HANDBOOK

Bio-T kit® *Besnoitia besnoiti*

Cat. N° BIOTK078 - 50 reactions

Detection of *Besnoitia besnoiti* (*B. besnoiti*)
by real-time PCR (qPCR)
with Endogenous internal positive control (IPC)

BOVINE

Sample types

- Whole blood (on EDTA)
- Skin biopsy
- Individual analysis

Recommended nucleic acids (NA) extractions

- Silica membrane columns extraction (ex: BioSellal – BioExtract® Column Cat. N° BEC050 or BEC250)
- Magnetic beads extraction (ex : BioSellal – BioExtract® SuperBall® Cat. N° BES384)

Veterinary use only

DOCUMENTS MANAGEMENT

The Bio-T kit® *Besnoitia besnoiti* has two technical handbooks:

- The extraction handbook shared between all the Bio-T kit® of the BLOOD line, displaying BioSellal's recommended extraction protocols for each type of sample.
- The Bio-T kit® *Besnoitia besnoiti* qPCR handbook, presenting the instruction information to perform the qPCR.

The last versions in use for each handbook are indicated on the certificate of analysis (CA) provided with the Bio-T kit® *Besnoitia besnoiti.*

Besides these two handbooks, a summary report of the validation file and a performances confirmation handbook are available on request, contact BioSellal (contact@biosellal.com).

MODIFICATIONS MANAGEMENT

BioSellal indicates modifications done to this document by highlighting them using the rules presented in the Table below:

MODIFICATIONS MANAGEMENT			
Type of modification Highlighting color	**Minor modifications**	**Type 1 Major modifications**	**Type 2 Major modifications**
Impact on revision / version	**Change of revision date** **No change of version**	**Change of revision date** **+ change of version**	**Change of revision date** **+ change of version**
Examples of modifications	Corrections: typographical, grammatical or turns of phrase	EPC reference modification	Modification of Master Mix composition
	Addition of new sample type for extraction	Exogenous IPC reference modification	Modification of validated extraction protocol
	Addition of information giving more details or alternative protocol		
	Addition/Suppression of optional information		

PRESENTATION

Recommendations for sampling, shipping and storage of samples

Real-time PCR is a powerful technique allowing the detection of few amounts of pathogen genome. Genome can be rapidly degraded depending on the pathogen nature (bacteria / parasites, enveloped viruses...), the genome nature (DNA / RNA) and the sample type (presence of DNase / RNase). Thus, BioSellal recommends the following instructions to guarantee an optimal diagnosis.

Sampling

To prevent cross-contamination between samples leading to false positive results, it is mandatory to use disposable materials for single use and to avoid direct contact between specimens.

Shipping

Except the whole blood samples, it is mandatory to ship immediately after sampling or by default to store it at ≤ -16°C. Shipment has to be done within 24h under cover of positive cold.

Storage after reception

Recommended storage of samples at 5°C ± 3 for a maximum of 7 days and ≤ -16°C beyond.

BLOOD Line

The BLOOD line gathers a set of kits dedicated to the detection on whole blood of a group of ruminant's pathogens called Haemoparasites: *Anaplasma phagocytophilum, Anaplasma marginale, Babesia-Theileria, Mycoplasma wenyonii, Anaplasma ovis, Mycoplasma ovis, Besnoitia besnoitii*. These pathogens are transmitted by insects or acarid and are responsible for marked hyperthermia syndromes that could be associated with anemia.
Another pathogen (OHV2 responsible for bovine malignant catarrhal fever) has been included in the BLOOD line since it may be part of the differential diagnosis of the non-specific febrile phase of Besnoitiosis. Indeed, this phase is characterized by marked hyperthermia, epiphora and serous nasal discharge discharge.

The BLOOD line gathers a set of kits sharing common extraction and qPCR protocols. It is compatible with BioSellal's other kits except with the ones belonging to the PIG and AVIAN lines.

In addition to the Bio-T kit® from the BLOOD line, BioSellal offers an ELISA kit for the detection of antibodies specific to *Besnoitia besnoiti* (BioLisa®kit *Besnoitia* AB). For more informations, contact BioSellal (contact@biosellal.com)

Description of the Bio-T kit® *Besnoitia besnoiti*

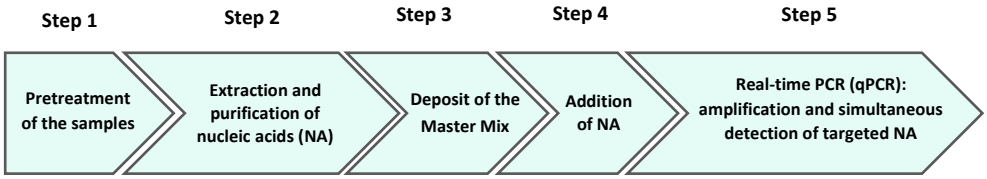
The **Bio-T kit® *Besnoitia besnoiti*** (Cat. N° BIOTK078) contains a ready to use **PCR Master Mix** allowing the detection **in the same reaction well of**:

- ***Besnoitia besnoiti* (*B. besnoiti*)** with a 6-FAM labelling
- **An Endogenous internal positive control IPC (gapdh)**, with a Cy5 labelling, to assess the presence of sufficient amount of host cells, sample integrity, nucleic acids extraction quality and absence of RT- PCR inhibitors.

This kit, based on qualitative dectection (detected or non detected) from whole blood (on EDTA) and skin biopsy samples (Individual analysis), was developed and validated according to the **French regulatory standard NF U47-600-2 edited by AFNOR**

Extraction protocols recommended by BioSellal are described in the extraction handbook of the BLOOD line.

Description of the whole process



BLOOD line Extraction handbook		qPCR handbook of the Bio-T kit® *Besnoitia besnoiti*		
Whole blood (on EDTA)* Skin biopsy*	BioExtract® Column BioExtract® SuperBall®	Ready-to-use Master Mix MMBesno-A	Samples NC/NCS Process positive control/MRI EPC (EPCBesno-A)	Dyes: FAM/Cy5 Passive reference: ROX Program: Classical program ± RT Standard or Fast ramping

* pretreatment mandatory

Kit contents and storage

Table 1. Description of the kit contents				
Description	**Reference**	**Volume/tube**	**Presentation**	**Storage**
Master Mix (MM) Ready to use	MMBesno-A	750 µl	**White** cap tube Bag A	≤-16°C Protected from light, « MIX » Area
External Positive Control (EPC) Positive PCR control of *B. besnoiti*	EPCBesno-A	110µl	Orange cap tube Bag B	≤-16°C « Addition of Nucleic acids » Area
Water RNase/DNase free	Aqua-A	1 ml	Blue cap tube Bag B	5°C ±3 or ≤-16°C « Addition of Nucleic acids » Area

Kit reagents are stable until the expiration date stated on the label, subject to compliance with good storage conditions.

List of consumables and reagents not included in kit

Table 2. Consumables and reagents not included in kit			
Consumables/ Reagents	**Description**	**Fournisseur**	**Cat. N°**
ATL Buffer	Lysis Buffer	BioSellal	ATL19076
BioExtract® Column	DNA/RNA column extraction kit (50)	BioSellal	BEC050
BioExtract® Column	DNA/RNA column extraction kit (250)	BioSellal	BEC250
BioExtract® SuperBall®	DNA/RNA Magnetic beads extraction kit (4 x 96)	BioSellal	BES384

For consumables related to the thermal cycler, refer to the user manual of the device.

List of reagents to confirm laboratory performances

To confirm performances of your thermal cycler(s), *B. besnoiti* DNA (quantified in GE copy number/qPCR) provided with the qPCR kit (**orange** cap tube) could be used

To confirm the performance of your complete method, an internal reference material (MRI) is also available. BioSellal provides this reagent under the following reference:

Table 3. Optional reagents*			
Reagent	**Description**	**Provider**	**Cat. N°**
MRI Whole blood (EDTA tube)	Whole blood (EDTA tube) *B. besnoiti*	BioSellal	MRI-Besno-001

* This reagent is available only on demand, please contact BioSellal (contact@biosellal.com).

Main critical points

- Wear appropriate personal protective equipment (lab coat, disposable gloves frequently changed).
- Work in dedicated and separate areas to avoid contamination: "Extraction" (unextracted samples storage, extraction equipment area), "Mix" (ready to use MM storage, qRT-PCR plates preparation), "Nucleic acids (NA) Addition" (Nucleic Acids storage and addition of extracted NA and controls in the qRT-PCR plate), "PCR" (final area containing the thermal cycler(s)).
- Use dedicated equipment for each working area (gloves, lab coat, pipettes, vortex, ...).
- Use filter tips.
- Before use, thaw all components at room temperature.
- Vortex and spin briefly (mini-centrifuge) all reagents before use.
- Avoid the repetition of freezing-thawing cycles for samples, lysates, extracted NA.
- **Working with RNA is more demanding than working with DNA** (RNA instability and omnipresence of the RNases). Genome(s) of pathogens of the BLOOD line are DNA but nucleic acids extract can also be used for RNA virus detection. For these reasons, special precautions must be taken:
    - Always wear gloves, change them frequently, especially after contact with skin or work surfaces.
    - Treat all surfaces and equipment with DNases inactivation agents (available commercially).
    - When wearing gloves and after material decontamination, minimize the contact with surfaces and equipment to avoid the reintroduction of RNases.
    - Use “RNase free” consumable.
    - It is recommended to store the RNA at ≤ 5 ±3°C during the manipulation and then freeze it as soon as possible, preferably at ≤ -65°C or by default at ≤ -16°C.
    - Open and close tubes one by one to limit the opening times and avoid any contact with RNases present in the environment (skin, dust, working surfaces…).

DETECTION OF *BESNOITIA BESNOITI* BY qPCR WITH BIOTK078

Global Procedure

1) Establish qPCR plate setup defining each sample position and **including the following controls**:

- **Negative Control Sample (NCS)**: water (or PBS) replaces the sample from the first step of sample preparation.
  This control is mandatory for each extraction series.

- **Negative Amplification Control (NC)**: 5 µl of water RNase/DNase free (Aqua-A, **blue** cap tube) replaces sample Nucleic Acids extract on qPCR plate.
  This control is recommended when using the kit for the first time or to verify the absence of Master Mix contamination.

- **External Positive Control of and *B. besnoiti* (EPC)** : Synthetic DNA provided (**EPCBesno-A**, **orange** cap tube), containing specific target of *B. besnoiti*)
  This control is mandatory.

⚠ CAUTION: *EPC tube handling represents nucleic acids contamination hazard, it is thus recommended to open and handle it in a restricted area, away from other PCR components and to take precautions to avoid cross-contamination with nucleic acids extracts during deposit on the qPCR plate.*

- If available, **a Process Positive Control (MRI),** a weak positive sample of whole blood (on EDTA) or Skin biopsy is extracted in parallel with tested samples. After qPCR, MRI Ct values will be monitored on a Shewhart control card. Obtaining conform Ct values validates the whole process. In this case, the use of the EPC, provided with the kit, is not mandatory.

2) qPCR plate preparation

In the "MIX" dedicated area

1. After thawing, vortex and rapid centrifugation, **transfer 15 µl Master Mix MMBesno-A (White** cap) in each well of interest (samples and controls).

In the "Nucleic Acids addition" dedicated area

2. **Add 5 µl of extracted nucleic acids (or NCS, water, MRI or EPC**: **EPCBesno-A orange** cap tube) in each well of interest. Make sure to pipet out in the bottom of the well, in the Master Mix, and to avoid the formation of bubbles.

3. **Seal the plate with an optically clear sealer** or **close the strip caps**.

In the "PCR" amplification dedicated area

4. **Define the thermal cycler parameters** (see Table 4, Table 5, Table 6, Table 7)

5. It is recommended to **spin the plate down prior to place it in the thermal cycler**, to prevent drops in the well pit walls.

6. Start the qPCR program. Approximate run time: 60 min.

3) Thermal cycler settings

This kit was developed and validated on AriaMx™ (Agilent Technologies, Fast ramping by default) and confirmed on ABI PRISM® 7500 Fast (Applied Biosystems) in standard ramping and fast ramping, but it is compatible with all thermal cyclers able to read 6-FAM and Cy5 channels in the same PCR well. For other thermal cyclers, contact our technical support.

Table 4. Thermal cycler configuration

	ABI PRISM® 7500 Fast	AriaMx™
Mode	Quantitation – Standard curve	Quantitative PCR, Fluorescence Probe
Ramping	Standard Ramping or Fast Ramping	Fast Ramping by default
Passive Reference	**ROX**	**ROX**

Table 5. Thermal cycler Settings			
Target	**Detectors**		**Final Volume / well**
	Reporter	**Quencher**	
B. besnoiti	FAM	NFQ-MGB ou None*	**20 µl**
Endogenous IPC	Cy5	NFQ-MGB ou None*	= 15 µl Master Mix + 5 µl extracted nucleic acids or controls†
To assign to samples and controls†			

* Depends on the thermal cycler model. Do not hesitate to contact the BioSellal Technical Support (tech@biosellal.com)
† Controls are NC (water), NCS (extracted water), EPC and/or extracted MRI.

Table 6. CLASSICAL Amplification program settings without RT†		
Standard or Fast Ramping		
Cycles	Time	Temperature
1 cycle	5 min	95°C
40 cycles	15 sec	95°C
	30 sec* + data acquisition	60°C

* Set 31s for some thermal cyclers such as ABI PRISM® 7500.
† optional step, in case of simultaneous detection of RNA genomes. Achieving a reverse-transcription (RT) step prior to PCR for the amplification of RNA genomes has no impact on the effectiveness of the Bio-T kit® *Besnoitia besnoiti* (see the summary of the validation file).
NB: This amplification program is compatible with all Bio-T kits® except for ones belonging to the PIG and AVIAN LINES.

For thermal cycler such as LightCycler®480 and LightCycler®96 (Roche Life Science), it is recommended to use the following program:

Table 7. PIG/AVIAN Amplification program settings without RT†		
Standard Ramping		
Cycles	**Time**	**Temperature**
1 cycle	5 min	95°C
40 cycles	10 sec	95°C
	45 sec + data acquisition	60°C

† optional step, in case of simultaneous detection of RNA genomes. Achieving a reverse-transcription (RT) step prior to PCR for the amplification of RNA genomes has no impact on the performances of the Bio-T kit®.
NB: This amplification program is compatible with all Bio-T kits® of the PIG and AVIAN LINES

RESULTS INTERPRETATION

To analyze and interpret the signals obtained by qPCR, the Threshold must be set up.

The threshold must be assigned carefully to obtain the most reproducible result between different manipulations according to the requirements defined in Annex C of the French Standard **NF U47-600 (part 1)**. A consistent set of positives controls, usually an In-house Reference Material (MRI) or the EPC, is used to set the threshold value above the baseline and in the exponential amplification phase of the plot.

The Threshold Cycle, named « Ct » or « Cq » (depending on thermal cyclers), corresponds to the intersection between the amplification curves and the threshold line. It allows the relative measurement of the concentration of the target in the PCR reaction when a calibrated extract is analyzed in the same series.

The qPCR series is validated if the controls (EPC, MRI, NCS and NC) present valid results, then the result of each sample can be interpreted.

Main Scenarios

Controls Reading

Table 8. PCR Controls results interpretation

	Targets		Interpretation
	B. besnoiti **(FAM)**	**Endogenous IPC (Cy5)**	
NCS Negative Control Sample	Neg	Neg	Valid
MANDATORY	At least one of the two targets Pos		Contamination with a positive/negative sample during extraction step or during qPCR plate preparation.
NC Negative PCR Control	Neg	Neg	Valid
OPTIONAL	At least one of the two targets Pos		Contamination with a positive/negative sample during extraction step or during qPCR plate preparation or Master Mix/water contamination
EPC ***B. besnoiti*** PCR external positive control	Pos*	Neg	Valid
	Neg	Neg	Problem during qPCR plate preparation: Master Mix error? EPC omission?
MANDATORY *IN ABSENCE OF MRSI*	Pos*	Pos	Contamination with a sample during qPCR plate preparation?
Sample process positive Control MRI	Pos†	Pos¥	Valid
RECOMMENDED *IF AVAILABLE*	Neg	Neg	Problem during qPCR plate preparation: Master Mix error? Nucleic acids extract omission or extract not in contact with Master Mix? Process drift: extraction and/or qPCR ? Degradation of the sample process positive control?

* The Ct value obtained must be conform with the value indicated on the Certificate of Analysis (CA).
† The Ct value must be included within control card limits.
¥ The obtained Ct value depends on the thermal cycler, the sample type and the used extraction protocol. Ct values for IPC using the recommended extraction protocols are available upon request. BioSellal recommends you determine your own maximal IPC Ct value depending on your own extraction method and thermal cycler.

Note:

Endogenous IPC targets a gene expressed by bovine cells, thus it cannot be detected in NCS, NC and EPC. Due to cross-reaction between ruminant GAPDH and human GAPDH, a slight signal can be observed for IPC in the controls, the Ct value of this signal must be over than 35.

Samples Reading

Table 9. Different types of results obtained for the samples		
Targets *B. besnoiti* (FAM)	Endogenous IPC (Cy5)	Interpretation
Neg	**Pos***	**Negative ou Undetected**
Pos		**Positive ou Detected**
Pos	**Neg or Ct>35**	**Positive ou Detected** Lack of host cells? Presence of inhibitors ǂ? Competition with the main target?
Neg	**Neg or Ct>35**	**Uninterpretable** = Repeat the analyse Problem during qPCR plate preparation: Master Mix error? Nucleic acids extract omission or extract not in contact with Master Mix? Presence of inhibitors ǂ? Nucleic acids degradation in the sample? Sampling problem: lack of cells? Extraction problem?

*The obtained Ct value depends on the thermal cycler, the sample type and the used extraction protocol. This value must be, at least, included within the specified range in the certificate of analysis (CA). Ct values for IPC using the recommended extraction protocols are available upon request. BioSellal recommends you determine your own maximal IPC Ct value depending on your own extraction method and thermal cycler.

ǂ In case of inhibition suspicion, 1) Repeat the qPCR with the dilution of extracted nucleic acids at 1/10 or 1/100 in the DNase/RNase free water. 2)Restart the analysis from the extraction step.

Notes:

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