



Adia^X Vet

Instruction manual
AD1045-PTUB_NO_(EN)_V01
06/2023

PARATB REAL TIME

Reference: AD1045-100

Test for the detection and quantification of *Mycobacterium avium* subsp *paratuberculosis* by real time enzymatic amplification

PCR Test – 100 reactions

For veterinary *in vitro* use only



Sample	Individual analysis	Pool of sample *, up to:
Faeces	✓	5
Environmental specimen (cow dung scraping, waiting areas...)	✓	✗
Milk	✓	✗
Bacterial culture	✓	✗
Tissue (ganglion, mucosa, ileocecal valve)	✓	✗

* Depending on the epidemiological case and on the quality of samples.

Kit composition

Content		AD1045-100 Kit
		100 Reactions
A5	Amplification solution	2 x 1000 µL tubes with green cap (Ready to use)
PARATB CTL+	PARATB positive control	1 tube with purple cap (To reconstitute)
EPC-Ext	Exogenous extraction control	2 x 300 µL tubes with yellow cap (Ready to use)
EPC-Amp	Exogenous control of amplification	1 x 100 µL tube with colorless cap (Ready to use)
NF-Water	Nuclease-Free Water	1 x 1000 µL tube with white cap (Ready to use)

Revision history

Date	Version	Modifications
01/2020	NE045-10	Former version
06/2023	AD1045-PTUB_NO_(EN)_V01	New format Addition of pre-filtration system with ADIAPREP Quantification using PARATB CTL+ Availability of additional kits

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

A. Introduction

Paratuberculosis disease is a contagious and fatal disease that mainly affects domestic ruminants such as sheep, cows, and goats. It is also known as Johne's disease. Johne's disease is caused by the bacterium *Mycobacterium avium subsp. paratuberculosis* (MAP) which multiplies in the intestines and is excreted in the faeces. The level of contamination of faeces varies according to the stage of the disease, from a few bacteria per gram of faeces in the early stages of infection, to 10^4 - 10^{10} bacteria per gram of faeces in the clinical phase (Collins *et al.*, 1993). The bacteria can then be disseminated into the animal's body via macrophages, and contamination through colostrum, milk and semen is possible.

The detection of MAP by culture is time consuming and tedious (6-8 weeks). Hence, PCR is the fastest and most specific method for MAP detection.

Quantification of the contamination level is of interest to classify animals and identify the heaviest shedders.

Bio-X Diagnostics offers 3 types of analysis:

- Qualitative PCR.
- Quantitative PCR. The Paratb CTL+ is used to produce a standard curve (genome equivalent/gram of faeces).
- Relative PCR with the use of a quantified *Mycobacterium avium subsp. paratuberculosis* positive faeces, on demand (ref. ADC04SQ01).

B. Test principle

ADIAVET™ PARATB REAL TIME test is based on gene amplification of *Mycobacterium avium subsp. paratuberculosis* specific DNA fragments. This test is intended to detect simultaneously, in one well:

- *Mycobacterium avium subsp. paratuberculosis* (FAM labelled probe).
- Exogenous control (HEX labelled probe or its equivalent), can be used as an:
 - Extraction and amplification control if the EPC-Ext is added to each specimen during nucleic acids extraction (can be used with the ADIAMAG extraction kit or silica columns).
 - Amplification control if the EPC-Amp is added to A5 solution (in the case where samples have been extracted from milk using ADIAPURE PARATB MILK kit).

The PARATB CTL+ positive control, included in the kit, enables the quantification of *Mycobacterium avium subsp. paratuberculosis*.

C. Storage conditions

After reception, the kit should be stored at $<-15^{\circ}\text{C}$ until the expiration date.

It is recommended to make aliquots of the A5 solution if it should be defrosted more than 3 times.

Do not thaw more than 3 times.

Store away from sunlight.

Do not mix reagents of two different batches.



D. Material required but not provided

- Real-time Thermal cycler and device.
- Instrument for homogenous mixing of tubes.
- Pipettes of 1 - 10 μL , 20 - 200 μL and 200 - 1000 μL .
- Nuclease-free filtered pipette tips.
- Nuclease-free microtubes of 1,5 mL and 2 mL.
- Powdered-free latex or nitrile gloves.
- Nuclease-free water.
- Faecal preparation system:
 - ADIAPREP™ (Bio-X Diagnostics, ref. ADPREP-200 (200 tests)).
 - ADIAFILTER™ (Bio-X Diagnostics, ref. ADIFIL100 (100 tests)).
- Grinding beads.

For Mixer Mill:

- ADIAPURE™ ALIQUOTED GLASS BEADS (Bio-X Diagnostics, ref. ADIADPBIA-480 (480 tests)).
- ADIAPURE™ GLASS BEADS RACKS 4x96 (Bio-X Diagnostics, ref. ADPBIAR-4x96).

For Fast Prep or Ribolyser:

- Lysing Matrix B (MP biomedical, 100 tubes, ref. 116911100).
- Kit for nucleic acids extraction.



Additional kits for method adoption and PCR

- **Extraction Positive Control PARATB (Ref.: ADC04EPC).** Supplier reference material for method adoption that can also be used as a sentinel (Calibrated between 1 and $100\times\text{LOD}_{\text{Method}}$).
- **LD_{PCR} Positive Control – PARATB (Ref.: ADC04LD)** Confirmation of performances – LOD_{PCR} of kit.
- **Quantified Extraction Positive Control PARATB Faecal (Ref.: ADC04SQ01).** Extraction positive faeces quantified in PARATB to identify high positive (calibrated to 10000 GE/gram of faeces according to Kralik, P. *et al.*, 2014 Beinhauerova, M. *et al.*, 2021).

E. Warnings and precautions

- For veterinary *in vitro* use only.
- For animal use only.
- For professional use only.
- All instructions must be read before performing the test and strictly respected.
- Do not use reagents after the expiration date.
- Do not use reagents if the packaging is damaged.
- Do not open PCR wells or tubes after amplification.
- Do not mix reagents from different batches.
- Used material must be disposed of in compliance with the legislation in force regarding environmental protection and biological waste management.
- This kit contains products of animal origin. Certified knowledge of the origin and/or sanitary state of the animals does not totally guarantee the absence of transmissible pathogenic agents. It is therefore recommended that these products be treated as potentially infectious and handled observing the usual safety precautions (do not ingest or inhale).

F. Preparation of faeces or environmental samples

Nucleic acids must be extracted from the samples before using the kit.

1. With ADIAPREP (ref. ADPREP-200)

- Collect 1 spoon of faecal matter and transfer it into the ADIAPREP.
- Vortex until a homogeneous suspension is obtained.
- Transfer 1 mL into a clean tube, centrifuge 5 minutes at 3000 g and discard the supernatant.
- Add 300 mg grinding beads and 500 μL sterile deionized water to the pellet.
- Grind for 5 minutes at 30 Hz on the Mixer Mill or 3 x 45 seconds on the Fast Prep/Ribolyser and centrifuge for 5 minutes at 3000 g.
- Use a volume of supernatant according to the extraction kit chosen.

2. With ADIAFILTER (ref. ADIFIL100)

- Collect 3 g (+/- 0.2) of faecal matter, vortex into 20 mL of sterile deionized water (or identical weight/volume ratio) and allow to settle for 10 to 20 minutes until sedimentation.
- Place 10 mL of supernatant in the ADIAFILTER, centrifuge for 5 minutes at 3000 g and discard the supernatant and ADIAFILTER.
- Add 500 μL sterile deionized water to the pellet, vortex, and transfer to a microtube containing 300 mg grinding beads.

- Grind for 10 minutes at 30 Hz on Mixer Mill or 3 x 45 seconds on Fast Prep/Ribolyser and centrifuge for 5 minutes at 15 000 g.
- Use a volume of supernatant according to the extraction kit chosen.

3. Without ADIAFILTER system

- Collect 3 g (+/- 0.2) of faecal matter (or identical weight/volume ratio), vortex into 20 mL of sterile deionized water and allow to settle for 10 to 20 minutes until sedimentation.
- Transfer 1 mL to a microtube containing 300 mg of grinding beads.
- Grind for 10 minutes at 30 Hz on Mixer Mill or 3 x 45 seconds on Fast Prep/Ribolyser and centrifuge for 5 minutes at 15 000 g.
- Use a volume of supernatant according to the extraction kit chosen.

G. Procedure

1. Use of EPC-Ext

EPC-Ext must be added to each sample and extraction controls (except with ADIAPURE™ PARATB MILK kit).

Aliquot and store the solution at a temperature below -15 °C according to the size of extraction series. Do not thaw more than 3 times.

Add **5 µL** of EPC-Ext in the first nucleic acids extraction lysis buffer.

2. Extraction kits

Nucleic acids must be extracted from the samples before using the amplification kit. The RNA/DNA purification kits listed below are recommended by Bio-X Diagnostics:

Product name	Extraction system	Number of tests and reference
ADIAMAG™	Magnetic beads	200 tests: ref. NADI003 800 tests: ref. NADI003-XL
ADIAPURE™ PARATB MILK	Capture on magnetic beads from milk	100 tests: ref. ADIADP04M1-100

The instruction manual for extraction is available on the website, all information are indicated on the certificate of analysis included in the PCR kit.

Other purification kits can be used if they have been validated by the user.

After extraction, nucleic acid extracts can be kept on ice or at +2/8 °C until use. For long term storage, they must be kept at a temperature below -15 °C.

3. Controls

Using controls allow to verify the reliability of the results. Controls can be included by series of analysis according to the recommendations defined by the standards.

Controls	Validation of	How to proceed
No Template Control (NTC)	Absence of amplification contamination	5 µL NF-Water in a well per run
PARATB CTL+ (Dilution pure to 1/100 000)	Target amplification and standard range	5 µL CTL+ for each range point in a well per run
Negative extraction control	Absence of contamination for the extraction and amplification	1 extraction (water or lysis buffer) per run
Positive extraction control	Extraction and amplification	1 extraction (Positive sample between 1 et 100x LOD _{Method}) per run
Quantified positive faeces control for relative PCR	Extraction and amplification	1 extraction (quantified positive sample) per extraction run

4. Use of EPC-Amp

The EPC-Amp is used when the EPC-Ext has not been added during nucleic acid extraction (example: using ADIAPURE™ PARATB MILK kit).

Aliquot and store the solution at a temperature below -15 °C according to the size of extraction series. Do not thaw more than 3 times.

For each PCR well, add **1 µL** of EPC-Amp to the amplification solution. (see § « Amplification », Step 1).



5. Use of CTL+

Add **200 µL** of « NF-Water » per tube.

Homogenize the tubes using a mixer, such as vortex, > 20 seconds and until dissolution of the blue pellet.

After reconstitution, aliquot and store the solution below -15 °C until the kit expiration date. Do not thaw more than 3 times.

For quantification, prepare the following ranges in Nuclease-free water (4 minimum range points including 1 at LQ_{PCR}).

Dilution	PARATB CTL+ concentration (copies/PCR)
Pure	10 ⁶
1/10	10 ⁵
1/100	10 ⁴
1/1000	10 ³
1/10000	10 ²
1/100000	10 (=LD-LQ PCR)

Use **5 µL** of each dilution in the dedicated wells (see § « Amplification », Step 2).

In the case of a qualitative or relative test, use **5 µL** of the **1/1000** dilution in one of the dedicated wells (see § « Amplification », Step 2).

6. Amplification

Warning:

- Before starting, thaw reagents at room temperature in the dark.
- Homogenize all reagents and samples before use.
- Store reagents at a temperature below -15 °C after use.

Step 1:

If EPC-Ext was used during extraction

Dispense **20 µL** of amplification solution (A5) in each well.

If EPC-Ext was not used during extraction

Place (n*+1) x **20 µL** of amplification solution (A5) in a microtube and add (n*+1) x **1 µL of EPC-Amp**. Dispense **20 µL** of the mixture into each well.

*n = number of PCR wells to run.

Step 2: Dispense **5 µL** of nucleic acids extracts and **5 µL** of controls in each dedicated well. Use NF-Water for the No Template Control (NTC).

Step 3: Cover the wells with an appropriate optical film or caps.

Step 4: Start the PCR analysis.

The following program is defined for ABI Prism thermocyclers (like 7500, QuantStudio5, Step-one...) from Applied Biosystems, for Mx3000, Mx3005P and AriaMx from Agilent, for LightCycler from Roche Diagnostics, for Rotor-Gene Q from Qiagen, for CFX96 and Chromo 4 from Biorad and for MIC from BioMolecular System.

DNA standard program		
2 min. 50 °C		
10 min. 95 °C		
15 sec. 95 °C*	45 cycles	
60 sec. 60 °C**		

*30 sec. 95 °C for MX3000 and MX3005P

** Reading and parameters for fluorescence acquisition:

Fluorochrome	Absorbance (nm)	Emission (nm)
FAM	494	520
HEX or equivalent	530	549
ROX	575	602

Note: The Quencher is non-fluorescent. The A5 solution contains a passive reference read in the same spectra as ROX for ABI machines.

For other thermal cycler instruments, please contact your sales representative or the customer relations department (support.pcr@biox.com).

H. Interpretation of results

1. Validation and interpretation of qualitative results

Display all curves and position the threshold line for each fluorochrome.

a. Test validation

Amplification is valid if the following results are obtained. Expected Ct (Threshold Cycle) values for the CTL+ are indicated on the certificate of analysis of the kit.

Controls	Amplification		Validation of
	FAM	HEX or equivalent	
No Template Control (NTC)	No	Yes/No*	Absence of amplification contamination
PARATB CTL+	Yes	Yes/No*	Target amplification
Extraction negative control	No	Yes	Absence of extraction contamination
Extraction positive control	Yes	Yes	Extraction and amplification steps
Quantified positive faeces control	Yes	Yes	Extraction and amplification steps

*According to the addition or not of EPC-Amp.

b. Results interpretation

Nucleic acids extraction and amplification are **valid** for each sample if at least one typical amplification curve is observed in FAM or HEX (or equivalent).

Amplification		Interpretation
FAM	HEX or equivalent	<i>Target Mycobacterium avium</i> subsp. <i>paratuberculosis</i>
No	Yes	Undetected
Yes	Yes/No	Detected
No	No	Undetermined

« **Undetermined** »: no characteristic amplification curve.

Possible causes:

Defective PCR due to inhibitors, set up error, absence of samples, degraded samples and/or issue with nucleic acids extraction (loss or destruction of nucleic acids).

Recommendations:

Set up a new PCR assay using pure nucleic acids extracts and 10x dilutions in Nuclease-free water.

If the assay is inconclusive, perform a new nucleic acids extraction.

2. Validation and interpretation of quantitative results

a. CTL+ range

PARATB CTL+ dilution	Concentration (Copies IS900/PCR)	FAM amplification	Validation of
Pure	10 ⁶	Yes	PARATB target amplification and calibration curve set up
1/10	10 ⁵	Yes	
1/100	10 ⁴	Yes	
1/1000	10 ³	Yes	
1/10 000	10 ²	Yes	
1/100 000	10	Yes	

To interpret quantitative results, set up a calibration curve (number of cycles = f (Log concentration)), determine the curve equation ($y = ax + b$) and check PCR efficiency ($Eff\% = (10^{\frac{-1}{a}} - 1) \times 100$).

The calibration curve is valid if:

- The 6 points of the range are amplified. However, one point of the range can be omitted if that point is not one of the extreme points.
- The coefficient of correlation R^2 is higher than 0,9.
- Efficiency between 75 et 125 %.

b. Quantification interpretation

Quantification of a positive sample is only possible in the quantification domain of the method use (see validation data).

FAM amplification	Sample status
No signal	Undetected Nucleic acid undetected
Signal < LOQ _{METHOD}	Detected Nucleic acid detected with a quantity under the LOQ _{METHOD}
LOQ _{METHOD} < signal < LOQ _{max}	Detected Quantifiable nucleic acid
Signal > LOQ _{max}	Detected Nucleic acid detected with a quantity over the LOQ _{MAX}

In the case of a "quantifiable" sample, the concentration is determined using the calibration curve equation:

$$x = 10^{\left(\frac{y-b}{a}\right)} \times F$$

Where x : concentration (in GE/g of faeces if F is used)
 y : Ct value in FAM for the positive sample to quantify
 b : intercept
 a : slope
 F : multiplying coefficient (optional)

The multiplying coefficient is determined according to the sample matrix and extraction method. Multiplying coefficient examples with ADIAMAG extraction kit according to the NEKF user manual:

ADIAMAG protocol with ADIAFILTER	$F=2.7$
ADIAMAG protocol with ADIAPREP	$F=5.35$

Bio-X Diagnostics offers, upon request (support.pcr@biox.com), an Excel file for quantitative analysis.

Bibliography

- Collins, J. D. *et al.*, Comparison of polymerase chain reaction tests and faecal culture for detecting *Mycobacterium paratuberculosis* in bovine faeces, *Vet. Microbiol.* (1993).
- Beinhauerova, M., *et al.* Development of a reference standard for the detection and quantification of *Mycobacterium avium* subsp. *paratuberculosis* by quantitative PCR. *Sci Rep* (2021).
- Kralik P., *et al.*, Evidence of passive faecal shedding of *Mycobacterium avium* subsp. *Paratuberculosis* in a Limousin cattle herd. *Vet J* (2014).

Symbols

Symbols	Signification
	Catalog number
	Manufacturer
	Temperature limitation
	Use by
	Batch code
	Consult Instructions for Use
	Contain sufficient for "n" tests
	For veterinary <i>in vitro</i> use only – For animal use only
	Keep away from sunlight

1 Extract nucleic acids with

**Adia^X
Mag**



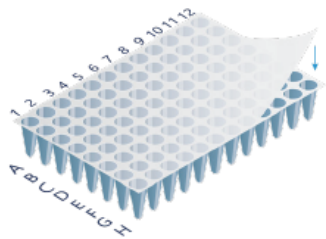
Scan me to
discover Adiamag™

If using the EPC-Ext:

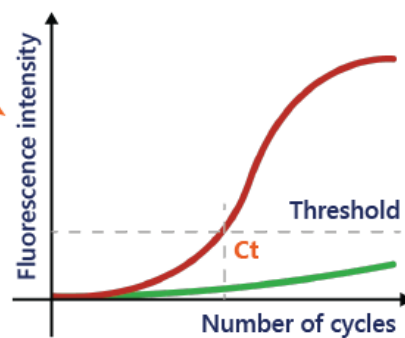
2 Distribute **20 µL** of **A5** amplification solution

3 Distribute **5 µL** of **nucleic acids**, **CTL+** and **NF-Water**

4 Seal the wells



5 Start PCR analysis



*The notes do not replace the instructions for use of which they are a summary.

If not using the EPC-Ext:

2 Prepare a premix of **20 µL** of **A5** amplification solution + **1 µL** of **EPC-Amp**
Dispense **20 µL** of the premix

