



Data validation
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06/2023

Adia^X
Vet

PARATUB REAL TIME

Reference: AD1045-100

Test for the detection and the quantification of *Mycobacterium avium* subsp. *paratuberculosis* by real time enzymatic gene amplification from faeces, tissue, and milk specimens of bovine, ovine, caprine and camelidae, as well as from environmental specimen and bacterial culture.



In vitro and strictly veterinary use

Validation data

ADIAVET PARATUB REAL TIME

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I. *Mycobacterium avium* subsp. *paratuberculosis*

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 are 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.

II. Method description

A. Intended use

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, caprine and camelidae, as well as from environmental specimen and bacterial culture.

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

* It depends on the epidemiological case and on the quality of the specimen, and country regulation.

B. Principle

ADIAVET™ PARATB REAL TIME 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).
- EPC-Ext or EPC-Amp: an exogenous internal control (HEX labelled probe or its equivalent) enabling:
 - **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-Amp is added to A5 solution. (To be used for the ADIAPURE™ PARATB MILK extraction kit or as a bacterial culture control). Refer to the "F. Procedure" section, for further details.

The test allows the amplification of the sequence of the IS900 gene specific of *Mycobacterium avium* subsp. *paratuberculosis* (Green, E.P., 1989).

C. Content of the kit

Material supplied		ADI045-100 Kit 100 reactions
A5	Amplification solution	2 x 1000 µL tubes with green cap (a ready-to-use reagent)
PARATB CTL+	<i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i> Positive control	1 tube with purple cap (To reconstitute)
EPC-Ext	External control of extraction	2 x 300 µL tubes with yellow cap (a ready-to-use reagent)
EPC-Amp	External control of amplification	1 x 100 µL tube with white cap (a ready-to-use reagent)
NF-Water	Nuclease free water	1 x 1000 µL tube with white cap (a ready-to-use reagent)

Reagent composition

- **A5:** Synthetic DNA (primers) – Synthetic DNA labelled with a fluorophore – Mineral and organic salts – Nucleotides – DNA polymerase enzyme- Glycerol.
- **PARATB CTL+:** Synthetic DNA – Salts – Carbohydrate.
- **EPC-Ext:** Inactivated bacteria – Salts.
- **EPC-Amp:** Synthetic DNA – Salts.
- **NF- Water:** Nuclease free water.

D. Storage conditions

- Store the kit at a temperature below -15 °C.
- Store away from sunlight.
- Do not thaw more than 3 times.
- In case of several small PCR assays, A5 reagent should be aliquoted in small volumes and then stored at a temperature below -15 °C in the dark, until the expiration date.

E. Material required but not provided

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.
- Class II Microbiological Safety Cabinet.
- Centrifuge for microtubes, tubes of 15 mL, 96-wells plate.
- Disruption equipment for bead beating (such as Mixer Mill, Fast Prep or Ribolyser).
- Incubator, water bath or heat block.
- Instrument for homogenous mixing of tube.
- 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.
- Sterile tube of 5, 10 or 15 mL.
- Latex or nitrile powder-free gloves.
- Scalpel.
- 96-100 % ethanol solution.
- Sterile distilled water.
- Pre-filtration system:
 - ADIAPREP (Bio-X Diagnostics, 200 tests ref. ADPREP-200).
 - ADIAFILTER (Bio-X Diagnostics, 100 tests ref. ADIFIL100).
- Grinding beads
 - For Mixer Mill:
 - ADIAPURE™ ALIQUOTED GLASS BEADS (Bio-X Diagnostics, 480 tests: ref. ADIADPBIA-480).
 - ADIAPURE GLASS BEADS RACKS 4x96 (Bio-X Diagnostics, 384 tests: ref. ADPBIA-4x96).
 - For Rybolyser and Fast prep
 - Lysing Matrix B (MP biomedical, 100 tubes, ref. 116911100).
- DNA extraction kits:

Technology	Extraction kit	References	Validated protocols	User Manual
Capture using magnetic beads	ADIAPURE™ PARATB MILK	Bio-X Diagnostics, 100 tests: ref. ADIADP04M1-100	Milk	NF04M1 www.biox.com
Magnetic beads	ADIAMAG	Bio-X Diagnostics, 200 tests: ref. NADI003 800 tests: ref. NADI003-XL	Faeces or environmental specimen: ✓ with ADIAPREP ✓ with ADIAFILTER ✓ without pre-filtration	NFKF www.biox.com
			Tissue	
Silica column	QIAamp® DNA Mini Kit	Qiagen, 50 tests: ref.51304 250 tests: ref.51306	Faeces or environmental specimen: ✓ with ADIAFILTER ✓ without pre-filtration	In the appendix of validation data
			Tissue	
	NucleoSpin® Tissue	Macherey-Nagel, 50 tests: ref.740952.50 250 tests: ref.740952.250	Faeces or environmental specimen: ✓ with ADIAFILTER ✓ without pre-filtration	
	NucleoSpin® 96 Tissue	Macherey-Nagel, 2x96 tests: ref.740741.2 4x96 tests: ref.740741.4	Faeces or environmental specimen: ✓ with ADIAFILTER ✓ without pre-filtration	
			Bacterial culture (without purification)	

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 100xLOD_{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 positives (calibrated to 10 000 GE/gram of faeces according to Kralik, M. *et al.*, 2014; Bienhauerova, M. *et al.*, 2021).

Validation of the ADIAVET™ PARATB REAL TIME kit was performed on Agilent Mx3005P and AriaMx, Applied Biosystems® 7500 and Quantistudio 5, Biorad CFX96, Qiagen's Rotor-Gene Q, Roche Lightcycler96 and BioMolecular System's MIC. Other thermal cyclers may be used. In all cases, an adoption of the PCR must be carried out (see the user manual).

F. Procedure

Prior to using this kit, nucleic acids must be extracted from the samples. The DNA/RNA extraction kits listed in chapter II-E.

The user manual of the amplification kit package insert is available at www.biox.com. The version of the user manual to use for each lot of amplification kit is indicated on the certificate of analysis included in each kit. The use of pre-filtration systems and grinding beads tubes is described in section "F. Sample Preparation" of the amplification kit package insert.

For extraction, refer to the version of the instructions available on the website www.biox.com, indicated on the certificate of analysis included in the amplification kit used for ADIAMAG or on the certificate of analysis of the extraction kit for ADIAPURE™ PARATB MILK.

Other extraction methods than those recommended can be used after validation by the user.

After extraction, the extracted nucleic acids can be stored at +2/8 °C for 24 hours before use. For longer storage, they should be stored below -15 °C or -65 °C.

Validated or evaluated extraction protocols are presented in the Appendix.

G. Additional information

1. Diagnostic kit manufacturing procedure

The manufacturing procedure is included in Bio-X Diagnostics's production process flow chart. Bio-X Diagnostics is an ISO 9001 certified company.

2. Equipment safety data sheet

See product MSDS sheet.

3. Precautions

- 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 amounts 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.
- The software used is included with the real-time thermal cycler. No additional software is required.
- Samples for analysis should be handled and disposed of as biological waste.
- 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 handled with gloves and into a chemical cabinet.
- See warnings and precautions of the user manual.

4. Troubleshooting guide

Contact your sales representative or customer service support.pcr@biox.com.

5. Manufacturing information

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22440 Ploufragan – France
RCS 417 876 299
Tél : +33(0)2 96 68 40 20
www.biox.com

III. Validation data

DNAs are extracted according to the extraction protocols presented in the appendix and PCR amplification is performed according to the user manual of ADIAVET™ PARATB REAL TIME kit.

A. Characterization of the PCR

1. Kit stability

Stability and performance of the ADIAVET™ PARATB REAL TIME kit have been assessed. The kit is stored at a temperature below -15 °C for 24 months.

The following table shows the results obtained for:

- A positive sample (PARATB CTL+) analysed pure with EPC-Amp.
- A positive sample near the limit of detection (LOD control), repeated 3 times.
- Two samples without PARATB including either the EPC-Amp or the EPC-Ext.
- reagent control (NTC).

The stability was evaluated with several batches of reagent. The same controls were used during the study. Ct values are shown for information only.

Stability study of three batch of reagent

		PARATB CTL+	LOD control	LOD control	LOD control	NTC	Sample with EPC-Amp	Sample with EPC-Ext
Expected results	Target (FAM)	28.4 (+/-1)	100 % positive			-	-	-
	IPC (HEX)	29.2 (+/-3)	29.2 (+/-3)			-	29.2 (+/-3)	29.2 (+/-3)
T=24 months	Batch 1	Target (FAM)	28.7	33.6	32.7	32.3	-	-
		IPC (HEX)	29.4	29.4	29.3	29.5	-	30.2
	Batch 2	Target (FAM)	28.8	36.1	35.6	34.5	-	-
		IPC (HEX)	29.5	29.4	29.2	29.2	-	28.2
	Batch 3	Target (FAM)	29.3	33.5	33.1	33.1	-	-
		IPC (HEX)	29.1	31.8	31.7	32.0	-	30.7

The Shelf life of ADIAVET™ PARATB REAL TIME is validated for 24 months when stored at a temperature below -15 °C.

2. Analytical specificity

The specificity of the PCR kit was assessed *in silico* and with experimental analysis from a panel of representative materials of the genetic diversity of *Mycobacterium avium* subsp. *paratuberculosis* and organisms preferentially found in the same ecological niche and/or close phylogenetically.

In silico analysis

An *in silico* specificity study has been carried out. All primers and probes used were verified with BLASTn software. No sequence other than *Mycobacterium avium* subsp. *paratuberculosis* showed a cross-reaction with these oligonucleotides.

Experimental analysis

Inclusivity and exclusivity tests were carried out to determine the ADIAVET™ PARATB REAL TIME kit specificity. Inclusivity was evaluated on 197 *Mycobacterium avium* subsp. *paratuberculosis* strains.

Inclusivity results

Strain	Origin	Number of strains tested	PCR Results
Reference strain	ANSES Maison Alfort	6	+
Field strain N° 61222	ANSES Maison Alfort	1	+
CIP 7912	INRA tour	1	+
ATCC 19698	INRA tour	1	+
MAP 54 milk cow 13-10-99	BFEL-Kiel Germany	1	+
MAP 52 milk cow 13-10-99	BFEL-Kiel Germany	1	+
MAP 65 faeces cow 25 -1-2000	BFEL-Kiel Germany	1	+
MAP 64 faeces cow 25 -1-2000	BFEL-Kiel Germany	1	+
MAP 63 faeces cow 13-10-99	BFEL-Kiel Germany	1	+
MAP 62 faeces cow 13-10-99	BFEL-Kiel Germany	1	+
MAP 61 faeces cow 13-10-99	BFEL-Kiel Germany	1	+
MAP 2966 OL faeces cow 23-1-97	BFEL-Kiel Germany	1	+
MAP 2318 OL faeces cow 23-1-97	BFEL-Kiel Germany	1	+
MAP 2276 OL faeces cow 23-1-97	BFEL-Kiel Germany	1	+
MAP 914 OL faeces cow 23-1-97	BFEL-Kiel Germany	1	+
MAP 2 OL faeces cow 23-1-97	BFEL-Kiel Germany	1	+
2008-01-6419-1	NVI- Oslo Norway	1	+
2007-04-20821-1	NVI- Oslo Norway	1	+
2007-04-20821-3	NVI- Oslo Norway	1	+
2007-04-20821	NVI- Oslo Norway	1	+
2007-04-19960-3	NVI- Oslo Norway	1	+
ATCC 19698	NVI- Oslo Norway	11	+
1984-P91 frozen as 84P	NVI- Oslo Norway	1	+
1985-P317 frozen as 90 P	NVI- Oslo Norway	1	+
1989-557 frozen as 164 P	NVI- Oslo Norway	1	+
1989-572 frozen as 167 P	NVI- Oslo Norway	1	+
1993-1278 frozen as 202 P	NVI- Oslo Norway	1	+
1998-04-417 frozen as 283 P	NVI- Oslo Norway	1	+
1998-01-978 frozen as 286 P	NVI- Oslo Norway	1	+
2009-01-2423-2	NVI- Oslo Norway	1	+
2009-01-2423-6	NVI- Oslo Norway	1	+

« + » detected

Strain	Origin	Number of strains tested	PCR Results
B4	QUB- Belfast-North Ireland UK	1	+
377 PS	QUB- Belfast-North Ireland UK	1	+
B2	QUB- Belfast-North Ireland UK	1	+
743 PSS	QUB- Belfast-North Ireland UK	1	+
796 PSS	QUB- Belfast-North Ireland UK	1	+
806 PSS	QUB- Belfast-North Ireland UK	1	+
519 PSS	QUB- Belfast-North Ireland UK	1	+
99 PW	QUB- Belfast-North Ireland UK	1	+
Niebuell	QUB- Belfast-North Ireland UK	1	+
L87 H ₂ O	QUB- Belfast-North Ireland UK	1	+
377 PW	QUB- Belfast-North Ireland UK	1	+
806 R	QUB- Belfast-North Ireland UK	1	+
NCTC 8578	QUB- Belfast-North Ireland UK	1	+
DVL 943	QUB- Belfast-North Ireland UK	1	+
637 PSS	QUB- Belfast-North Ireland UK	1	+
ATCC 43015	QUB- Belfast-North Ireland UK	1	+
806 PS	QUB- Belfast-North Ireland UK	1	+
L85 H ₂ O	QUB- Belfast-North Ireland UK	1	+
759 R	QUB- Belfast-North Ireland UK	1	+
ATCC 19698	QUB- Belfast-North Ireland UK	1	+
808 R	QUB- Belfast-North Ireland UK	1	+
102C Cork	QUB- Belfast-North Ireland UK	1	+
Field strain	LVD 35	49	+
Field strain	LDA 22	10	+
Field strain	LVD limoges	25	+
Field strain (bovine)	INRA tour	30	+
Field strain (ovine)	INRA tour	16	+
Field strain (rabbit)	INRA tour	1	+
Field strain (dear)	INRA tour	3	+
Field strain (caprine)	INRA tour	4	+
Field strain (humane)	INRA tour	1	+

Exclusivity was determined using samples from microorganisms found preferentially in the same ecologic niche and/or phylogenetically close to *Mycobacterium avium* subsp. *paratuberculosis*.

Exclusivity results

Mycobacterium	Number of strains tested	PCR results
<i>Mycobacterium bovis</i> (dont BCG 1173P2 pasteur)	10	-
<i>Mycobacterium celatum</i>	1	-
<i>Mycobacterium chelonae</i>	2	-
<i>Mycobacterium elephantis</i>	1	-
<i>Mycobacterium fortuitum</i>	1	-
<i>Mycobacterium genavense</i>	1	-
<i>Mycobacterium goodii</i>	2	-
<i>Mycobacterium intermedium</i>	1	-
<i>Mycobacterium intracellulare</i> (dont ATCC 13950)	2	-
<i>Mycobacterium kansasii</i>	2	-
<i>Mycobacterium Mageritense</i>	1	-
<i>Mycobacterium nonchromogenicum</i>	1	-
<i>Mycobacterium peregrinum</i>	1	-
<i>Mycobacterium porcinum</i>	1	-
<i>Mycobacterium scrofulaceum</i>	1	-
<i>Mycobacterium simiae</i>	1	-
<i>Mycobacterium smegmatis</i>	3	-
<i>Mycobacterium szulgai</i>	2	-
<i>Mycobacterium terrae</i>	1	-
<i>Mycobacterium thermoresistibile</i>	1	-
<i>Mycobacterium tuberculosis</i>	1	-
<i>Mycobacterium abscessus</i>	2	-
<i>Mycobacterium avium</i> subsp. <i>avium</i> (dont ATCC 25291)	11	-
<i>Mycobacterium avium</i> hominis	2	-
<i>Mycobacterium</i> no MAP	7	-
<i>Mycobacterium</i> sp. SP2333 (IS900-like)	3	-
<i>Mycobacterium</i> no MAP WA-1	1	+/-
<i>Mycobacterium</i> no MAP WA-2	1	-
IS1613	1	-
IS1626	1	-
Microorganismes	Number of strains tested	PCR results
<i>Actinomyces</i>	3	-
<i>Anaplasma phagocytophilum</i>	1	-
<i>Bordetella bronchiseptica</i>	2	-
<i>Borrelia burgdorferi</i>	1	-
Bovine fungus	4	-
Bovine herpesvirus type 1 (IBR)	1	-
<i>Brucella inactivé</i>	2	-
BTV type 1	1	-
BTV type 8	1	-
BVD	1	-
<i>Campylobacter</i>	10	-
<i>Chlamydia abortus</i>	2	-
<i>Clostridium perfringens</i>	1	-
<i>Coxiella burnetii</i>	1	-
<i>E. Coli</i>	1	-
<i>Enterobacter agglomerans</i>	2	-
<i>Escherichia coli</i>	5	-
<i>Haemophilus parasuis</i>	1	-
<i>Leptospira</i>	3	-
<i>Listeria</i>	12	-
<i>Neospora caninum</i>	1	-
<i>Proteus</i>	3	-
<i>Pseudomonas aeruginosa</i>	4	-
<i>Salmonella</i>	14	-
<i>Staphylococcus</i>	1	-
<i>Streptococcus</i>	5	-
<i>Actinomyces</i>	3	-

« - » undetected; « + » detected.

The ADIAVET™ PARATB REAL TIME kit does not cross-react with any samples tested other than *Mycobacterium avium* subsp. *paratuberculosis*. All *Mycobacterium avium* subsp. *paratuberculosis* field strains are detected. The kit is specific for *Mycobacterium avium* subsp. *paratuberculosis*.

3. Determination of the limit of detection of the PCR

The limit of detection PCR (LOD_{PCR}) is the smallest number of copies of target nucleic acid per unit of volume that can be detected in 95 % of cases. It was determined in accordance with the French AFNOR NF U47-600-2 standard.

The limit of detection was determined using a plasmid of known concentrations containing the Paratb target. According to the experimental model, 4 ranges of DNA from 2.5 to 20 copies/PCR (5 μ L) were tested during 3 independent experiments. Each point was tested 8 times.

Experimental plan

Number of sessions	Thermal cyclers used	Different operators	Dilutions tested	Replicates number per dilution
3	3	1	4	8

LOD_{PCR} determination results

	20 copies/PCR	10 copies/PCR	5 copies/PCR	2.5 copies/PCR
Total	24	24	24	24
# Positive	24	24	16	9
# Negative	0	0	8	15
% Detected	100	100	66.7	37.5

LOD_{PCR} corresponds to the target concentration producing at least 23 positive results out of 24 attempts. The LOD_{PCR} of the ADIAVET™ PARATB REAL TIME kit is 10 copies IS900/PCR.

4. Determination of the linear dynamic range and of the limit of quantification of the PCR

The linearity of a quantitative test and its capacity to generate results is proportional to the concentrations of the targets present in a given range and which can be modelled by a linear function.

Linear regression exists between the instrumental Ct response and the common logarithm of the target quantity (copies/ PCR). In order to establish the parameters of the regression line ($y = ax' + b$) and validate the linear module, an evaluation protocol must be complied with, and a data analysis carried out. This analysis has several objectives:

- Establish the curve equation.
- Determine efficiency.
- Determine and validate the linear regression's performances.
- Determine the limit of quantification PCR (LOQ_{PCR}).

Five separate sessions were carried out. For each session, a range of 6 levels of constant dilution was prepared and each point of the range analysed once.

Experimental plan

Number of sessions	Thermal cyclers used	Different operators	Dilutions tested	Replicates number per dilution
5	3	1	6	1

PCR efficiency calculation results

	Level A	Level B	Level C	Level D	Level E	Level F					
x_i	1 000 000	100 000	10 000	1 000	100	10					
$\log x'_i$	6.00	5.00	4.00	3.00	2.00	1.00	a	b	R^2	eff (%)	Validity efficacy
Session 1	19.1	22.6	26.0	29.1	33.0	35.9	-3.38	39.46	0.9991	97.58	yes
Session 2	20.5	24.0	27.5	31.0	33.9	37.8	-3.42	41.06	0.9990	96.24	yes
Session 3	18.2	21.6	24.8	28.5	31.7	35.9	-3.50	39.03	0.9986	93.12	yes
Session 4	18.0	21.4	25.0	28.4	32.1	35.9	-3.57	39.31	0.9995	90.44	yes
Session 5	17.5	21.2	24.6	27.7	30.7	33.8	-3.23	37.24	0.9984	103.98	yes

x_i = expected amount of target /PCR; $x'_i = \log_{10}(\text{expected amount of target /PCR})$

The efficiencies of the 5 ranges are validated since they are comprised between the required 75 and 125 % to support the precision (reliability and accuracy).

Performance criteria taken on to define the linearity domain and LOQ_{PCR}. The maximum discrepancy tolerated between the lower and higher x' value (quantity measured in log₁₀ of the number of copies) is fixed at 0.5 log₁₀ copies/PCR which corresponds to a critical value of the *deviate* ≤ 0.25 log₁₀ copies/PCR in relation to the expected level.

Deviante, linearity uncertainty and limit of quantification calculation

	Level A	Level B	Level C	Level D	Level E	Level F
Level x_i in Copies IS900/PCR	1 000 000	100 000	10 000	1 000	100	10
x'_i	6,00	5,00	4,00	3,00	2,00	1,00
$\wedge x'_{ij}$	6,01	4,98	4,00	3,05	1,90	1,05
	6,01	5,01	3,96	2,96	2,11	0,95
	5,95	4,99	4,06	3,01	2,09	0,90
	5,95	5,02	4,02	3,05	2,01	0,95
	6,10	4,97	3,90	2,94	2,04	1,05
<i>Deviante</i>	-0,01	0,02	0,00	-0,05	0,10	-0,05
	-0,01	-0,01	0,04	0,04	-0,11	0,05
	0,05	0,01	-0,06	-0,01	-0,09	0,10
	0,05	-0,02	-0,02	-0,05	-0,01	0,05
	-0,10	0,03	0,10	0,06	-0,04	-0,05
$\Sigma \wedge x'_{ij}$	30,03	24,97	19,93	15,02	10,15	4,91
Average $\wedge x'_i$	6,01	4,99	3,99	3,00	2,03	0,98
Average	-0,01	0,01	0,01	0,00	-0,03	0,02
S'_i	0,06	0,02	0,06	0,05	0,08	0,07
U_{LINi}	0,12	0,04	0,12	0,10	0,17	0,14
U_{LINi}^2	0,01	0,00	0,01	0,01	0,03	0,02
Validity	yes	yes	yes	yes	yes	yes
x_i calculated	1012490	98719	9698	1007	107	10
$U_{LIN}(\log_{10})$	0,12					
LOQ PCR in Copies IS900 /PCR	10					

x_i = expected amount of target /PCR; x'_i = log₁₀(expected amount of target /PCR); $\wedge x'_{ij}$ = log₁₀(measured amount of target /PCR); S'_i = standard deviation values $\wedge x'_{ij}$ per level; U_{LINi} = uncertainty of linearity extended per level; $U_{LIN}(\log_{10})$ = uncertainty of linearity extended linear range.

For each session, the absolute value of the *Deviante_i* is below the critical value regardless of the level. Hence, linearity is validated for the whole range tested from 10 to 1 000 000 copies IS900 /PCR in Paratb target.

For the defined calibration range, the uncertainty of linearity extended (U_{LIN}), taking into account the linearity deviate (lack of accuracy) and the precision (dispersion) of all the results, is 0.12 log₁₀ copies/PCR.

The LOQ_{PCR} corresponds to the last level of the range at which linearity is validated. The LOQ_{PCR} is therefore 10 copies IS900/PCR (5 µL).

5. Robustness

Robustness is a measure of an assay's capacity to remain unaffected by small, but deliberate, variations in method parameters, and provides an indication on its reliability during normal usage.

The major parameter which can alter the amplification performances is temperature. Temperature determines the efficiency of enzymatic activities involved in amplification and the good matching of oligonucleotides included in the mix.

To check temperature robustness, the usual 60 °C annealing temperature was changed by +/- 1 °C. For each temperature tested, DNA solutions close to the LOD_{PCR} were amplified in three replicates.

Robustness validation criteria is a Coefficient of Variation (CV) < 5 %.

Experimental plan

Number of sessions	Thermal cyclers used	Different operators	Dilutions tested	Replicates number / dilution
1	1	1	1	3

Detection of target according to the annealing temperature

	Ct PARATB (FAM) / Annealing temperature		
	Tm = 59 °C	Tm = 60 °C	Tm = 61 °C
Repeat 1	36.0	38.0	37.6
Repeat 2	39.0	35.6	38.0
Repeat 3	39.2	35.4	38.2
Average	37.4		
Standard	1.4		
CV %	4 %		

The critical second point is the volume of DNA added to the mix. The kit was developed and validated with 5 µL of DNA solution. The robustness was evaluated by changing by 10 % the volume of DNA added to the amplification reagent. For each volume tested, the DNA solution close to the LOD_{PCR} was amplified in three replicates.

Detection of target according to the volume of DNA solution added to the mix

	Ct PARATB (FAM) / DNA solution volume		
	4.5 µL	5 µL	5.5 µL
Repeat 1	35.3	38.0	34.2
Repeat 2	36.1	35.6	34.6
Repeat 3	36.7	35.3	33.6
Average	35.5		
Standard	1.3		
CV %	4 %		

The robustness of the ADIAVET™ PARATB REAL TIME kit is verified by varying the annealing temperature by +/- 1 °C and the volume of DNA solution added to the mix by +/- 10 %.

6. PCR repeatability and reproducibility

The repeatability corresponds to the narrowness of the agreement of the results during analyses carried out under the same conditions of measurement. The reproducibility corresponds to the narrowness of the agreement of the results during analyses carried out under varying conditions of measurement. The narrowness of the chord is determined by the calculation of the Coefficient of Variation.

Repeatability experimental plan

Number of sessions	Thermal cyclers used	Different operators	Dilutions tested	Replicates number / dilution
1	1	1	5	3

Determination of the repeatability of the PCR (Ct PARATB results)

	Copies IS900 / PCR				
	450 000	45 000	4 500	450	45
Repeat 1	21.6	25.3	28.4	32.1	37.2
Repeat 2	21.6	25.0	28.7	32.2	35.3
Repeat 3	21.6	25.0	27.9	32.1	35.4
Average	21.6	25.1	28.3	32.1	36.0
Standard dev	0.03	0.16	0.32	0.04	0.90
CV %	0.15	0.63	1.13	0.13	2.49

Reproducibility experimental plan

Number of sessions	Thermal cyclers used	Different operators	Dilutions tested	Replicates number / dilution
3	3	1	5	1

Determination of the reproducibility of the PCR (Ct PARATB results)

	Copies IS900 / PCR				
	450 000	45 000	4 500	450	45
Session 1	21.6	25.3	28.4	32.1	37.2
Session 2	21.9	24.8	28.0	32.3	36.4
Session 3	21.2	24.2	27.8	31.6	35.4
Average	21.55	24.78	28.03	31.99	36.33
Standard dev	0.29	0.46	0.25	0.29	0.75
CV %	1.34	1.86	0.89	0.90	2.07

Coefficients of variation of PCR repeatability and reproducibility in PARATB target are included in the acceptance criteria (repeatability CV < 5 % and reproducibility CV < 10 %).

B. Characterization of the analysis method from faeces

The analytical method takes into account all the steps (assay, sample preparation, extraction, gene amplification, including analysis of the result) necessary for the diagnosis.

Two complete methods have been characterized (ADIAMAG with ADIAFILTER and ADIAMAG with ADIAPREP).



For each method, the limit of detection, diagnostic sensitivity and specificity, limit of quantification, repeatability, reproducibility, and determination of relative PCR method fidelity with EPCQ Paratb Faeces (Bio-X Diagnostics: ref ADC04SQ01) have been characterized.

1. Limit of detection of the method

The limit of detection of the method (LOD_{METHOD}) determines the lowest quantity of the target substance per sample required to produce 100 % of positive result.

The detection limits of the method are characterized according to a methodology that consists in diluting a biological sample naturally contaminated by the biological agent of interest into a negative sample.

For this purpose, a naturally positive bovine faeces sample was titrated by digital PCR at $6.2 \cdot 10^6$ *Mycobacterium avium* subsp. *paratuberculosis* genome equivalent (GE) per gram. The suspension of this positive faeces was prepared and diluted in a suspension of negative faeces. The negative faeces were obtained from a free of PARATB cattle farm (> 18 months) and monitored for 3 years by PCR. Different levels of GE/g were prepared.

Each matrix of interest was spiked with a known quantity of *Mycobacterium avium* subsp. *paratuberculosis* strain and each spiking level was tested 4 times. The analytical method was repeated twice.

Experimental plan

Number of sessions	Different operators	Dilutions tested	Replicates number per dilution
2	1	3	4

Determination of the LOD_{METHOD} from faeces with the ADIAMAG with ADIAFILTER

Spiking level (GE/g)	Replicates session 1				Replicates session 2				% Detected
	1	2	3	4	1	2	3	4	
310	31.8	31.3	31.5	32.3	29.7	30.5	30.8	30.0	100 %
62	35.9	34.3	37.2	33.5	36.1	34.4	35.4	34.6	100 %
6.2	-	-	nd	nd	-	36.0	nd	nd	25 %

« - »: undetected; nd: not determined.

Determination of the LOD_{METHOD} from faeces with the ADIAMAG with ADIAPREP

Spiking level (GE/g)	Replicates session 1				Replicates session 2				% Detected
	1	2	3	4	1	2	3	4	
620	32.1	32.3	31.0	31.6	31.5	33.4	32.2	32.2	100 %
310	33.3	32.8	32.9	32.9	32.5	33.3	35.2	36.2	100 %
62	-	34.4	nd	nd	39.7	-	Nd	nd	50 %

« - »: undetected; nd: not determined.

The LOD_{METHOD} is the last level at which all 8 repeats produce a positive result. After DNA purification from faeces, the LOD_{METHOD} in *Mycobacterium avium subsp. paratuberculosis* target is 62 GE/g with ADIAMAG protocol with ADIAFILTER and 310 GE/g with ADIAMAG protocol with ADIAPREP.

2. Diagnostic sensitivity and specificity determination

Ninety-three samples with a status defined by another PCR method were extracted with ADIAMAG methods with ADIAFILTER or with ADIAPREP, and the nucleic acids were amplified with the ADIAVET™ PARATB REAL TIME kit.

Diagnostic sensitivity and specificity determination with protocol ADIAMAG with ADIAFILTER

		Status		
		Positive	Negative	Total
ADIAMAG with ADIAFILTER	Detected	67	2	69
	Not detected	1	23	24
	Total	68	25	93

Diagnostic sensitivity and specificity determination with protocol ADIAMAG with ADIAPREP

		Status		
		Positive	Negative	Total
ADIAMAG with ADIAPREP	Detected	64	1	65
	Not detected	4	24	28
	Total	68	25	93

For the ADIAMAG method with ADIAFILTER, the diagnostic sensitivity is 98.6 % [96 and 100 %] and the diagnostic specificity 92 % [86 and 98 %]. For the ADIAMAG method with ADIAPREP, the diagnostic sensitivity is 94 % [89 and 100 %] and the diagnostic specificity is 96 % [91 and 101 %].

Discordant results correspond to samples at the limit of detection and below the LOD_{METHOD} where the results are not reproducible.

3. Limit of quantification of the method

Characterization procedure for a quantitative method is based on the construction and interpretation of an accuracy profile. The accuracy profile is a graph of several tolerance intervals calculated at different concentration levels and previously defined acceptance limits. The interpretation is based on both the trueness and precision of measurements.

The limit of quantification of the method (LOQ_{METHOD}) was determined using *Mycobacterium avium subsp. paratuberculosis* faeces of known concentrations (6.2×10^6 GE/g).

Three independent sessions were carried out, where the matrix of interest was spiked with a known quantity of the *Mycobacterium avium subsp. paratuberculosis*. Four or five spiked levels were tested, and the experiments were realized in duplicates.

Experimental plan

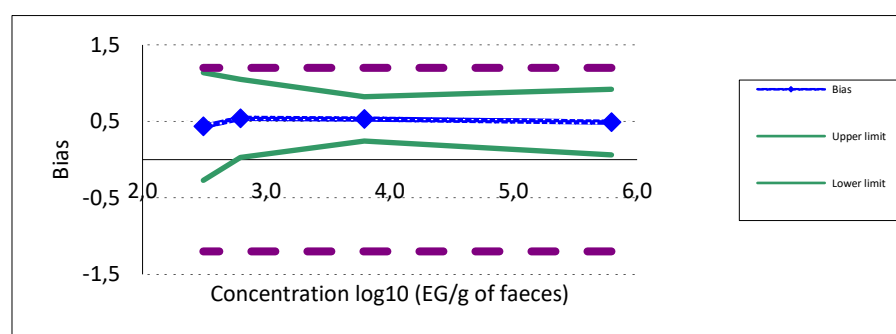
Number of sessions	Different operators	Dilutions tested	Replicates number per dilution
3	1	≥ 4	2

The PCR quantification range being valid (each point of the range is amplified; $R^2 > 0.9$; $75\% < \text{Efficiency} < 125\%$; points homogeneously spread), the samples can be analyzed. The Ct values obtained fit in the PCR quantification range.

The accuracy profile is constructed from the results obtained. In order to validate an infection level, the bias and the upper and lower limits must be included in the acceptance limits (set at 1 for ADIAPREP; 1.2 for ADIAFILTER).

Accuracy profiles on faeces with ADIAMAG with ADIAFILTER

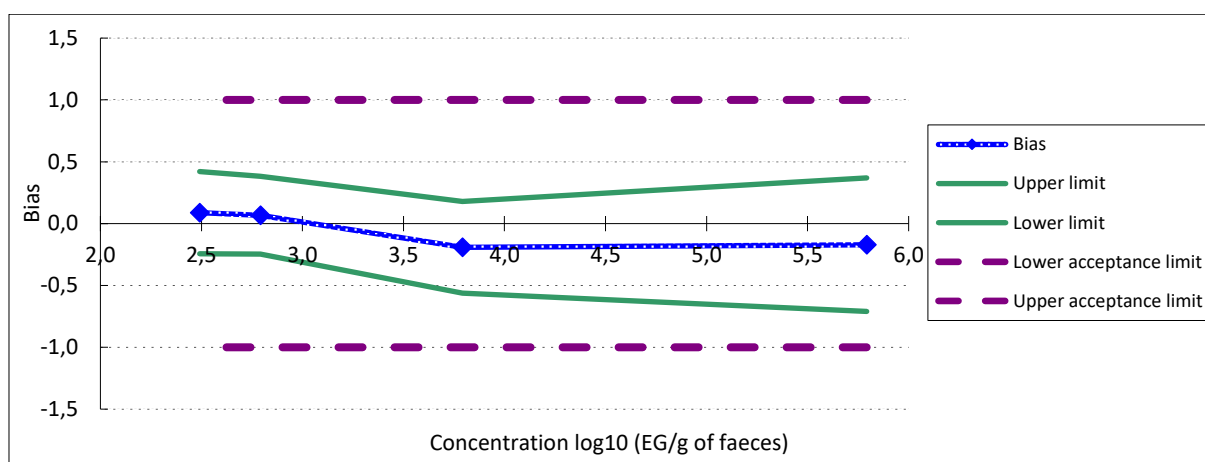
		Level A	Level B	Level C	Level D
	Average theoretical concentration in TCID ₅₀ /extraction	620 000	6 200	620	310
	Average theoretical concentration (in log ₁₀)	5,79	3,79	2,79	2,49
	Average concentration calculated (in log ₁₀)	6,28	4,33	3,33	2,92
Precision	Repeatability standard deviation (sr)	0,02	0,04	0,11	0,14
	Inter-assay standard deviation (sB)	0,21	0,14	0,23	0,32
	Precision standard deviation (sFI)	0,21	0,14	0,26	0,35
	Intermediate precision coefficient of variation	3,69	3,82	9,16	14,13
Trueness	Bias	0,49	0,53	0,54	0,43
Tolerance interval	Lower limit	0,06	0,24	0,03	-0,27
	Upper limit	0,92	0,82	1,05	1,14
	Lower acceptance limit	-1,20	-1,20	-1,20	-1,20
	Upper acceptance limit	1,20	1,20	1,20	1,20



The tolerance limits are within the acceptance limits in a range between 310 and 620 000 GE/g (2.49 to 5.78 log₁₀ GE/g), which corresponds to the quantification domain. The LOQ_{METHOD} is 310 GE/g (2.49 log₁₀ GE/g) of faeces with ADIAMAG with ADIAFILTER.

Accuracy profiles on faeces with ADIAMAG with ADIAPREP

		Level A	Level B	Level C	Level D
	Average theoretical concentration in TCID ₅₀ /extraction	620 000	6 200	620	310
	Average theoretical concentration (in log ₁₀)	5.79	3.79	2.79	2.49
	Average concentration calculated (in log ₁₀)	5.62	3.60	2.86	2.58
Precision	Repeatability standard deviation (sr)	0.18	0.19	0.11	0.11
	Inter-assay standard deviation (sB)	0.20	0.00	0.12	0.13
	Precision standard deviation (sFI)	0.27	0.19	0.16	0.17
	Intermediate precision coefficient of variation	4.66	4.89	5.62	6.67
Trueness	Bias	-0.17	-0.19	0.07	0.09
Tolerance interval	Lower limit	-0.71	-0.56	-0.25	-0.24
	Upper limit	0.37	0.18	0.38	0.42
	Lower acceptance limit	-1.00	-1.00	-1.00	-1.00
	Upper acceptance limit	1.00	1.00	1.00	1.00



The tolerance limits are within the acceptance limits in a range between 310 and 620 000 GE/g, (2.49 to 5.78 log₁₀ GE/g) which corresponds to the quantification domain. The LOQ_{METHOD} is 310 GE/g (2.49 log₁₀ GE/g) of faeces with ADIAMAG with ADIAPREP.

4. Repeatability and reproducibility method

The repeatability corresponds to the narrowness of the agreement of the results during analyses carried out under the same conditions of measurement. The reproducibility corresponds to the narrowness of the agreement of the results during analyses carried out under varying conditions of measurement. The narrowness of the chord is determined by the calculation of the Coefficient of Variation.

Reproducibility and repeatability have been determined using samples carrying 3 different PARATB loads (strong, moderate, or light positive). Each level was amplified twice on 2 independent runs according to the kit instructions.

Experimental plan

Number of sessions	Different operators	Dilutions tested	Replicates number per dilution
2	1	3	2

Repeatability and reproducibility of ADIAMAG with ADIAFILTER method (Quantitative analysis Log₁₀ GE/g)

		Level 1 (Strong)				Level 2 (Moderate)				Level 3 (Light)			
		session 1		session 2		session 1		session 2		session 1		session 2	
		N°1	N°2	N°1	N°2	N°1	N°2	N°1	N°2	N°1	N°2	N°1	N°2
	Log ₁₀ GE/g	5.83	5.87	5.52	5.55	3.89	3.92	3.95	3.85	3.03	2.95	2.95	2.93
Repeatability	Average	5.8		5.5		3.9		3.9		3.0		2.9	
	Standard deviation	0.03		0.02		0.02		0.07		0.1		0.0	
	Coefficient of variation %	0.5 %		0.4 %		0.5 %		1.7 %		1.9 %		0.6 %	
Reproducibility	Average	5.7				3.9				3.0			
	Standard deviation	0.20				0.04				0.04			
	Coefficient of variation %	3.2 %				1.1 %				1.5 %			

Repeatability and reproducibility of ADIAMAG with ADIAPREP method (Quantitative analysis Log₁₀ GE/g)

		Level 1 (Strong)				Level 2 (Moderate)				Level 3 (light)			
		session 2		session 1		session 2		session 2		session 1		session 2	
		N°1	N°2	N°1	N°2	N°1	N°2	N°1	N°2	N°1	N°2	N°1	N°2
	Log ₁₀ GE/g	5.96	6.23	5.81	5.54	3.83	3.81	3.83	3.82	2.89	2.92	3.19	3.01
Repeatability	Average	6.1		5.7		3.8		3.8		2,9		3,1	
	Standard deviation	0.19		0.19		0.01		0.01		0.02		0,1	
	Coefficient of variation %	3.1 %		3.4 %		0.3 %		0.3 %		0.7 %		4.1 %	
Reproducibility	Average	5.9				3.8				3.0			
	Standard deviation	0.30				0.01				0.1			
	Coefficient of variation %	4.9 %				0.3 %				4.5 %			

The coefficients of variation of the repeatability and reproducibility of the evaluated methods are included in the acceptance criteria (repeatability CV < 5 % and reproducibility CV < 10 %).

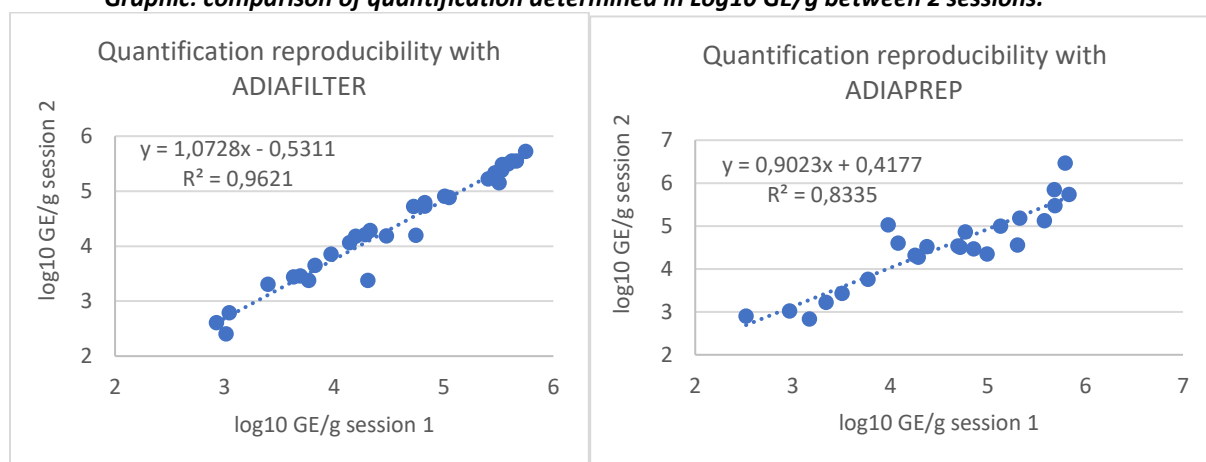
In a second step, a reproducibility study was performed on 51 positive samples (8 goats, 3 environments and 40 cattle) of different infection levels. Each sample was extracted on 2 independent sessions with each of the quantitative methods.

Experimental plan

Number of sessions	Different operators	Sample tested	Replicates number per sample
2	1	51	1

The quantification results are represented in the graphs below, where the results of session 1 are compared to session 2.

Graphic: comparison of quantification determined in Log10 GE/g between 2 sessions.



For both methods, the determination coefficients are consistent since R^2 is close to 1 (>0.83) in the quantification domain (between 2.49 and 5.78 $\log_{10}$ GE/g).

C. Evaluation from a mixture of 5 faeces

9 faeces identified as positive (# 1 to 9), and 2 faeces identified as negative (# 10 and 11) for MAP were extracted individually and in mixtures of 5 (1 positive + 4 negative or 5 negative). To make the mixtures of faeces by 5, each faeces was treated individually and then 2 mL of each supernatant was mixed and extracted. The results are presented in the table below:

Results obtained on individual faeces and in a mixture of 5

Sample #	Ct value target (FAM)	
	Individual results	Mixture of 5 results
1	19.0	22.7
2	23.0	27.6
3	24.4	25.4
4	24.9	28.0
5	26.8	35.5
6	32.1	35.9
7	34.7	38.7
8	36.0	-
9	37.2	-
10	-	-
11	-	-

« - » undetected; « + » detected.

All 9 positive samples are found positive individually and both negative samples are found negative for MAP. Out of the 9 individual positive samples, 2 with late Cts individually (36.0 and 37.2) were found negative for MAP in a mixture of 5, probably due to the dilution effect.

D. Evaluation from alpaca faeces

Twenty *Mycobacterium avium* subsp. *paratuberculosis* negative alpaca faeces were collected; six of these faeces were spiked with EPCQ (expected Ct 31+/-3 Ct). All were extracted with the ADIAPREP protocol in ADIAMAG magnetic beads and then amplified with the ADIAVET™ PARATB REAL TIME kit. The results obtained are presented in the table below.

PCR results obtained from DNA extracted from alpaca faeces.

		Ct Target (FAM)	Ct IPC (HEX)
Alpaca faeces spiked	E2284-2 spiked	30.6	23.2
	E2284-6 spiked	30.7	23.7
	E2284-11 spiked	31.1	22.9
	E2284-12 spiked	31.3	23.5
	E2284-16 spiked	31.4	22.8
	E2284-3 spiked	32.3	23.6
Alpaca faeces	E2284-1	-	34.1
	E2284-2	-	30.8
	E2284-3	-	30.2
	E2284-4	-	32.6
	E2284-5	-	31.0
	E2284-6	-	32.5
	E2284-7	-	28.2
	E2284-8	-	30.1
	E2284-9	-	28.7
	E2284-10	-	30.6
	E2284-11	-	31.1
	E2284-12	-	31.7
	E2284-13	-	31.0
	E2284-14	-	31.7
	E2284-15	-	31.0
	E2284-16	-	30.0
	E2284-18	-	30.0
	E2284-19	-	31.5
	E2284-20	-	30.8

« - » undetected

ADIAVET™ PARATB REAL TIME kit enables detection of *Mycobacterium avium* subsp. *paratuberculosis* in alpaca faeces.

E. Other validation data from milk and tissue

1. From milk

The following results come from of inter-laboratory tests published by Donaghy *et al.* in 2008. Negative bovine tank milk was infected with a titrated *Mycobacterium avium* subsp. *paratuberculosis* positive faeces. Six levels of infection were tested, between 0 and 600 MAP / mL of milk respectively. Each level of infection was extracted five times according to ADIAPURE™ PARATB MILK protocol. Each DNA solution was amplified with the ADIAVET™ PARATB REAL TIME kit. Results are presented in the table below:

Determination of LOD METHOD from milk

Spiking Levels GE/mL	Results
0	0 Positive / 5
0.6	4 Positives / 5
6	5 Positives / 5
60	5 Positives / 5
300	5 Positives / 5
600	5 Positives / 5

The LOD_{METHOD} from milk is estimated at 6 GE/mL, which is the last dilution level at which 5 repeated experiments test positive.

Additional data using the ADIAPURE™ PARATB MILK kit on experimental cheese and milk powder infections are available in the publication by Donaghy J.A., *et al.*, (2011).

2. From tissue

Sixteen samples of known *Mycobacterium avium* subsp. *paratuberculosis* status were collected. They were extracted using silica columns or ADIAMAG. Amplification was performed with the ADIAVET™ PARATB REAL TIME kit. The results obtained are presented in the table below.

PCR results obtained on DNA extracted from tissue

			ADIAMAG extraction		QIAamp® DNA extraction	
		Status	Ct target (FAM)	Ct IPC (HEX)	Ct target (FAM)	Ct IPC (HEX)
Ileum	1	Negative	-	+	-	+
Ileo cecal valve	2	Negative	-	+	-	+
Jejunum	3	Negative	-	+	-	+
Ileum	4	Negative	-	+	-	+
Ileo cecal valve	5	Negative	-	+	-	+
Jejunum	6	Negative	-	+	-	+
Ileo cecal valve	7	Negative	-	+	-	+
Duodenum	8	Negative	-	+	-	+
Ileum	9	Negative	-	+	-	+
Ileo cecal valve	10	Negative	-	+	-	+
Jejunum	11	Negative	-	+	-	+
Ganglion	12	Positive	25.4	+	23.5	+
Ileum	13	Positive	23.4	+	26.1	+
Ganglion	14	Positive	24.1	+	25.6	+
Ileum	15	Positive	21.2	+	24.8	+
Ileum	16	Negative	-	+	-	+

« - » undetected; « + » detected.

The ADIAVET™ PARATB REAL TIME kit allows the detection of *Mycobacterium avium* subsp. *paratuberculosis* in tissue.

IV. Conclusion

The characterization of ADIAVET™PARATB REAL TIME kit is defined according to the AFNOR NF U47-600 standard.

Performance criteria	Controls / acceptance criteria	Results obtained
Analytical specificity	Inclusivity	197 detected
	Exclusivity	- No cross-reaction with tested pathogens: 66 <i>Mycobacterium</i> other than <i>paratuberculosis</i> 86 other microorganisms
LOD _{PCR}	8 repetitions / 4 levels /3 sessions	10 copies IS900/PCR
LOQ _{PCR}	5 ranges of dilution/ 5 repetitions	10 copies IS900/PCR
Efficiency PCR	5 ranges of dilution / 5 repetitions Efficiency between 75 and 125 %	90 % < Efficiency < 106 % and R ² >0,997
PCR Repetability	3 repetitions / 5 levels /1 session CV <5 %	CV < 2.49 %
PCR Reproducibility	1 repetition / 5 levels /3 sessions CV <10 %	CV < 2,07 %
PCR Robustness	3 repetitions in limit of detection	100 % detection with: - Assay of 5 µL +/- 0,5 µL - Tm at 60 °C+/- 1 °C
Protocol ADIAMAG with ADIAFILTER from faeces		
LOD _{method faeces}	4 repetitions / 3 levels / 2 sessions	62 GE/gram of faeces
LOQ _{method faeces}	2 repetitions / 3 levels / 3 sessions	Quantification domain: 310 to 620 000 GE/gram of faeces LOQ method: 310 GE/gram of faeces
Diagnostic sensitivity and specificity	93 faeces of known status assessed by methods other than Bio-X Diagnostics	SE=99 % [96-100 %] SP=92 % [86-98 %]
Quantification repeatability/reproducibility	3 levels /2 repetitions / 2 sessions Repeatability<5 %; reproducibility<10 %	Repeatability CV % < 2 % Reproducibility CV < 3 %
Method quantification reproducibility	51 faeces / 1 repetition /2 sessions Expected: R ² > 80 %	R ² = 98.6 %
Protocol ADIAMAG with ADIAPREP from faeces		
LOD _{method faeces}	4 repetitions / 3 levels / 2 sessions	310 GE/g of faeces
LOQ _{method faeces}	2 repetitions / 3 levels / 3 sessions	Quantification domain: 310 to 620 000 GE/g LOQ method: 310 GE/gram of faeces
Diagnostic sensitivity and specificity	93 faeces known status assessed by methods other than Bio-X Diagnostics	SE=94 % [89-100 %] SP=96 % [91-101 %]
Quantification repeatability/reproducibility	3 level /2 repetitions / 2 sessions Repeatability<5 %; reproducibility<10 %	Repeatability CV % < 4.1 % Reproducibility CV < 5 %
Method quantification reproducibility	51 faeces /1 repetitions /2 sessions Expected R ² > 80 %	R ² = 83.4 %

V. Communication

2014

12th International colloquium on Paratuberculosis - Parma (Italy) - 22-26 June 2014:

- Evaluation and comparison of two methods for the extraction of *Mycobacterium avium* spp. *paratuberculosis* (MAP) specific DNA from feces for PCR. Jäckel S., Muluneh A., Blanchard B., Yilmaz M.
- Evaluation of diagnostic tests to classify cattle, in control plans, according to their levels of excretion of *Mycobacterium avium paratuberculosis*. Meens E., Rambaud T., Arnaud D.
- Detection limit of boot Swab and liquide manure sampling for herd level screening. Dnat K; Eisenberg T.; Hahn N.; Schlez K.; Kohler H.; Wolter W.; Lenz M.; Noll I.; Rene P.; Klaus F.; Zschock M.
- Facing interpretation difficulties of positive serology in presupposed paratuberculosis free herds/ longitudinal study and result comparison of commercial ELISAS. Comtet L., Brugel C., Olagon L., Pourquier P., Fouchas G.

2012

2nd Congress of the European Association of veterinary laboratory Diagnosticians (EAVLD). Kazimierz-Poland S1P05:

- Development and evaluation of a new and original extraction protocol to detect *Mycobacterium avium* subsp. *paratuberculosis* in bovine feces by real time PCR. Blanchard Béatrice, Versmisse Yann, Rouillard Tony.

Journée nationale de coordination de la paratuberculosis (30 mars) à Paris :

- MEENS Eric (GDS 76) ; Résultats préliminaires de l'étude de la paratuberculose bovine excréteurs de MAP en Normandie.

The 11th International Colloquium on Paratuberculosis.; Sydney, Australia. 2012:

- Johansen TB, Nilsen S, Lindheim D, Sølverød L, Olsen I, Djønne B, Detection of ***Mycobacterium avium* subsp. Paratuberculosis** in caprine faeces; validation using ADIAVET™ PARATB REAL TIME KIT.
- Geisbauer E, Liebhart M, Altmann M, Evaluation of an ELISA for the detection of antibodies against ***Mycobacterium avium* subsp. Paratuberculosis**.

VI. List of files available on demand

File 1: EIL FLI 2017 Diagnostic specificity versus culture.

File 2: Qualitative comparison of ADIAMAG methods with and without ADIAFILTER.

File 3: Field studies.

VII. Bibliography

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VIII. Appendix: Validated and evaluated extraction protocols

Technology	Extraction kit	References	Validated protocols	User Manual
Capture using magnetic beads	ADIAPURE™ PARATB MILK	Bio-X Diagnostics, 100 tests: ref. ADIADP04M1-100	Milk	NE04M1 www.biox.com
Magnetic beads	ADIAMAG™	Bio-X Diagnostics, 200 tests: ref. NADI003 800 tests: ref. NADI003-XL	Faeces or environmental specimen: ✓ with ADIAPREP ✓ with ADIAFILTER ✓ without pre-filtration Tissue	NEKF www.biox.com
Silica column	QIAamp® DNA Mini Kit	Qiagen, 50 tests: ref. 51304 250 tests: ref. 51306	Faeces or environmental specimen: ✓ with ADIAFILTER ✓ without pre-filtration Tissue	In the appendix of validation data
	NucleoSpin® Tissue	Macherey-Nagel, 50 tests: ref. 740952.50 250 tests: ref. 740952.250	Faeces or environmental specimen: ✓ with ADIAFILTER ✓ without pre-filtration	
	NucleoSpin® 96 Tissue	Macherey-Nagel, 2x96 tests: ref. 740741.2 4x96 tests: ref. 740741.4	Faeces or environmental specimen: ✓ with ADIAFILTER ✓ without pre-filtration	
			Bacterial culture (without purification)	

A. Extraction using ADIAPURE™ PARATB MILK kit

1. Mycobacteria capture

- Place **10 mL** of raw milk as fresh as possible in a 15 mL tube and add **1 mL** of L0 buffer. Mix by vortexing.
- Add **25 µL** of magnetic beads Mb suspension and gently mix for capture, for example on a rotating wheel at 10 rpm, for 30 minutes.
- Magnetize during 10 minutes to catch all beads on one side of the tube. Carefully discard the liquid phase with a pipette.
- Resuspend the beads pellet in **600 µL** of L1 Lysis buffer.

2. Grinding with the Mixer Mill (Werder)

- Put the whole mix into a microtube containing **300 mg** of glass beads.
- Disrupt at 30 Hz for 10 minutes using a mixer mill. Centrifuge 5 minutes at 7500 g.
- Place **300 µL** of the supernatant into a new 1.5 mL microtube and add **20 µL** of L2 buffer.
- Incubate 10 minutes at 70 °C then 15 minutes at +95 °C.

The obtained DNA extract can be stored 24 hours at +2/8 °C or several months at -20 °C +/- 5 °C.

3. Grinding with the Fast Prep (MP Biomedical)

- Put the whole mix into a Lysing Matrix B tube containing glass beads.
- Disrupt at 4 m/sec for 3 x 45 seconds. Centrifuge 5 minutes at 7500 g.
- Place **300 µL** of the supernatant into a new 1.5 mL microtube and add **20 µL** of L2 buffer.
- Incubate 10 minutes at +70 °C then 15 minutes at +95 °C.

The obtained DNA extract can be stored 24 hours at +2/8 °C or several months at -20 °C +/- 5 °C.

B. Extraction using ADIAMAG kit

1. From faeces

Note: Overnight rehydration at room temperature may be recommended for **goat and sheep faeces**.

Environmental samples (for example soil scrapings from different breeding areas, ...) must be treated as faecal samples. Dilute 3-10 g of sample in water.

Three ADIAMAG protocols are applicable:

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.
- Place **100 µL** of supernatant in Well 1 / Line 1 / Plate 1.
- Add **100 µL LB1 + 10 µL Proteinase K + 5 µL EPC-Ext.**
- Run the instrument.

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 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.
- Place **100 µL** of supernatant in Well 1 / Line 1 / Plate 1.
- Add **100 µL LB1 + 10 µL Proteinase K + 5 µL EPC-Ext.**
- Run the instrument.

Without pre-filtration

- 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.
- Place **100 µL** of supernatant in Well 1 / Line 1 / Plate 1.
- Add **100 µL LB1 + 10 µL Proteinase K + 5 µL EPC-Ext.**
- Run the instrument.

2. From tissues

- Place **20 mg** of **sample** in a microtube.
- Add **1 mL** of **sterile saline water**.
- Grind (e.g., with a grinder like Mixer Mill), add a metal bead (3 mm) + 300 mg of glass beads (Bio-X Diagnostics, 480 tests: ref ADIADPBA1-480) and grind 10 minutes at 30 Hz.
- Centrifuge 3 000 g/5 minutes.
- Transfer **100 µL** of **the supernatant** in Well 1 / Line 1 / Plate 1.
- Add **100 µL LB1 + 10 µL Proteinase K + 5 µL EPC-Ext.**
- Run the instrument.

3. Loading of the instrument

According to the KingFisher™ instrument used, the loading is conducted as follow:

- KingFisher™ mL: one tube comb per sample.
- KingFisher™ DUO: one 96-plate.
- KingFisher™ 96/Flex: one 96-plate per step.

Distribute the buffer in each well/line/plate as mentioned in the following table :

	Buffer to add	KingFisher™ mL	KingFisher™ DUO	KingFisher™ 96/Flex
Preparation of reagents	600 µL binding buffer (600 µL B2 Binding Buffer + 13 µL ADIAMAG beads)	Well 1 	Line B 	Plate 1 
	350 µL W3 Wash Buffer	Well 2 	Line C 	Plate 2 
	350 µL W4 Wash Buffer	Well 3 	Line D 	Plate 3 
	350 µL Ethanol 80 %	Well 4 	Line E 	Plate 4 
	100 µL E6 Elution Buffer	Well 5 	Line F 	Plate 5 
	Tip	On rail 	Line A 	Plate 6 
Loading of samples and run of the instrument	Transfer the sample prepared according to §V, in the Well 1/Line B/Plate 1. Turn on the instrument. Select the program. Place the comb or plates in the instrument. Press Start. After extraction, collect the purified nucleic acids in Well 5/Line F/Plate 5.			

4. Instrument programs

All instrument programs listed below are compatible with the PCR kit range ADIAVET™ or ADIALYO™.

	Short program (21 minutes)
KingFisher™ 96/Flex	KF96V4-2
KingFisher™ Duo	KFDOV4-1
KingFisher™ mL	KFMLV4

For program files request: support.pcr@bio.com

C. Extraction with silica column kits

1. 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 higher the faecal sample quantity, the better are sensitivity and reproducibility.

Environmental samples (stool scraping, wait areas ...) 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 proposed.

Sample preparation WITH ADIAFILTER :

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. Note: 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 1.5 mL-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	

* 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

Sample preparation without pre-filtration:

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	

* 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. 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 to obtain about 10 g +/-0.5 g of organ in a blender bag containing 40 mL of demineralized water.	Scrape the lining of the mucous membranes with a scalpel blade, to obtain about 5 g +/-0.5 g of organ in a blender bag containing 40 mL of demineralized water.

3. Purification using QIAamp® DNA Mini kit

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

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 (SIV-1) in a microtube. Add 180 µL of AL buffer + 20 µL of proteinase K + 5 µL of EPC-Ext . Mix.	Transfer 200 µL of the organ solution (SIV-5-B) in a microtube. Add 360 µL of ATL buffer 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 AL buffer , 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. <i>If the whole sample has not been loaded once, apply the residual volume onto the column and centrifuge 1 minute at 10 000 g.</i>	
1 st wash	Change the collection tube and add 500 µL of AW1 buffer to the column. Centrifuge 1 minute at 10 000 g.	
2 nd 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.

4. Purification 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 (SIV-1) in a microtube. Add 180 µL of B3 buffer + 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. <i>If the whole sample has not been loaded once, apply the residual volume onto the column and centrifuge 1 minute at 10 000 g.</i>
1 st wash	Change the collection tube and add 500 µL of BW buffer to the column. Centrifuge 1 minute at 10 000 g.
2 nd 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.

5. Purification using NucleoSpin® 96 Tissue with centrifugation

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

Note: 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 BQ1 buffer + 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 5 600 g. Note: in case of plugging, centrifuge 3 minutes at 5 600 g once again.
1 st wash	Add 500 µL of BW buffer . Centrifuge 2 minutes at 5 600 g.
2 nd wash	Add 700 µL of B5 buffer . Centrifuge 2 minutes at 5 600 g.
Plate dry step	Place the 96 NucleoSpin® Tissue Binding plate on the new 96 plate. Centrifuge 10 minutes at 5 600 g.
Elution in 96 wells plate	Place the 96 NucleoSpin® Tissue Binding Plate on the Rack of MN Tube Strips. Add 100 µL of BE buffer (previously heated at +56 °C). Important: Incubate 10 minutes . Centrifuge 2 minutes at 5 600 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.

D. Without purification – Bacterial Culture

With a sterile single use material, place 1 colony in 50 µL of sterile demineralised water. Boil 10 minutes. Centrifuge at 1 000 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 the same day. For long term storage, keep at <-15 °C.

Use EPC-Amp during the Amplification step.

IX. Revision history

Version	Date	Description
R045-D-en	01/2020	Old format
ADI045-PTUB_DV_(EN)_V01	06/2023	Format Change Addition of validation data: ▪ Efficiency verification and PCR QL determination ▪ Characterization of method fidelity with relative PCR ▪ Characterization of analytical methods using faeces ▪ Evaluation from alpaca faeces ▪ Appendix: Extraction protocols other than ADIAMAG