (IC Ct)av value by more than 2, the result is regarded as equivocal. A repeated analysis of the sample, starting with the DNA isolation step is necessary.

10.8 The sample is considered positive and containing *Borrelia burgdorferi s.l.* DNA, if Ct value via ROX channel for this sample is less than or equals to 40.

10.9 In case of contamination all positive results of this individual PCR run are considered equivocal. Actions are required to identify and eliminate the source of contamination, and repeat the analysis of all Samples of this run that were identified as positive. Samples that showed negative results in this run should be considered as negative.

11. STORAGE AND TRANSPORTATION

- Store the assay kit at (2 - 8) °C in the manufacturer's packing.
- Transport at (2 – 8) °C; transportation up to 25 °C for up to 10 days is allowed.
- Do not freeze the kit!
- Do not pool reagents from different lots or from different vials of the same lot.
- Strictly follow the Instruction manual for reliable results.
- Do not use kits with damaged inner packages and get in contact with BIORON Diagnostics GmbH.

- **Storage and shelf life of solutions and components of the kit after initial opening:**
  Positive Control sample: 1 month at (2 – 8) ° C or in 50 µl aliquots at (-18 … -24) °C for not more than 3 months.
  Ready Master Mix (RMM): 3 months at (2 – 8) °C.

Technical Support: techsupport@bioron.de

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ANNEX I: PREPARATION OF TICK SUSPENSIONS

Equipment required:

- Laminar box;
- 1.5 ml tube rack;
- IsoFreeze double-sided refrigerator SSI-5610-43 or floating stand SSI-5100-43 (“SSI”, USA);
- Cooling element or a container with ice;
- Metal pestles for grinding ticks.

Place ticks into the numbered 1.5 ml Eppendorf type tubes.

To clean the ticks from contamination by substances used for the removal of attached insects, wash them before preparation of suspension (see p. a). In case of analysis of free ticks, the suspension can be prepared immediately, (see p. b or c).

a) Preliminary washing of ticks:

Add 300 µl of 96% ethanol to each tube with a tick, vortex the tubes, and then centrifuge briefly. Remove ethanol using a pipette or an aspirator without touching the tick, using separate tips for each specimen. Add 500 µl of 0.15 M sodium chloride to tubes, vortex the tubes and spin for 5 sec at 7000 rpm to collect any drops; discard the supernatant using a pipette or an aspirator with a new tip for each specimen.

b) Preparation of tick suspensions:

Attention! *Ticks that died recently as well as dried or crushed ticks are suitable for PCR analysis.*
To prevent degradation of nucleic acids (NA) extracted from the ticks, avoid heating of the sample to a temperature above 8 °C at all stages of ticks suspension preparation.
Before analysis, full ticks, which diameter is comparable to that of the tube, should be pierced with a disposable needle or a pipette tip.
When preparing the ticks for the analysis use SSP (Solution for Sample Preparation (SSP) – is a component of some of the kits) pre-cooled to a temperature of (2 - 8) °C.

Method 1. Freeze the tubes with ticks in liquid nitrogen (for at least 5 min). Take one frozen tube and immediately carefully grind the tick with a separate sterile pestle combining rotational movements and pressing. Without removing the pestle, put the tube with the crushed tick into the rack, placed on ice. Add 250 µl of pre-chilled SSP to the tube. Gently rinse the pestle in the tube and discard it into disinfectant solution.
Vortex the tube for 5-10 sec. Centrifuge briefly to collect the drops from the inside tube walls. Perform the grinding procedure with other specimens. Without touching the pellet, take 100 µl of the specimen for nucleic acids extraction and further assay.

Method 2. Add 30 µl of SSP to the tubes with ticks (for full or large ticks add 50 µl of SSP). Place the tubes into liquid nitrogen or pre-chilled thermal rack and keep until completely frozen in a freezer at minus (20-30) °C. Take one tube with the tick frozen in the SSP, and crush the tick thoroughly with a sterile pestle before the solution thaws. Without removing the pestle, put the tube with the crushed tick into the rack, placed on ice. Add 200 µl of pre-chilled SSP to the tube. Gently rinse the pestle in the tube and discard it into disinfectant solution.

Vortex the tube for 5-10 sec. Centrifuge briefly to collect any drops from the inside tube walls. Without touching the pellet, take 100 µl of the specimen for nucleic acids extraction and further assay. Perform the grinding procedure with other specimens.

c) **Method 3**. Tick suspension preparation using MagNA Lyser (Roche Diagnostics, Switzerland) or similar homogenizer:

Prepare a tube with ceramic beads for each specimen; place the tubes into pre-chilled thermal rack. Add a tick and 300 µl of SSP to each tube and close the tubes tightly. Place the tubes into the homogenizer's rotor (up to 16 tubes) and fix the rotor according to the Instruction manual. Homogenize specimens for 90 sec at 7000 rpm.

Attention! To ensure biological safety and prevent contamination, before opening the homogenizer, check through the window in the lid that no leakage of material has occurred.

If droplets or aerosols appear in the working chamber, the homogenizer should be moved to the laminar box and kept with closed lid for 30-40 min. Open the homogenizer and process the device in accordance with the instructions for handling potentially infectious material and the disinfection instruction given in the device manual.

After disinfection, remove the tubes from the device and place into pre-chilled thermal rack. In case of insufficient grinding of individual specimens, repeat the homogenization procedure.

Discard the drops of the suspension from the walls of the tubes by brief centrifugation. To extract nucleic acids using the **RealLine Extraction 100** kit, take 100 µl of the supernatant.

Attention! Add additional 300 µl SSP after homogenization when working with large ticks (i.e. ticks larger than 5 mm in diameter, representatives of the genera *Dermacentor* and *Hyalomma*, full ticks).

Transportation and storage of ticks and tick suspension specimens:

- At (2 - 8) °C – for no more than 24 hours;
- At minus (18 - 60) °C – for no more than 2 weeks.
- At minus 70 °C and below – for no more than 1 year.

Do not freeze - thaw specimens repeatedly!

ANNEX II: NOTE: Settings for RealLine Cycler and DT96:

for these cyclers the measurement exposure must be adjusted. Choose the **Operation with the device** mode in the **Settings** menu, select the item **Measurement exposition:**

- **FAM** to **250**
- **ROX** to **1000**

Confirm that the current exposure value is saved by pressing **YES**

Attention! The specified exposure values are applicable only for RealLine kits and, if necessary, must be changed for other purposes.

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