# Brucella Detection Kit v1

# USER MANUAL

For *in vitro* Diagnostic Use

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IVD CE

**Contents**

## 1. PRODUCT DESCRIPTION

Bosphore® Brucella Detection Kit v1 detects B.abortus, B.melitensis, B.canis, B.suis, B.ovis and B.microti DNA in human biological samples. The analytic sensitivity is $7.5x10^2$ copies/ml. A region within the BCSP31gene is amplified and fluorescence detection is accomplished using the FAM filter.

An internal control has been integrated into the kit in order to check PCR inhibition. The amplification data of the internal control is detected with the HEX filter. The internal control is added in PCR step.

## 2. CONTENT

Bosphore® Brucella Detection Kit v1 is composed of Real-Time PCR reagents.

| Component | REAGENT | 100 Reactions | 50 Reactions | 25 Reactions |
|---|---|---|---|---|
| 1 | $dH_2O$ | (1000 µl) | (500 µl) | (500 µl) |
| 2 | PCR Mix | (1375 µl) | (688 µl) | (344 µl) |
| 3 | Detection Mix1 | (165µl) | (83 µl) | (42 µl) |
| 4 | Detection Mix2 | (28 µl) | (15 µl) | (15µl) |
| 5 | Internal Control | (15µl) | (15 µl) | (15 µl) |
| 6 | Positive Control | (88 µl) | (44 µl) | (22 µl) |

## 3. STORAGE

Bosphore® Brucella Detection Kit v1 PCR reagents should be stored at -20°C. Repeated thawing and freezing (>3x) should be avoided since it may reduce sensitivity. If the components are to be used in small amounts, they should be frozen in aliquots.

While preparing the PCR; the components should not be exposed to room temperature for more than 10 min., the PCR master mixes and detection mix components should not be exposed to light or air more than necessary, vials must be kept closed except during pipetting. We recommend preparing the PCR on a cooling block and keeping the detection mixes within a closed container.

The components maintain their stability until the expiry dates on the labels, if they are stored at advised conditions.

## 4. REQUIRED MATERIALS AND DEVICES

- Montania® 483 or Montania® 4896 Real-Time PCR Instrument (Anatolia Geneworks), or another Real-Time PCR system with FAM and HEX filters (such as iCycler, iQ5, CFX–BioRad, LightCycler 2.0, 480-Roche, 7300, 7500 Real-Time PCR System, ABI StepOne or StepOne Plus systems -ABI, Stratagene Mx3005P, Mx3000P-Agilent, LineGeneK, LineGene 9600-Bioer, Rotorgene 2000, 3000, 6000, Q-Qiagen)
- 0.2 ml Thin-Wall PCR tubes, PCR plates or strips
- Magnesia® 16 Nucleic Acid Extraction System / Magnesia® Bacterial Genomic DNA Extraction Kit/ Bosphore® Bacterial DNA Extraction Spin Kit (Anatolia Geneworks), or other high quality bacterial DNA extraction kits and systems
- Deep freezer (-20°C)
- Desktop centrifuge with rotor for 2 ml. microcentrifuge tubes
- Calibrated adjustable micropipettes
- DNAse, RNAse, pyrogen free micropipette tips with filters
- DNAse, RNAse, pyrogen free 1.5 or 2 ml. microcentrifuge tubes

- Disposable laboratory gloves

## 5. IMPORTANT NOTES AND SAFETY INSTRUCTIONS

**Important!:**

- The product should be delivered on dry ice. Check for presence of dry ice upon arrival.
- Check for the expiry dates on the box and tube labels, upon arrival. Do not use expired products or components.
- Calibrated or verified micropipettes, DNAse, RNAse, pyrogen free micropipette tips with filters, and DNAse, RNAse, pyrogen free microcentrifuge tubes should be used.
- Before starting a test procedure, all components should be thoroughly thawed. After thawing, all components should be centrifuged briefly (spin-down for 3-5 seconds), and mixed well to ensure homogeneity prior to use.
- The kit components should be kept on ice or a cooling block until the reaction is prepared, and they should be quickly returned to -20°C.
- PCR and nucleic acid isolation must be performed in different compartments. Samples should be stored separately to avoid contact with the kit components.
- Pathogen information should be reviewed to be aware of the health related risks.
- Biological samples should be handled with extreme caution: Physical contact with pathogens should be avoided by; wearing lab coats and gloves, no allowance for eating or drinking within the workspace, prevention of unauthorized individuals' access to the working area.
- All the pathogenic wastes produced during the nucleic acid isolation step; including the biological samples and material contacted with them, should be discarded into medical waste and disposed safely.

## 6. PRODUCT USE LIMITATIONS

- All the components may exclusively be used for in vitro diagnostics.
- This product should be used in accordance with this user manual, by personnel specially trained to perform in vitro diagnostic procedures.

## 7. PATHOGEN

### Causative Agents

Brucella is a gram-negative, facultative, intracellular bacteria infecting many species of animals and humans. It is distinguished from most other pathogens since it has no obvious virulence factors such as capsules, fimbriae etc. It is the causative agent of Brucellosis (a zootonic disease that can also be transmitted to humans) and also recognized as a bioterrorism agent. The genus Brucella has at least six species, where Brucella *abortus* (affecting primarily cattle), *Brucella suis* (affecting primarily swine) and *Brucella melitensis* (affecting primarily sheep and goats.) are of the major concern, which are all 'not host-specific' and may be transmit to other animal species and humans under appropriate conditions. [1], [2]

**Epidemiology**

Brucellosis occurs worldwide, except in countries where bovine brucellosis (B. abortus) has been eliminated (Australia, Canada, Cyprus, Denmark, Finland, the Netherlands, New Zealand, Norway, Sweden and the United Kingdom).The Mediterranean countries of Europe, northern and eastern Africa, Near East countries, India, Central Asia, Mexico and Central and South America are especially affected. It is usually either an occupational or a food-borne infection. Sporadic and epidemics both occur in humans; however the disease or infection is most often either unrecognized or, if diagnosed, not reported to the public authorities. [3]

**Modes of Transmission**

Transmission of infection to humans (with an incubation period of generally 1-2 months) occurs; through breaks in the skin, by direct contact with placental tissues or vaginal discharges from infected animals (lesser transmission degree of contact with blood or urine). Food-borne infection occurred via unpasteurized milk and other dairy products are rarely seen. Occupational airborne infection (laboratories and abattoirs) has also been reported. Also cases of venereal and congenital infection in humans are reported. [3]

## 8. METHOD

Bosphore® Brucella Detection Kit v1 is based on the Real-Time PCR method. Polymerase chain reaction is a technique that is used for amplification of a DNA region. The reaction occurs by the repeating cycles of heating and cooling. The main components of PCR are primers, dNTPs, Taq polymerase enzyme, buffer solution and template. As a brief explanation, primers are small synthetic DNA those anneal to the specific regions of the template in order to start the synthesis. dNTPs are the building blocks of the amplified products. Taq polymerase amplifies the DNA template. Buffer solution provides the pH adjustment required for the reaction and template, as referred, is the target region for synthesis.

In Real Time PCR technique, in contrast to conventional PCR, PCR product can be monitored during the reaction. Therefore Real-Time PCR obviates the need for further analysis methods like gel electrophoresis, whereby minimizing the risk of contamination. Dual labeled probes employed in the reaction in addition to the conventional PCR reagents, enable detection of the amplified target with increased sensitivity.

The assay utilizes the 5' exonuclease activity of Taq Polymerase to cleave a dual-labeled fluorescent probe during the extension phase of PCR. The probe is labeled at the 5' end with a fluorescent 'reporter' molecule, and at the 3'end with another fluorescent molecule that acts as a 'quencher' for the 'reporter'. When the two fluorophores are in close proximity, and the reporter is excited by light, no reporter fluorescence can be detected. During the elongation step of PCR, Taq Polymerase encounters and cleaves the probe bound to the template. As the reporter is freed from the suppressing effect of the quencher, fluorescence signal can be detected.

The fluorescence generated by the reporter increases as the PCR product is accumulated; the point at which the signal rises above background level and becomes distinguishable, is called the threshold cycle ($C_T$). There is a linear relationship between the log of the starting amount of a template and its threshold cycle, thus starting amount of unknown templates can be determined using standard curves constructed using $C_T$ values of the known starting amounts of target templates.

Bosphore Brucella Detection Kit v1 employs multiplex PCR, and an internal control is incorporated into the system in order to control the isolation procedure and to check for possible PCR inhibition. Brucella DNA and an internal control are co-amplified in a single reaction, using sequence-specific primers. The fluorescent signal generated by the Brucella amplification is detected by a probe labeled at the 3′ end with FAM, through the FAM channel. The fluorescent signal generated by the internal control amplification, is detected by a second probe (labeled at the 5′ end with a different reporter molecule, HEX) through the HEX channel.

## 9. PROCEDURE

### 9.1. DNA Isolation

We recommend that the Magnesia® 16 Nucleic Acid Extraction System / Magnesia® Bacterial DNA Extraction Kit/ Bosphore® Bacterial DNA Extraction Spin Kit (Anatolia Geneworks) isolation system is used with Bosphore® Brucella Detection Kit v1. The DNA isolation should be performed according to the manufacturers' instructions. The starting volume is 200 µl, the elution volume is 60 µl. Other high quality bacterial DNA extraction kits may also be used to isolate DNA from blood, serum and plasma samples. Please see Pathogen Section for health risks.

### 9.2. Kit Components

#### 9.2.1. PCR Mix

HotStarTaq DNA Polymerase: HotStarTaq DNA Polymerase is a modified form of a recombinant 94 kDa DNA polymerase, originally isolated from *Thermus aquaticus*, cloned into E.Coli. The enzyme is provided in an inactive form. It is activated by a 15-minute 95 ºC incubation step. This prevents the formation of misprimed products and primer-dimers during reaction setup and the first denaturation step, leading to high PCR specificity and accurate quantification. PCR Buffer: contains Tris-Cl, KCl, $(NH_4)_2SO_4$, 8 mM $MgCl_2$, pH 8.7 (20ºC ). dNTP Mix: Contains ultrapure quality dATP, dGTP, dCTP ve dTTP/dUTP.

#### 9.2.2. Detection Mix 1

Detection Mix 1 contains Brucella -specific forward and reverse primers and a dual-labeled probe.

#### 9.2.3. Detection Mix 2

Detection Mix 2 contains internal control-specific forward and reverse primers and a dual-labeled probe.

#### 9.2.4. Internal Control

An internal control is included in the kit to check PCR inhibition. The internal control is a synthetic DNA molecule. It is added into the PCR master mix to control the PCR inhibition exclusively. For this purpose, 0.1 µl of internal control should be added for each reaction into the master mix. Lack of internal control amplification in the FAM negative samples, may indicate a problem in isolation or PCR inhibition. In this case, isolation and PCR should be repeated. In samples that contain a high bacterial load, internal control can be suppressed and no increase of the signal is detected. Please use the table below for the interpretation of internal control data:

| Brucella (FAM) | Internal Control (HEX) | Interpretation |
|---|---|---|
| + | + | Sample positive |
| - | + | Sample negative |
| + | - | Sample positive |
| - | - | Repeat the test! |

### 9.2.5. Positive Control

The positive control contains Brucella DNA. It can be included in the PCR to test the efficiency of the PCR exclusively. The threshold cycle for the positive control is given in the acceptance criteria table (Section 10. Analysis). Threshold cycles higher than the acceptance criteria may indicate an efficiency loss in the reaction.

## 9.3. Preparing the PCR

Positive control should be added into the PCR reaction together with the samples and the negative control (PCR-grade water). Make sure that all the kit components are thawed before use. Refer to the table below for preparing the PCR. It is for only one reaction, multiply these values with the sample number to find the values required for the master mix. While preparing master mixes for more than 5 samples, an extra 10% should be added to the total sample number.

| | |
|---|---|
| **PCR Mix** | 12.5 µl |
| **Detection Mix 1** | 1.5 µl |
| **Detection Mix 2** | 0.25 µl |
| **Internal Control** | 0.1 µl |
| **$dH_2O$** | 0.65 µl |
| **Sample DNA** | |
| **Negative/Positive Control** | 10 µl |
| **Total Volume** | 25 µl |

Pipette 15 µl of the master mix into the PCR tubes or strips, and add 10 µl of DNA (sample/ positive or negative control). Close the tube cap. Make sure that the solution in each tube is at the bottom of the tube. Centrifuge if necessary.

## 9.4. Programming the Real-Time PCR Instrument

The thermal protocol for Bosphore® Brucella Detection Kit v1 is composed of an initial denaturation for activation the HotStarTaq DNA Polymerase, a two-step amplification cycle and a terminal hold. The real-time data is collected at the second step of the amplification cycle.

| | | | |
|---|---|---|---|
| Initial denaturation | 95°C | 14:30 min. | |
| Denaturation | 97°C | 00:30 min. | 50 cycles |
| Annealing and Synthesis (Data Collection) | 54°C | 01:30 min. | |
| Hold | 22°C | 05:00 | |

Before starting a Real-Time PCR reaction using the Bosphore® Kits, the following steps should be completed:

- Choose the filter pairs to be used (FAM and HEX),
- Identify unknown samples, positive and negative controls,
- Select the correct thermal protocol.
- Start the experiment.

# 10. ANALYSIS

By the end of the thermal protocol, the Real-Time PCR Instrument software automatically calculates the baseline cycles and the threshold.

Example of an amplification curve is given in Fig. 1.

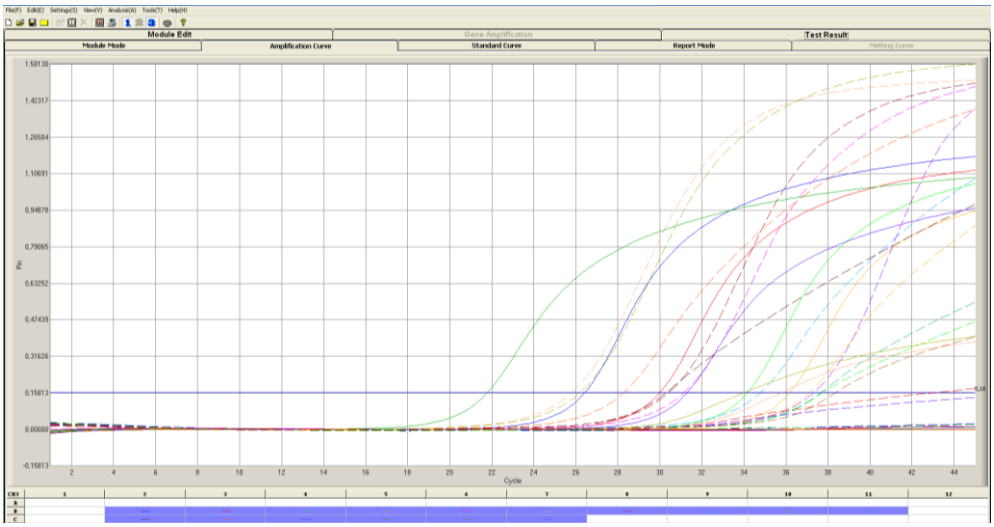

Fig. 1: Amplification Curve of a Bosphore® Brucella v1 test

Analysis of the results should be performed by trained personnel who have received the required training for analyzing Real-Time PCR data. We recommend that the test results must be evaluated by an expert clinician, taking the patient's clinical findings and the results of other tests into consideration.

All analysis is done automatically in routine use. However, when the trained personnel, who have received the required training from manufacturer, consider it as necessary, if the system allows pulling down the threshold as much as possible in order to detect slight amplifications, attention should be paid to keep the threshold line above the background.

The table below displays the acceptance criteria for Bosphore® Brucella v1.

| Component/Parameter | Cycle Threshold ($C_T$) |
|---|---|
| Positive Control | 31±4 |

Test results should not be reported unless the assay results meet the criteria stated above. Please contact the manufacturer if an impairment in the product's performance is observed (See the last page for contact information).

The samples that cross the threshold in FAM are displayed with a Ct, samples that do not cut the threshold are displayed as "No Ct". These samples are regarded as negative or having a bacterial load below the detection limit of the assay. For these undetectable samples, the HEX data of the internal control should also be checked to avoid false negative results.

The following table shows the possible results and their interpretation:

| | | |
|---|---|---|
| Signal detected in FAM filter pair | The sample contains Brucella DNA, the result is positive | No need to check the internal control since the sample is positive (high positive samples may suppress the signal from the internal control) |
| No signal in FAM, signal in HEX | The Brucella DNA in the sample is not detectable | Signal from HEX filter pair rules out the possibility of PCR inhibition |
| No signal in FAM and HEX | The diagnosis is inconclusive | No signal in HEX points out to PCR inhibition or to a problem in DNA isolation |

## 11. SPECIFICATIONS

### 11.1. Sensitivity

Analytical sensitivity may be expressed as the limit of detection: i.e. the smallest amount of the target marker that can be precisely detected. The detection limit of an individual analytical procedure is the lowest amount of nucleic acid in a sample which can be detected but not necessarily quantitated as an exact value. The analytical sensitivity or detection limit for NAT assays is expressed by the 95% positive cut-off value.

The analytical detection limit for Bosphore® Brucella v1 was found to be $7.5x10^2$ IU/ml (p=0.05). The sensitivity was determined using serial dilutions of Brucella DNA calibrated with the positive control. The dilutions were tested in different runs in replicates. The results were analyzed by probit method.

### 11.2. Cross-Reactivity

To eliminate potential cross-reactivity, both assay design evidence and experimental studies were employed. Primer and probe sequences were checked for possible homology to other known pathogen sequences by sequence comparison analysis using database alignment. Samples of M.tuberculosis, C.trachomatis and H. pylori with known high positivity were tested, and found negative.

### 11.3. Reproducibility

Reproducibility data (on $C_T$ value basis) were obtained by the analysis of positive control of the Bosphore® Brucella Detection Kit v1. Test was performed in at least 4 replicates by 3 different operators, on multiple days, using 3 different lots. The resulting data is given in Table 1:

Table 1: Reproducibility Data.

| Brucella ($10^4$ copies/ml) | Standard deviation | Variance | Coefficient of variation [%] |
|---|---|---|---|
| **Intra-assay Variability N=4** | 0.043 | 0,0018 | 0,13 |
| **Inter-lot Variability N=3** | 0,070 | 0,0052 | 0,21 |
| **Inter-operator Variability N=3** | 0,017 | 0.0002 | 0.05 |
| **Total Inter-assay Variability N=5** | 0.043 | 0,0018 | 0,13 |

## 12. REFERENCES


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## 13. SYMBOLS

 Use by

 Lot/Batch

 Catalog number

 Temperature limitation

 Caution, consult accompanying documents

 Manufacturer

 In Vitro Diagnostic Medical Device

## 14. CONTACT INFORMATION

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