Real-time PCR detection of *Borrelia burgdorferi* Sensu Lato

**DESCRIPTION**

Real-Time PCR kit is dedicated for a detection of *Borrelia burgdorferi* Sensu Lato, *B. afzelii*, *B. garinii* and *B. valaisiana*. Recommended input material is mainly urinary sediment, liquid, punctate, full blood (EDTA/citrate). We recommend process the biological material using any common commercial procedures for DNA purification. Kit was optimized for the purification of nucleic acids using Qiagen QIAamp Mini Kit. Real Time PCR detection is based on the gene sequence for the synthesis of Flagelin, which is commonly used for similar purposes (Schwaiger *et al.*, 2001; Mommert *et al.*, 2001; Pahl *et al.*, 1999).

**STORAGE CONDITIONS**

The components of the kit should be stored at -20°C (or lower) and repeated thawing and freezing should be avoided, as this may reduce assay performance. PMX II is light-sensitive and it is highly recommended to expose it to the light only for the time needed for assay preparation.

**GENERAL RECOMMENDATIONS FOR PCR DIAGNOSTICS**

PCR is a highly sensitive method capable of detecting even very small amounts of nucleic acid which can serve as a template to amplify and cause contamination analysis.

The source of contamination PCR may be in particular:
- Laboratory table accessories and pipettes contaminated from previous isolation of nucleic acids
- Contacts between samples
- PCR products from previous analyzes
- STD/positive controls

Diagnostics using molecular genetic analyzes can be performed only by qualified persons for this purpose trained and technically qualified. Manufacturer is not liable for erroneous results caused by improper handling.

Physical separation of spaces/areas for:
- Nucleic acids purification
- Preparation of reagents for amplification
- PCR analysis and evaluation

Each area must be equipped with instruments and tools that are specifically meant for the space (room or laminar box), you must use only tools in the appropriate space and do not transfer them between the outlined areas. Extraction of nucleic acids and preparation of PCR reagents must be performed in a reserved safety cabinet and PCR laminar flow hood or other sterile place which is equipped with an UV lamp. The equipment should be emitted at least 15 minutes with UV light before the use, the use of substances degrading the DNA/RNA (thermo-DNA-Tor, etc.) is recommended.
For storage it is recommended to physically separate reagents into individual boxes for nucleic acid extraction, preparation of PCR reactions, especially you have to put into different boxes positive controls, which are the most common source of contamination.

When handling reagents and accessories it is recommended:

- Maintain strict physical separation of individual practices
- Do not use expired or otherwise depreciated products
- Reagents can be in a clean place aliquoted for daily use and properly stored
- Use RNase and DNase-free solutions and sterile filter tips
- Before and after analysis use chemical nucleic acid decontamination method for all surfaces, pipettes, racks and accessories, or use UV lamps.
- Avoid aerosol formation
- Use disposable gloves and change them regularly.
- Empty containers for biological waste after each procedure
- During the extraction of nucleic acids and preparation of PCR left gaps in the rack between the tubes to reduce the risk of contamination between samples.
- When preparing PCR pipette in following order: 1st - negative control, 2nd - analyzed samples, 3rd - positive control

DISPOSAL OF THE REST OF THE KIT

The individual kit components and consumables are recommended to be treated as infectious material. Waste is appropriate to place into containers for the disposal of infectious biological material.

KIT CONTAINS:

Amplification reagents for the preparation of 50 assays for Real-Time PCR analysis:

- PMX I (600µl)
- PMX II (24µl)
- PMX III (408µl)
- PMX IV (24µl)
- Polymeráza TAQ (24µl)
- BBSL STD (55µl) – positive control

REQUIRED REAGENTS AND CONSUMABLES, WHICH ARE NOT INCLUDED IN THE KIT

Purification kit
Filter tips sterile
SmartCycler Tubes
Tubes about volume 1.5-2ml for preparation of Mastermix

REQUIRED INSTRUMENTATION

Centrifuge with minimal centrifugal acceleration of 14,000 x g
Thermoblock or water bath maintained at 56°C
Biohazard box, box PCR
Pipettes with adjustable volume 0.5 - 1000µl
Vortex, chilled block
Real Time Cycler with an optical system for reading FAM, TxR fluorochromes (kit has been optimized for devices SmartCycler/Cepheid and RotorGene/Qiagen).
Refrigerator, freezer

BIOLOGICAL MATERIAL

The recommended material is urine, whip, punctate, in acute conditions as well as whole blood (EDTA/citrate). Biological material is recommended to keep at 0 ° to 4 ° C. If necessary, the sample can be frozen and stored long term at -20°C. The recommended urine volume for urinary sedimentation is at least 8 ml, optimally 10 ml or more, urine after centrifugation at maximum speed (14,000 g or more) for 10 minutes creates sediment, which is suitable to be washed twice with PBS buffer and then dissolved in 100 µl of saline. In case of analysis of liquor, puncts or blood, it is advisable to use at least 200µl sample, the recommended amount is 400-500µl sample.
The method was tested for species *Borrelia burgdorferi* Sensu Stricto, *Borrelia afzelii*, *Borrelia garinii*, *Borrelia valaisiana*. Specificity was verified on the above-mentioned BBSL species and related organisms (Treponema) and a number of other bacterial strains. Estimated sensitivity: 10E1 - 10E2/ml.

**PROCEDURE FOR DNA PURIFICATION FROM CSF, SERUM AND SWAB**

Suggested commercial kits for the processing of the above mentioned samples:
- **QIAamp DNA Mini Kit (50)** REF: QIA-51304 / (250) REF: QIA-51306
- **QIAamp MinElute Virus Spin Kit(50)** REF: QIA-57704
- **QIAamp DNA Blood Mini Kit(50)** REF: QIA-51104 / (250) REF: QIA-51106

Samples are recommended to process according to the manufacturer's instructions of the particular kit. The treated sample can be directly used for amplification or stored in a refrigerator at 2 to 8 °C for 24h. For longer storage temperature range -20 °C to -80 °C is recommended.

**REAL-TIME PCR ANALYSIS – SMART CYCLER**

After a spontaneous thawing all tubes necessary to prepare the analysis should be vortex thoroughly (It is inappropriate exhibit the solutions in higher temperatures than ambient for quicker thawing!). Subsequently, all the components in the required volume needed for a mastermix preparation for particular sample count (n+1) and one positive and one negative control are pipetted into a clean sterile minicentrifuge tubes (1.5 - 2 ml) according to the protocols:

**PCR/1reaction (20μl):**
- 2 μl DNA sample
- 10 μl PMX I
- 0,4 μl PMX II
- 6,8 μl PMX III
- 0,4 μl PMX IV
- 0,4 μl Polymerase (5U/ μl)
- 20 μl total volume sample

The tubes with the prepared mastermix should be vortex thoroughly and then 18μl aliquots of the mastermix are pipetted into the Smart tubes placed in a chilled block. It is recommended to first pipette the negative controls (2 μl dd H₂O or TE buffer), prepared DNA samples (2μl), and as the last the positive controls.

After addition of the negative control, sample and the positive control it is recommended close immediately Smart tubes to not increase the risk of contamination between the tubes. Subsequently, Smart tubes should be centrifuged for about 10 seconds in the SMART centrifuge and then placed in a thermal cycler.

**PROTOCOL REAL-TIME PCR SET-UP IN SC SOFTWARE:**

In the software menu new temperature-time analysis profile is created over the "New protocol" button:

Subsequently, enters the number of test samples and defines their location in specific positions cycler. Despite icon "Start Run ” to start the analysis.
Treshold values for the individual optical channels:
Channel 1 (FAM) 30 RFU
Channel 3 (TxR) 30 RFU

RESULTS EVALUATION

The detection of Borrelia burgdorferi SL takes place on the basis of FAM signal detection in the first channel (channel1). Detection The internal control is based on detection of the TxR signal in the third channel (channel3).

Borrelia burgdorferi (FAM) + Internal control(TxR)

The results are judged as valid only if the positive control gives a positive result (RFU > 30), a negative control is negative in the analysis result (RFU < 30).

A sample was considered positive when fluorescence exceeded an increase threshold value Threshold 30RFU. A sample is considered negative only if the analysis shows a positive internal control signal.

If the increase in fluorescence exceeded Threshold later than the 42th cycle, it is recommended to repeat the analysis. In case of positive samples Listeria monocytogenes, the high intensity of positivity can cause the inhibition of amplification of internal controls - such samples are positive despite the negative signal of internal control.

TROUBLESHOOTINGS

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Suggestions</th>
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</thead>
<tbody>
<tr>
<td>1. Problems during nucleic acid purification</td>
<td>See the instructions of used purification kit</td>
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</tr>
<tr>
<td>2. Positive samples and positive controls are negative</td>
<td>a) enzyme was not added to the reaction or is inactive</td>
<td>Ensure that the enzyme was added to the correct tube and repeat the analysis</td>
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</tbody>
</table>
### 3. Negative controls or negative samples are positive

<table>
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<th>b) the wash buffers has been not completely removed during the purification using the Qiagen kit</th>
<th>Make sure that the centrifugation speed was properly set during the purification, repeat the purification and subsequent steps</th>
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<tbody>
<tr>
<td>b) positive control and positive samples were repeatedly thawed and re-frozen</td>
<td>Avoid repeated thawing, repeat the procedure with new controls/samples</td>
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<tr>
<td>d) positive control or positive samples were stored at improper temperature</td>
<td>Samples and controls should be stored at temperature recommended by the manufacturer, repeat the analysis with new controls/samples</td>
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<tr>
<td>e) part or the whole kit has been stored incorrectly or is expired</td>
<td>Make sure that all the components were stored at the correct temperature and check the lifetime of the kit</td>
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<tr>
<td>f) during the purification using the Qiagen kit ethanol was not added in the respective buffers</td>
<td>Make sure that ethanol was added to the appropriate buffers, repeat the purification and subsequent analysis</td>
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### 4. Lower result values than expected

<table>
<thead>
<tr>
<th>a) contamination from previous PCR analysis or contamination during the analysis</th>
<th>Change the tip after each pipetting</th>
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<tbody>
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<td></td>
<td>Use the filter tips</td>
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<td>Maintain the recommended separation of individual practices</td>
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<td>Use UV lamps and disinfectants for all desktops</td>
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<td>Change gloves regularly</td>
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<td>Handle all reagents with care, avoid unnecessary contact the individual tubes and avoid aerosol formation</td>
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<td>When pipetting, avoid the contact with the inner surface of the tube or lid</td>
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<td>Test tubes in the stand and in a centrifuge alternate with empty positions</td>
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<td></td>
<td>Repeat the analysis with new controls and samples</td>
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<thead>
<tr>
<th>a) isolate was stored too long before being analyzed</th>
<th>Isolated samples are recommended to analyze as soon as possible after purification, repeat the purification and perform Real-Time PCR immediately</th>
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<tbody>
<tr>
<td>b) purification was too slow or inappropriately discontinued</td>
<td>Samples are recommended process continuously without unnecessary time delays, repeat the isolation and subsequent analysis</td>
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<tr>
<td>c) PCR was not performed properly due to poor handling kit</td>
<td>Make sure the kit is stored in optimal conditions recommended by the manufacturer and repeat the analysis</td>
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