ADIAVET™ BESNOITIA REAL TIME

TEST FOR THE DETECTION OF *BESNOITIA BESNOITI* BY REAL-TIME ENZYMATIC GENE AMPLIFICATION (PCR TEST)

Reference:
ADI451-100 (100 reactions)

VET

NOTE
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English version
NE451-05
2016/07

ADIAVET™ BESNOITIA REAL TIME

I. Revision history

N/A	Not Applicable (first publication)
Correction	Correction of document anomalies
Technical change	Addition, revision and/or removal of information related to the product
Administrative	Implementation of non-technical changes noticeable to the user

Note: minor typographical, grammar and formatting changes are not included in the revision history.

Release Date	Part Number	Change type	Change summary
2012/03	NE451-03	Technical change	Addition of "Recommandation before the analysis of samples" section, in page 6, § IV.
2012/03	NE451-03	Technical change	Addition of "Extraction and purification" section, in page 7, § V.
2014/12	NE451-04	Technical change	Addition of "Index of symbols" section, in page 12.
2014/12	NE451-04	Technical change	Removal of reference ADI451-50 (50 reactions)
2016/07	NE451-05	Administrative	Changing logos
2016/07	NE451-05	Administrative	Biosearch legal mention
2016/07	NE451-05	Administrative	Addition of table "Analysis options according to the specimen"
2016/07	NE451-05	Technical change	Modification of centrifugation temperature §V

II. General information

1. Purpose of the test

ADIAVET™ BESNOITIA REAL TIME kit is intended to detect *Besnoitia besnoiti* using real-time Polymerase Chain Reaction (PCR) technology from skin and whole blood specimens of bovine.

2. Pathogen

Besnoitia besnoiti, the causative agent of bovine besnoitiosis, is an obligate intracellular protozoan. The disease affects mainly young cattle. Besnoitiosis is epizootic in the south of France, but is now widely distributed in Africa, Asia and in Southwestern Europe. The most likely pathway of transmission would be transcutaneous, by stinging insects (tabanids, stomox).

During infection, an incubation stage of 3 to 6 days is followed by 3 successive clinical stages:

- A febrile stage of 3 to 7 days; the tachyzoites multiplication in endothelial cells of blood vessels increases the animal temprerature
- A second stage of 1 to 2 weeks; the bradyzoites cysts generate subcutaneous oedema
- A chronic stage of several months, characterised by alopecia and sclerodermia. The skin becomes then markedly thickened and wrinkled, and parasitic cysts are observed on conjunctiva and sclera. This ultimate phase leads generally to the death of the animal or to its euthanasia.

Serologic tests are available for the detection of the specific antibodies of *Besnoitia besnoiti* present in the chronic stage. In order to avoid the transfers contaminated animals and to control the spread of the bovine besnoitiosis, it is essential to use diagnostic tools that detect the pathogens at the early stages of the disease.
PCR tests are able to detect the parasites by the detection of tachyzoites of *Besnoitia besnoiti* during febrile stage in the monocytes of blood and in the skin during oedema and chronic stages.

3. Description and purpose of the test

This test is based on enzymatic gene amplification or PCR technology.
Amplified products are detected in real-time thanks to a specific labelled hydrolysis probe (5'-exonulease technology).

The ADIAVET™ BESNOITIA REAL TIME kit enables the simultaneous detection of:

- *Besnoitia besnoiti* (probe labelled in FAM),
- GAPDH, an internal control of extraction and amplification steps specific from an endogenous DNA (probe labelled with a fluorochrome with the same spectra as VIC or HEX).

ADIAGENE validated the test using DNA purification kits (Qiagen, Macherey-Nagel). Other purification kits can be used if they have been validated by the user.

Analysis options according to the specimen:

Specimen	Individual analysis
Skin	☑
Whole blood	☑

III. Material and reagents

1. Reagents provided with the kit

Designation	Reagent	ADI451-100
A5	Amplification solution	2 x 1000 µl green tubes
Bb CTL+	Positive control *Besnoitia besnoiti*	1 purple tube

2. Validity and storage

On receipt, the kit should be stored at **<-15°C**.
It is recommended to make fractions of A5 solution if it should be defrosted more than 3 times.
Do not defrost reagents more than 3 times.
Realtime reagents are susceptible to light: store them in the darkness.
The A5 reagent is ready to use for PCR reaction.
Do not mix reagents of two different batches.

3. Use of Bb CTL+

Add **200 µl** of Nuclease-free water to the Bb CTL+ tube included in the kit. Homogenize tube contents using a mixer such as vortex, at least 20 seconds. Aliquot this solution by 6 or 12 µl and store them to <-15°C.
For each analysis, we recommend to use 5 µl of Bb CTL+ in a well.

4. Equipment required but not supplied

Material should be Nuclease-free (e.g. autoclaved 25 minutes twice at +120°C or once 60 minutes at +121°C)

- Thermal cycler with consumables for real-time PCR: 0.2 ml PCR tubes or closed 96-wells PCR plates with optical quality.
- Centrifuge for microtubes, tubes of 10 or 15 ml, 96-wells plate
- Etuve, heating baths or block heaters
- Instrument for homogenous mixing of tubes
- 1 - 10 µl pipette, 20 - 200 µl pipette and 200 - 1000 µl pipette
- Nuclease-free filter tips
- Nuclease-free microtubes: 1.5 ml and 2 ml
- Powder-free Latex gloves
- Scalpel blades
- 96-100% ethanol solution
- Sterile distilled water

- DNA extraction kit (individual columns)

- QIAamp® DNA Mini Kit (Qiagen, 50 tests: ref. 51304 or 250 tests: ref. 51306)
- NucleoSpin® Tissue (Macherey-Nagel, 50 tests: ref.740952.50 or 250 tests: ref. 740952.250)

IV. Recommendation before the analysis of samples

Before starting the test, read the entire protocol and scrupulously respect it.

1. Precautions

Adiagène has elaborated this PCR test with the use of Qiagen and Macherey-Nagel extraction kits. Other extraction kits can be used with a previous validation.

Follow the supplier's instructions for the storage, the preparation and the use of the extraction reagents.

Some kits include and/or need the use of toxic reagents. These reagents should be use with gloves and into chemical cabinet.

We strongly recommend that only appropriately trained personnel perform this test. Ensure the accuracy and precision of the micropipettes used. The quality of the obtained results depends upon rigorous respect of good laboratory practices.

The PCR generates large amount of amplified DNA. A few molecules of amplified products are sufficient to generate a positive result. It is important to reserve 2 rooms, one for manipulation of samples to be tested, and another one for amplified products analysis. Do not open the PCR tubes after amplification.
Samples for analysis should be handled and disposed of as biological waste. **Take all measures of security and confinement required for the manipulation of the concerned biological agents.**

We recommend using fractions of demineralised and saline water and to autoclave them 25 minutes at +120°C. Take a new fraction for each new manipulation to avoid contamination.

2. Storage of samples and DNA extracts

Samples can be stored a couple of days at +2/8°C. After 2 days, we recommend to store them at -20°C +/-5°C or -70°C +/-10°C. Samples of blood with anticoagulant reagent must not be freezed.
Extracted DNAs are quite sensitive molecules. Crude extracts should be stored at the end of extraction on melting ice or at +2/8°C for 24 hours, then at -20°C +/-5°C.

3. Controls to include

The use of control allows verifying the reliability of the results.
The controls are included per trial of analysis. A trial is defined as all the samples treated in the same conditions.
The use of the control follows the recommendation of the the normative requirements and recommendations for the development and the validation of veterinary PCR (NF U47-600).

All the steps of the analysis procedure (extraction+amplification), for the the type of sample, are validated with the association of the controls included in the kit.

- The internal endogenous control (GAPDH) naturally found in the samples allows verifying the extraction and amplification steps of each sample.
- The Bb CTL+ allows validating the amplification of the target.

Other controls must or could be added.

- **Negative control of extraction (obligatory)**

To verify the absence of cross-contamination, at least one negative control must be included per trial. The control is a negative sample, for example a buffer used for dilution.

- **Positive control of extraction (recommended)**

A positif control could be added in each trial. The control is a sample incuding *Besnoitia besnoiti*. It could come from a positive sample available in the laboratory or from a negative sample spiked with a solution of *Besnoitia besnoiti*. This positive control will be closed to the limit of detection of the method. It will inform about the fidelity of the obtained results between different trials.

V. Extraction and purification

1. Using QIAamp® DNA Mini kit

All the centrifugations are performed at room temperature.
Before the beginning of extraction, turn on one or two heating systems at +56°C or +70°C.

	Blood	**Skin**
Preparation of the sample	Place **1 ml** of **blood collected with EDTA** in a 2 ml-microtube. Add **1 ml of sterile distilled water**. Homogenize. Incubate **10 minutes on ice** mixing now and then. Centrifuge 5 minutes at 6 000 g. Discard the supernatant. Add **1 ml of sterile distilled water**. Homogenize. Centrifuge 5 minutes at 6 000 g. Discard the supernatant.	Place **20 mg** of **skin** in a microtube.
Lysis	Add **180 µl** of **ATL buffer**, **20 µl** of **proteinase K**. Homogenize. Incubate **30 minutes** at **+70°C** OR a **night** at **+56°C**.	
	Add **200 µl** of **AL buffer**. Homogenize. Incubate **10 minutes** at **+70°C**.	
Binding preparation	Add **200 µl** of **ethanol 100%**. Homogenize the mixture by pipeting (~10 times) or by using a mixer such as vortex (~15 secondes).	
Transfer to columns and binding to the membrane	Identify columns, apply the the **whole** obtained solution to the corresponding column. Centrifuge at 10 000 g/1 minute. *If all the sample has not been loaded once, apply the residual volume onto the column and centrifuge 1 minute at 10 000 g.*	
1st wash	Change the collection tube and add **500 µl** of **AW1 buffer** to the column. Centrifuge 1 minute at 10 000 g.	
2nd wash	Change the collection tube and add **500 µl** of **AW2 buffer** to the column. Centrifuge 1 minute at 10 000 g.	
Column dry step	Change the collection tube. Centrifuge 3 minutes at 10 000 g.	
Elution	Transfer the column to a microtube. Add **200 µl** of **AE buffer**. Incubate ~1 minute at room temperature and centrifuge 1 minute at 10 000 g.	
Storage	Close the tubes, identify and store at +2/8°C for 24 hours, then at <-15°C.	

2. Using NucleoSpin® Tissue kit

All the centrifugations are performed at room temperature.
Before the beginning of extraction, turn on one or two heating systems at +56°C or +70°C.

	Blood	Skin
Preparation of the sample	Place **1 ml** of **blood collected with EDTA** in a 2 ml microtube. Add **1 ml of sterile distilled water**. Homogenize. Incubate **10 minutes on ice** mixing now and then. Centrifuge 5 minutes at 6 000 g. Discard the supernatant. Add **1 ml of sterile distilled water**. Homogenize. Centrifuge 5 minutes at 6 000 g. Discard the supernatant.	Place **20 mg** of **skin** in a microtube.
Lysis	Add **180 µl** of **T1 buffer**, **25 µl** of **proteinase K**. Homogenize. Incubate **30 minutes** at **+70°C** OR a **night** at **+56°C**.	
	Add **200 µl** of **B3 buffer**. Homogenize. Incubate **10 minutes** at **+70°C**.	
Binding preparation	Add **200 µl** of **ethanol 100%**. Homogenize the mixture by pipeting (~10 times) or by using a mixer such as vortex (~15 secondes).	
Transfer to columns and binding to the membrane	Identify columns, apply the the **whole** obtained solution to the corresponding column. Centrifuge at 10 000 g/1 minute. *If all the sample has not been loaded once, apply the residual volume onto the column and centrifuge 1 minute at 10 000 g.*	
1st wash	Change the collection tube and add **500 µl** of **BW buffer** to the column. Centrifuge 1 minute at 10 000 g.	
2nd wash	Change the collection tube and add **600 µl** of **B5 buffer** to the column. Centrifuge 1 minute at 10 000 g.	
Column dry step	Change the collection tube. Centrifuge 3 minutes at 10 000 g.	
Elution	Transfer the column to a microtube. Add **200 µl** of **BE buffer**. Incubate ~1 minute at room temperature and centrifuge 1 minute at 10 000 g.	
Storage	Close the tubes, identify and store at +2/8°C for 24 hours, then at <-15°C.	

VI. Amplification

a- Determine the number samples analysed including the control (e.g. positive and negative extraction control, positive control of amplification (CTL+) and reagent PCR control (NTC)).

b- Defrost A5 solution at room temperature. Homogenize. Dispense **20 µl** of A5 solution in each PCR tubes or PCR plate wells with a micropipette with a Nuclease-free tip.

c- **Immediately replace A5 solution tube at <-15°C and in darkness.**

d- For each sample, the negative control (obligatory) and the CTL+ (recommended) add **5 µl** of purified extract to the 20 µl of A5 solution.
For the CTL+, add **5 µl** of the solution obtained in § II-3 to the 20 µl of A5 solution.
For the PCR reagent control (NTC), nothing is added to the A5 solution.
Immediately replace purified DNA extracts at +2/8°C or at <-15°C. Take care to have no bubbles in the bottom of the wells.

e- Once all the tubes have been prepared, run real-time PCR amplification.

The *Besnoitia besnoiti* target is read in FAM. The Internal Control is read in VIC or HEX. The Quencher is non fluorescent. The solution contains a passive reference ROX for the ABI machines. Fluorescence is read during the elongation step (1 minute at 60°C).

The following program is defined for **ABI Prism** thermalcyclers (like 7500, StepOne...) from **Applied Biosystems** (check the "emulation 9600" option if available), for the **Rotorgene** from **Qiagen** and for the **Chromo 4** from **Biorad**:
2 minutes 50°C
10 minutes 95°C
15 seconds at 95°C and 1 minute at 60°C during 45 cycles

This program is concerning the **MX3000P** and **MX3005P** of **Stratagene**:
2 minutes 50°C
10 minutes 95°C
30 seconds at 95°C and 1 minute at 60°C during 45 cycles

Roche diagnostic: LightCycler 2*, LightCycler 480*
**** NOTE:*** *The use of LightCycler thermalcyclers requires a calibration manipulation. Adiagene will furnish process chart and reagents required for this calibration.*

Contact us if you wish to use other thermalcyclers.

VII. Interpretation of results

1. Definitions

The **« base line »** corresponds to the background of fluorescence and qualifies the non-characteristic part of the curve observed during the first cycles.

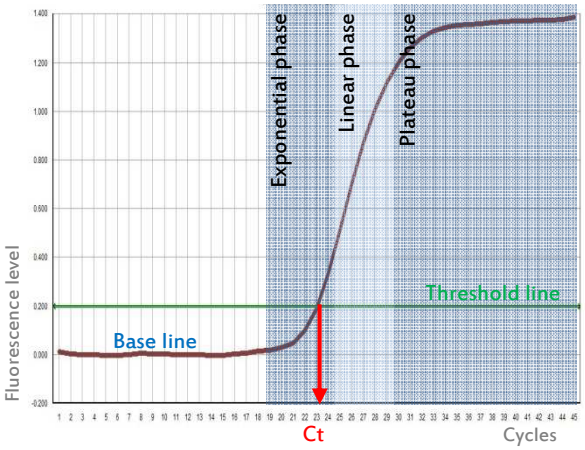
The **« Characteristic amplification curve »** qualifies a fluorescence curve with an exponential phase, a linear phase and a plateau phase.

The **« threshold line »** has to be placed over the background in the exponential phase of a characteristic amplification curve or a group of characteristic amplification curves.

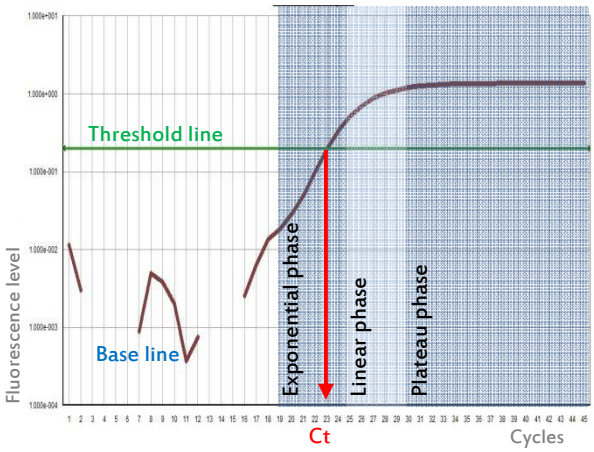
The **« threshold cycle » (Ct)** of a well corresponds, for each detected fluorophore, at the crossing point of the threshold line with the fluorescent curve. The Ct value expressed by the machine for each well depends on the threshold position and on the quantity of target sequences initially present in the PCR well.

Example of characteristic amplification curve

Arithmetic scale of Y axis



Logarithmic scale of Y axis



2. Validation and interpretation of results

Display the FAM curves from the plate and set the threshold value as indicated above.
Proceed in the same mean for the VIC or HEX curves.

A. Validation of the run

Amplification is considered to be **valid** if the following results are obtained for the controls:

Controls	Reagent control (NTC)	Bb CTL+	Extraction negative control	Extraction positive control *
FAM amplification	no	yes	no	yes
VIC/HEX amplification	no	no/yes	no	no/yes
Validation of	Absence of contamination for amplification	Amplification of the target	Absence of contamination for extraction	Extraction and amplification steps

* Optional

The indicative Ct values (FAM and VIC/HEX dyes) of the Bb CTL+ were indicated in the certificate of analysis of the kit.

B. Result interpretation

DNA extraction and amplification for each sample are considered to be **valid** if at least a characteristic amplification curve is observed for *Besnoitia besnoiti* (FAM) or for the internal control (VIC or HEX).

Example	A	B	C	D
FAM amplification	no	yes	yes	no
VIC/HEX amplification	yes	no	yes	no
Result	negative	positive	positive	undetermined

The sample is considered as **negative** if a characteristic amplification curve is observed in VIC or HEX without any amplification in FAM (A example).

The sample is considered as **positive** if a characteristic amplification curve is observed in FAM (B example). Internal control can be co-amplified (C example).

A total absence of characteristic amplification curve for a sample (example D) shows a defective DNA extraction (lost or destruction of DNA) or a deficient real-time PCR (inhibitors in the sample, program error or no template added). In this case, we recommend first to repeat the test with pure and tenfold diluted DNA in Nuclease-free water. Then, if the test is still not valid, a new extraction is recommended.

VIII. Index of symbols

Symbol	Meaning
	Catalogue number
	Manufacturer
	Upper temperature limit
	Use by date
	Batch code
	Consult Instructions for Use
	Contains sufficient for <n> tests
	Keep away from sunlight
	For veterinary in vitro use only – For animal use only

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