Instruction manual
ADL04Y1_PTUB_NO_(EN)_V01
03/2024

PARATB

Reference: ADL04Y1-100

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 possible*, up to:
Faeces	✓	4
Environmental specimen (cow dung scraping, waiting areas…)	✓	✗
Milk	✓	✗
Tissue (ganglion, mucosa, ileocaecal valve)	✓	✗
Bacterial culture	✓	✗

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

Kit composition

Content		ADL04Y1-100 Kit 100 reactions
A6	Amplification solution	1 lyophilized vial with blank caps (To reconstitute)
Rehydration buffer	Rehydration solution	1 x 6 mL vial (Ready to use)
PARATB CTL+	*Mycobacterium avium* subsp. *paratuberculosis* positive control	1 tube with purple cap (To reconstitute)
EPC-Ext	Exogenous extraction control	1 tube with yellow cap (To reconstitute)
NF-Water	Nuclease-Free Water	1 x 1000 µL tube with white cap (Ready to use)

Revision history

Date	Version	Modifications
03/2024	V01	First version

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 heavy 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

ADIALYO™ PARATB test is based on the amplification of specific *Mycobacterium avium* subsp. *paratuberculosis* DNA. This test is intended to detect simultaneously, in one well:

- *Mycobacterium avium* subsp. *paratuberculosis* (FAM labelled probe)
- Exogenous internal control (HEX labelled probe or its equivalent)
  - Either extraction and amplification control if the EPC-Ext is added to each specimen during nucleic acids extraction steps.
  - Or amplification control if the EPC-Ext is added to amplification solution.

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

C. Storage conditions

- Store the kit at a temperature below +2/8 °C after reception.
- Store away from sunlight and keep dry.
- After reconstitution, prepare aliquots and store them at a temperature below -15 °C until the expiration date.
- Do not thaw more than 3 times.

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)).
- 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 biomedicals, 100 tubes, ref. 116911100).

- Kit for nucleic acids extraction.

Additional kits for method adoption and PCR

- **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).
- **LD_{PCR} Positive Control – PARATB (Ref.: ADC04YLD) Confirmation of performances – LOD_{PCR} of kit.** Confirmation of performances – LOD_{PCR} of kit.
- **Extraction Positive Control PARATB (Ref.: ADC04EPC).** Supplier reference material for method adoption that can also be used as a sentinel (Calibrated between 1 and 100xLOD_{Method}).

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. Nucleic acids extraction

1. Faeces and environmental samples preparation

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

Instruction for faeces preparation with ADIAPREP disposal (ref ADIAPREP-200)

- Collect 1 spoon of fecal 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 (or Lysis Buffer LF1 of ADIAPURE Lysis Flex kit) 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.
- Extract a volume of supernatant using a recommended extraction kit.

2. Extraction kits

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™ Lysis FLEX	Direct lysis	500 mL : ref. ADPLF1-500
ADIAPURE™ PARATB MILK	Capture from milk using magnetic beads	500 mL: ref. ADPLF1-500

For the extraction, consult the user manual version, available on the website, indicated on the certificate of analysis included in the PCR kit of interest.

Extraction protocols are described in validation data. 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 for a few hours or until use. For long term storage, they must be kept at a temperature below -15 °C or -65 °C.

3. Controls

Using controls allow to verify the reliability of the results. Controls can be included.

Control	Validation of	Usage
No Template Control (NTC)	Absence of amplification contamination	5 µL NF-Water in a well per run
PARATB CTL+ (Dilution pure to 1/10 000)	PARATUB 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

G. Procedure

1. Amplification solution A6 preparation

- Add **1000 µL** of **« Rehydration buffer »** per A6 tube.
- Homogenize tube contents using a mixer, such as vortex, at least 20 seconds.
- After reconstitution, aliquot the solution and store at a temperature below -15 °C until the expiration date. Do not thaw more than 3 times.
- To use the A6, please refer to §« Amplification », Step 1.

2. Preparation of controls

a. Use of EPC-Ext

EPC-Ext must be added to each sample and extraction controls.

- Add **1000 µL** of **« NF-Water »** per tube.
- Homogenize the tube contents using a shaker such as a vortex, > 20 seconds.
- After reconstitution, aliquot and store the solution at a temperature below -15 °C until the kit expiration date. Do not thaw more than 3 times.
- For use, 2 solutions are possible:
  - Either add **5 µL** of EPC-Ext in the first lysis buffer during the extraction of nucleic acids in magnetic beads or silica columns.
  - Or add **0.5 µL** of EPC-Ext to each PCR well (if using ADIAPURE™ PARATB MILK). See § "Amplification", Step 1.

b. 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 at a temperature below -15 °C until the kit expiration date. Do not thaw more than 3 times.
- If quantification is needed, prepare the following ranges in Nuclease-free water:

Dilution	PARATB CTL+ concentration (IS900 copies/PCR)
Pure	10^6
1/10	10^5
1/100	10^4
1/1000	10^3
1/10000	10^2

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

3. Amplification

Warning:

- Before starting, rehydrate or 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: Dispense **10 µL** of amplification solution (A6) per well.
If use of EPC-Ext:
Dispense **10 µL** of amplification solution (A6) in each well.
If no use of EPC-Ext:
Place (n+1) x 10 µL of amplification solution (A6) in a microtube and add (n+1) x 0.5 µL of EPC-Amp. Dispense **10 µL** of the mixture into each well.
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: Set up the amplification program.

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/RNA Program	
10 min. 45 °C	
2 min. 95 °C	
5 sec. 95 °C	40 cycles
30 sec. 60 °C*	

*Reading and parameters for fluorescence acquisition:

Fluorochrome	Absorbance (nm)	Emission (nm)
FAM	494	520
HEX or equivalent	538	554
ROX	575	602

Note: The Quencher is non-fluorescent. The A6 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.

H. Reading and interpretation

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	No/Yes*	Absence of amplification contamination
PARATB CTL+	Yes	No/Yes*	Target amplification
Extraction negative control	No	Yes	Absence of extraction contamination
Extraction positive control	Yes	Yes	Extraction and amplification steps

*According to the addition or not of EPC-Ext during the amplification step.

b. Results interpretation

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

Amplification		Interpretation
FAM	HEX or equivalent	*Mycobacterium avium* subsp. *paratuberculosis*
No	Yes	Undetected
Yes	Yes	Detected
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 (IS900/PCR)	Target amplification	Validation of
Pure	10^6	Yes	PARATUB target amplification and calibration curve set up
1/10	10^5	Yes	
1/100	10^4	Yes	
1/1000	10^3	Yes	
1/10000	10^2	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 ($Eff\% = \left(10^{\left(\frac{-1}{a}\right)} - 1\right) \times 100$).

The calibration curve is valid if:

- The 5 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 %.
- Points of the range are spread homogenously.

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 for PARATUB
No signal	Undetected Nucleic acid undetected
Signal < LQ_{METHOD}	Detected Nucleic acid detected with a quantity under the LQ_{METHOD}
LQ_{METHOD} < signal < LQ_{max}	Detected Quantifiable nucleic acid
Signal > LQ_{max}	Detected Nucleic acid detected with a quantity over the LQ_{MAX}

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

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

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

The multiplying coefficient is determined according to the sample matrix and extraction method.

ADIAMAG (with ADIAPREP) protocol	F=6,4
ADIAPURE Lysis Flex (with ADIAPREP) protocol	F=9,4

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).
- U47-600 : Méthodes d'analyse en santé animale - PCR (réaction de polymérisation en chaîne).

Symbols

Symbole	Signification
	Catalog number
	Manufacturer
	Temperature limitation
	Use by
	Batch code
	Consult Instructions for Use
	Contain sufficient for "n" tests
	For veterinary *in vitro* use only – For animal use only
	Keep away from sunlight
	Keep dry

Notes

1 Extract nucleic acids with

Adia X Mag

Scan me to discover Adiamag™

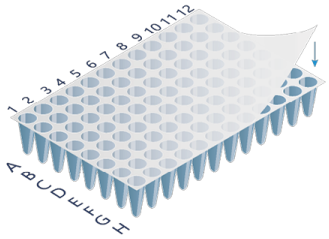
2 Add **1000 µL** of Rehydration buffer to the **A6** amplification solution

If using the EPC at the extraction step:

3 Distribute **10 µL** of **A6** amplification solution

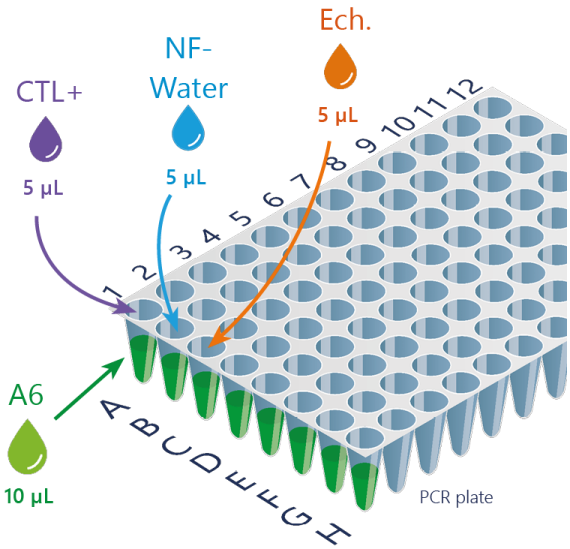
4 Distribute **5 µL** of nucleic acids, **CTL+** and **NF-Water**

5 Seal the wells

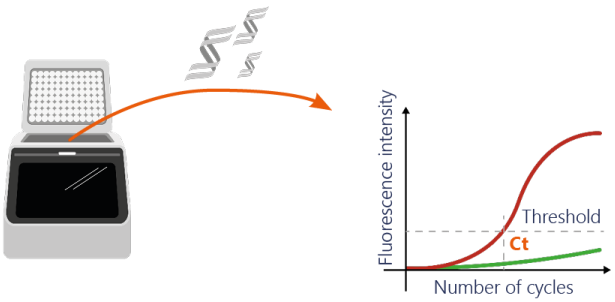


If not using the EPC at the extraction step:

3 Prepare a premix of **10 µL** of **A6** amplification solution + **0,5 µL** of **EPC**
Dispense **10 µL** of the premix



6 Start PCR analysis



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

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