ADIAVET™ PARATB REAL TIME

TEST FOR THE DETECTION OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* BY REAL-TIME ENZYMATIC GENE AMPLIFICATION (PCR TEST)

Reference:
ADI045-100 (100 reactions)

VET

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English version
NE045-09
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ADIAVET™ PARATB 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
2013/07	NE045-06	Technical change	Addition of technical details related to the used and unused ADIAFILTER protocol, in page 10, § V-1.
2014/12	NE045-07	Technical change	Addition of "Index of symbols" section, in page 20.
2014/12	NE045-07	Technical change	Removal of reference ADI045-50 (50 reactions)
2016/07	NF045-08	Administrative	Changing logos
2016/07	NF045-08	Administrative	Biosearch legal mention
2016/07	NF045-08	Administrative	Addition of table "Analysis options according to the specimen"
2016/07	NE045-08	Technical change	Remove of QIAextractor protocol
2018/09	NE045-09	Technical change	Remove of ADIAPURE GLASS BEADS references (Bio-X Diagnostics, ref ADIADPBI1-192 and ADIADPBI1-480) Remove of ADIAPURE™ PURIFICATION reference (BioX Diagnostics 192 tests: ref. ADIADP001-192)

II. General information

1. Purpose of the test

ADIAVET™ PARATB REAL TIME kit is intended to detect *Mycobacterium avium* subsp *paratuberculosis* (PARATB) using real-time Polymerase Chain Reaction (PCR) technology from faeces, tissue and milk specimens of bovine, ovine and caprine, as well as from environmental specimen and bacterial culture.

2. Pathogen

Mycobacterium avium subsp. *paratuberculosis* is the etiological agent of bovine paratuberculosis. The disease is characterized by diarrhoeas, a decrease of the production level (milk, reproduction) and a loss of weight leading to death.
In 1898, Johne and Frothingham detect acid-fast bacillus in intestinal mucosa of affected animals. These bacteria, similar to tuberculosis bacillus, are responsible for a thickening of intestinal mucosa corresponding to an enteropathy called paratuberculosis or Johne's disease (Thorel and *al.*, 1990). The incubation of illness is slow (between 2 and 5 years) so the majority of affected animals present clinical symptoms between 2 and 7 years. The most important mode of transmission of paratuberculosis is the faecal-oral route, although transmission can occur in utero, via infected semen, colustrum and milk. In the infected animal organisms, the bacteria can then spread through the macrophages.
Affected animals can shed varying numbers of *M. paratuberculosis* organisms in their faeces (from some bacteria / g of faecal material to 10^4- 10^{10}germs / g at the clinical stage).
Feacal culture for the causative organisms is the definitive method of diagnosis but it is very slow, requiring 6-8 weeks. Immunologically-based tests for Johne's disease are rapid but lack of specificity and sensitivity.
A shift of biologists of St George's Hospital of London conducted by the Dr J. Hermon-Taylor identified in 1985 a repetitive genomic fragment called IS900, specific of *M. paratuberculosis* strains (Green *and al.* 1990). Since this sequence has been used as probe in molecular diagnostic test in particular for PCR test (Guillou *and al.*, 1993).

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™ PARATB REAL TIME kit enables the simultaneous detection of:

- *Mycobacterium avium* subsp *paratuberculosis* (probe labelled in FAM),
- External Control (probe labelled with a fluorochrome with the same spectra as VIC and HEX).

According to the extraction protocol retained, two external controls are available:

- An External Positive Control of extraction named **"EPC–Ext"**, it will be added during the extraction step, will follow all the step of extraction and will check the whole extraction process and the absence of inhibitors.

or

- An External Positive Control of amplification named **"EPC–Amp"**; it will be added in the "A5 solution" before the amplification step and will only control the absence of amplification inhibition.

ADIAGENE recommends DNA purification kits coming from ADIAGENE, Qiagen, Macherey-Nagel suppliers. Other purification kits can be used if they have been validated by the user.

Analysis options according to the specimen:

Specimen	Individual analysis	Pool of sample is possible*, up to
Faeces**	☑	10
Tissue (ganglion, mucosa, ileocaecal valve)	☑	☒
Milk	☑	☒
Environmental specimen (cow dung scraping, waiting areas…)**	☑	10
Bacterial culture	☑	☒

***** It depends on the epidemiological case and on the quality of the specimen.
****** Concentration of the sample using the ADIAFILTER (Bio-X Diagnostics, ref. ADIFIL100) is proposed to increase the level of sensitivity and reproducibility of the global detection method (especially for weak positive samples).

III. Material and reagents

1. Reagents provided with the kit

Designation	Reagent	ADI045-100
A5	Amplification solution	2 x 1000 µl green tubes
PARATB CTL+	Positive control *Mycobacterium avium* subsp. *paratuberculosis*	1 purple tube
EPC-Ext	external control of extraction	2 x 300 µl yellow tubes
EPC-Amp	external control of amplification	1 x 100 µl white 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 controls

According to the extraction solution chosen, use **EPC-Ext or EPC-Amp**

A. Used of EPC-Ext

Aliquot this solution and store it to <-15°C.
For each extraction, use **5 µl of EPC-Ext per samples**.
Do not defreeze each aliquot more than 3 times

B. Used of EPC-Amp (only if EPC-Ext is not used):

Aliquot this solution and store it to <-15°C.
For each PCR reaction, mix **1 µl of EPC-Amp** and 20 µl of « A5 solution ».
Do not defreeze each aliquot more than 3 times.

C. Used of PARATB CTL+

Add **200 µl** of Nuclease-free water to PARATB 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 PCR analysis, we recommend to use **5 µl of PARATB 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 +120°C)

- Thermal cycler with consumables for real-time PCR: PCR tubes or closed 96-wells PCR plates with optical quality
- A centrifuge for microtubes
- Universal laboratory mixer mill
- Instrument for homogenous mixing of tubes
- Etuve, heating baths or block heaters
- Blender with paddles and blender bags for analysis from tissue
- Sterile microtubes: 1.5 ml and 2 ml
- Powder-free latex gloves
- 1 - 10 µl, 20 - 200 µl and 200 - 1000 µl pipettes
- Filter tips
- 100% Ethanol solution
- Sterile demineralised water
- Grinding beads :
  - ADIAPURE™ ALIQUOTED GLASS BEADS (BioX Diagnostics, ref. ADIADPBIA-192 (192 tests), ref. ADIADPBIA-480 (480 tests)), only for disruption equipment such as Mixer Mill
  - Lysing Matrix B (MP biomedicals, 100 tubes, ref. 116911100), only for disruption equipment such as Fast Prep or Ribolyser
- ADIAFILTER : PACK ADIAFILTER (Bio-X Diagnotics, ref. ADIFIL100)

- DNA extraction kit (individual silica 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)

- DNA extraction kit (96-plate columns)

- NucleoSpin® 96 Tissue (Macherey-Nagel, 2x96 tests: ref. 740741.2 or 4x96 tests : ref. 740741.4)
- ADIAPURE™ PURIFICATION (Bio-X Diagnostics, 480 tests: ref. ADIADP001-480)
- ADIAPURE™ PARATB MILK (Bio-X Diagnostics, 100 tests: ref. ADIADP04M1-100)

-Automated DNA/RNA extraction kit using magnetic beads

See the NEKF user manual available on the web site mentioned on the certificate of analysis included in the used ADIAVET™ kit.

IV. Recommendation before the analysis of samples

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

1. Precautions

ADIAGENE has elaborated this PCR test with the use of ADIAGENE, 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 used with gloves and into a 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 <-15°C. 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 <-15°C.

3. Extraction and purification kits recommended

Samples	**Faecal**					**Tissue**	**Milk**	**Culture**
	1 to 10 gram				**1 gram**			
Name of the Kit	QIAamp® DNA mini Kit	NucleoSpin® Tissue	NucleoSpin® 96 Tissue	NucleoMag® 96 Tissue	ADIAPURE™ PURIFICATION	QIAamp® DNA mini Kit	ADIAPURE™ PARATB MILK	/
Nucleic Acid capture technology	Silica columns	Silica columns	Silica columns	Magnetic beads	Ultra-filtration	Silica columns	/	/
Format of extraction	Individual		96 wells	96 wells or 15 places	96 wells	Individual	Individual	Individual
EPC to be added	**EPC-Ext**				**EPC-Amp**			

4. Controls to include

The use of controls 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 controls follows the recommendation of the normative requirement and recommendation for the development and the validation of veterinary PCR (AFNOR NF U47-600-1).

All the steps of the analysis procedure (Extraction + Amplification), for all the types of samples, are validated with the association of the controls included in the kit.

- The EPC-Ext allows verifying the extraction and amplification steps of each sample.
- The EPC-Amp allows verifying only amplification steps.
- The PARATB CTL+ allows validating the amplification of the target.

Other controls could be added:

- **Negative control of extraction**

To verify the absence of cross-contamination, one negative control could be included per trial. The control could be a negative sample, made with the dilution buffer.

- **Positive control of extraction**

A positive control could be added in each trial. The control is a sample containing *Mycobacterium avium* subsp. *paratuberculosis*. It could come from a positive sample available in the laboratory or from a negative sample spiked with a solution of *Mycobacterium avium* subsp. *paratuberculosis*. 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.

5. Samples preparation

A. Faeces

Faeces quantity can vary from 1 to 10 g of faeces (=X). Dilute 1 quantity of faecal sample in 7 volumes of sterile demineralised water (dilution (w/v) 1/7); for example, 3 g with 20 ml or 6 g with 40 ml or 10 g with 70 ml. Respect the weight/volume ratio. The sensitivity and reproducibility are better when the quantity of faecal sample is greats.

Environmental samples (stool scraping, areas waiting,....) are treated as a faecal sample. Take 3-10 g of samples and make a dilution in water.

Biological samples:	**Bovine faeces**	**Caprine and ovine faeces**
Dilution of sample	Place **X g +/- 0.2 g of faeces** in a bottle with **sterile demineralised water** and homogenize at least 15 seconds. Allow to settle 10 to 20 minutes.	Crush **X g+/- 0.2 g** of **faeces** in a bottle with **sterile demineralised water** and homogenize about 15 seconds. It is recommended to keep the mixture over night at room temperature for a complete homogenisation. Homogenize at least 15 seconds. Allow to settle 10 to 20 minutes.

A concentration using a special device, ADIAFILTER is proposed to increase the sensibility and reproducibility. A faeces preparation without the ADIAFILTER concentration is also propose.

B. Tissues

Biological samples:	Ganglia	Ileo-caecal mucous membranes and valvules
Sample treatment	Cut the organ with a scalpel blade, and lacerate the inside of the ganglion in order to obtain about **10 g +/-0.5 g of organ** in a blender bag containing **40 ml** of **demineralised water**.	Scrape the lining of the mucous membranes with a scalpel blade, in order to obtain about **5 g +/-0.5 g of organ** in a blender bag containing **40 ml** of **demineralised water.**
	Disrupt the bags in a blender with paddles 1 to 2 minutes in order to obtain a homogeneous organ solution.	

C. Milk

See the user manual available on the web site mentioned on the certificate of analysis included in the used ADIAPURE™ PARATB MILK kit.

D. Culture

With a sterile single use material, place 1 colony in 50 µl of sterile demineralised water. Put it to boil 10 minutes. Centrifuge 1000 g at room temperature. Transfer the supernatant in another microtube.
Close the tubes, identify and store at +2/8°C if PCR reaction is performed in the day, then they should be stored at <-15°C.

V. Extraction and purification

All the centrifugations are performed at room temperature.
After incubate and disruption, centrifuge few seconds to remove drops inside the lid.

1. Faeces preparation WITH or WITHOUT ADIAFILTER

A. Sample preparation WITH ADIAFILTER concentration:

Biological samples:	**Bovine faeces**	**Caprine and ovine faeces**
Cleaning and concentration	Transfer **10 ml** of **the obtained supernatant (§IV-5-A)** on the ADIAFILTER. Centrifuge 5 minutes at 3 000 g. Discard the filter and the supernatant. **Remark:** the pellet can be stored a week at +2/8°C before extraction.	
Disruption	Add **500 µl** of **sterile demineralised water** on the pellet and mix in order to obtain a homogeneous solution. Transfer the obtained solution in a 1.5ml-microtube containing **300 mg** of **glass beads** and disrupt 10 minutes at 30 Hz with a Mixer Mill*. Centrifuge 5 minutes at 15 000 g.	
Transfer	Transfer **200 µl of supernatant** for QIAamp® DNA Mini Kit, NucleoSpin® Tissue and NucleoSpin® 96 tissue Transfer **100 µl of supernatant** for Magnetic beads kit	

* or transfer the obtained solution in a Lysis Matrix B tube and disrupt 3 x 45 seconds at 4 m/sec with the Fast Prep

B. Sample preparation WITHOUT ADIAFILTER concentration:

Biological samples:	**Bovine faeces**	**Caprine and ovine faeces**
Disruption	Transfer **1 ml** of **the obtained supernatant (§IV-5-A)** in a microtube containing **300 mg** of **glass beads** and disrupt 10 minutes at 30 Hz with a Mixer Mill*. Centrifuge 5 minutes at 15 000 g.	
Transfer	Transfer **200 µl** of supernatant for QIAamp® DNA Mini Kit, NucleoSpin® Tissue and NucleoSpin® 96 tissue Transfer **100 µl** of supernatant for Magnetic beads kit	

* or transfer the obtained solution in a Lysis Matrix B tube and disrupt 3 x 45 seconds at 4 m/sec with the Fast Prep

2. Using QIAamp® DNA Mini kit

Before extraction switch on incubators, block heaters and/or heating baths at +56°C or +70°C or +95°C.

Biological samples	Faeces	Tissue
Lysis	Transfer **200 µl** of the **supernatant (§IV-1)** in a microtube. Add **180 µl** of buffer **AL** + **20 µl** of **proteinase K** **+5 µl of EPC-Ext** Mix.	Transfer **200 µl** of the **organ solution (§IV-5-B)** in a microtube. Add **360 µl** of buffer **ATL** and **40 µl** of **proteinase K**. Mix and incubate 1 hour at +56°C. Add about **300 mg** of **glass beads** and disrupt 10 minutes at 30 Hz in the Mixer Mill*. In a new microtube containing **200 µl** of buffer **AL,** add **300 µl** of **supernatant**. Mix.
	Incubate **10 minutes** at **+70°C**.	Incubate **30 minutes** at **+95°C**.
Binding preparation	Add **210 µl** of **ethanol 100%**. Homogenize the mixture by pipetting (~10 times) or by using a mixer such as vortex (~15 seconds).	
Transfer to columns and binding to the membrane	Identify columns, apply the **whole** obtained solution to the corresponding column. Centrifuge 1 minute at 10 000 g. *If the whole 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.	

* or transfer the obtained solution in a Lysis Matrix B tube and disrupt 3 x 45 seconds at 4 m/sec with the Fast Prep

3. Using NucleoSpin® Tissue

Before extraction switch on incubators, block heaters and/or heating baths at +70°C.

Biological samples	**Faeces**
Lysis	Transfer **200 µl** of the **supernatant (§IV-1)** in a microtube. Add **180 µL** of buffer **B3** + **25 µl** of **proteinase K** + **5 µl** of **EPC-Ext**. Mix.
	Incubate **10 minutes** at **+70°C**.
Binding preparation	Add **210 µl** of **ethanol 100%**. Homogenize the mixture by pipetting (~10 times) or by using a mixer such as vortex (~15 seconds).
Transfer to columns and binding to the membrane	Identify columns, apply the **whole** obtained solution to the corresponding column. Centrifuge at 10 000 g/1 minute. *If the whole 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 **500 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 **100 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.

4. Using NucleoSpin® 96 Tissue with centrifugation

Before extraction switch on incubators, block heaters and/or heating baths at +56°C.
Remark: the 96 well can be used in 3 times at the most. Put an adhesive film on non-used wells.

Biological samples	**Faeces**
Lysis	Transfer **200 µl** of the **supernatant (§IV-1)** to the corresponding well of a MN Square-well Block. Add **200 µl** of buffer **BQ1** + **25 µl** of **proteinase K** + **5 µl** of **EPC-Ext.** Seal the plate. Put the plate under agitation.
Incubation	Incubate 15 minutes at +56°C.
Nucleic acids polarisation	Add **210 µl** of **Ethanol 100%** in each well of the MN Square-well Block. Mix well by pipetting at least 10 times.
Transfer to plate and binding to the membrane	Place the 96 NucleoSpin® Tissue Binding Plate (green) of the MN Square-well Block.
	Apply the whole obtained solution to the corresponding well of the 96 NucleoSpin® Tissue Binding Plate. Centrifuge 2 minutes at 5600 g. Remark: in case of plugging, centrifuge 3 minutes at 5600 g once again.
1st wash	Add **500 µl** of buffer **BW.** Centrifuge 2 minutes at 5600 g.
2nd wash	Add **700 µl** of buffer **B5.** Centrifuge 2 minutes at 5600g.
Plate dry step	Place the 96 NucleoSpin® Tissue Binding plate on the new 96 plate. Centrifuge 10 minutes at 5600 g.
Elution in 96 wells plate	Place the 96 NucleoSpin® Tissue Binding Plate on the Rack of MN Tube Strips. Add **100 µl** of buffer **BE** (previously heated at +56°C). **Important : Incubate 10 minutes.** Centrifuge 2 minutes at 5600 g.
Storage	Close the Rack with MN Tube Strips with Caps Strips, identify and store at +2/8°C if PCR reaction is performed in the day, then it should be stored at <-15°C.

5. Using DNA/RNA extraction magnetic beads kit

See the NEKF user manual available on the web site mentioned on the certificate of analysis included in the used ADIAVET™ kit.

6. Using ADIAPURE™ PURIFICATION

See the user manual available on the web site mentioned on the certificate of analysis included in the used ADIAPURE™ PURIFICATION kit.

VI. Amplification

a- Determine the number of analysed samples including the controls (e.g. positive and negative extraction controls, positive control of amplification (positive control) and No Template Control (NTC)).

b- Preparation of the "A5 solution": Defrost the A5 solution at room temperature.

1. **When EPC-Ext has been added**

Transfer **20 µl** of A5 solution to each PCR tube or PCR plate well. Immediately store A5 solution tube at <-15°C and in darkness.

2. **When EPC-Amp has been added**

Take (n+2)*** 20 µl** of Amplification solution **A5** and add (n+2)***1 µl EPC-Amp**. Homogenize. Dispense **20 µl** of the Amplification solution **A5** in each PCR tube or PCR plate well with a micropipette with a Nuclease-free tip.

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

d- For each sample, add **5 µl** of purified extract (all samples except culture) or add **1 µl** of culture extract to the 20 µl of Amplification solution **A5**.
For the extraction negative control and the extraction positive control (recommended), add **5 µl** to the 20 µl of Amplification solution **A5**.
For the "PARATB CTL+", add **5 µl** of the solution obtained in § II-3 to the 20 µl of « A5 » solution.
For the No Template Control (NTC), nothing is added to the Amplification solution **A5**.
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 the real-time PCR amplification.

The *Mycobacterium avium* subsp. *paratuberculosis* target is read in FAM. The External 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.

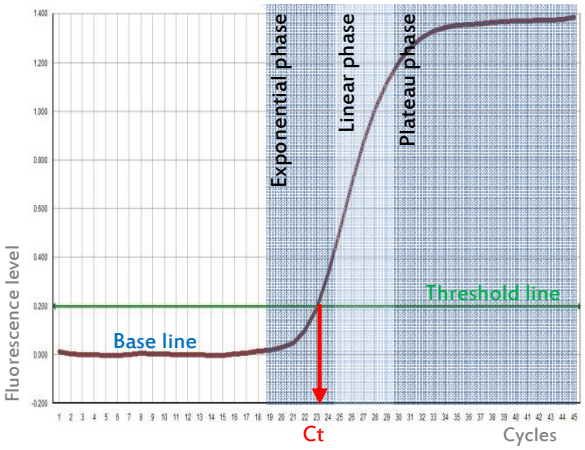
The **« Characteristic amplification curve »** qualifies a fluorescence curve with an exponential phase, a linear phase and a plateau phase.

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.

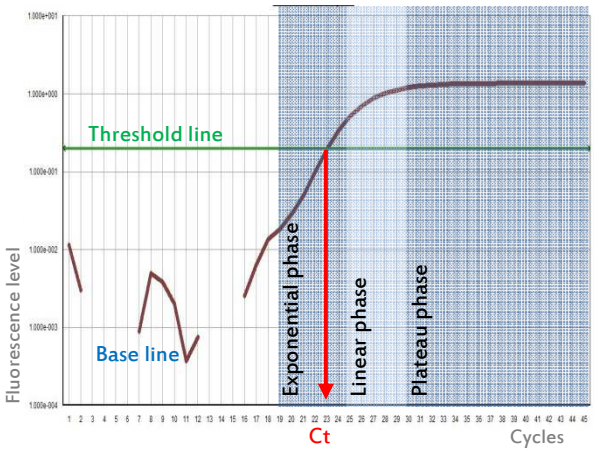
The **« threshold cycle » (Ct)** of a well corresponds, for each detected fluorophore, at the crossing point of the threshold line with the fluorescent curve. The Ct value expressed by the machine for each well depends on the threshold position and on the quantity of target sequences initially present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

The test is considered as **valid** if:

Controls	No Template Control (NTC)	Positive control	Extraction negative control *	Extraction positive control *
FAM amplification	no	yes	no	yes
VIC/HEX amplification	no	no/yes	yes	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 PARATB CTL+ are indicated in the certificate of analysis of the kit.

B. Validation of the run with EPC-Amp

The test is considered as **valid** if:

Controls	No Template Control (NTC)	PARATB CTL+	Extraction negative control *	Extraction positive control *
FAM amplification	no	yes	no	yes
VIC/HEX amplification	yes	no/yes	yes	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 PARATB CTL+ are indicated in the certificate of analysis of the kit.

C. Result interpretation

DNA extraction and amplification for each sample are considered to be **valid** if at least a characteristic amplification curve is observed for *Mycobacterium avium* subsp. *paratuberculosis* (FAM) or for the external control (VIC or HEX).

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

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

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

A total absence of characteristic amplification curve for a sample (example C) 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. References

Green E. P. *& al.* (1989) Sequence and characteristics of IS900, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. Nucleic Acids Res. 17 (22) : 9063-9073

Guillou J. -P. *& al.* (1993) Utilisation de la méthode d'amplification enzymatique in vitro pour la détection de *Mycobacterium paratuberculosis* dans les fèces. Rev. Sci. Tech. Off. Int. Epiz. 12 (2) : 605-615

Thorel M. -F. *& al.* (1990) Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium*, and description of *Mycobacterium avium* subsp. *avium* subsp. *nov.*, *Mycobacterium avium* subsp. *paratuberculosis* subsp. *nov.*, and *Mycobacterium avium* subsp. *silvaticum* subsp. *nov.* Int. J. Syst. Bacteriol. 40 (3) : 254-260

IX. 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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