Instructions for Use

Actiphage® TB Blood Test

A rapid bacteriophage assay for the detection of mycobacteria in human blood samples.

For research use only

100 Reactions

Actiphage® Test Catalogue No. PBD-PC-001

PBD Biotech Ltd., Grosvenor House, 11 St Pauls Square, Birmingham UK, B3 1RB
www.pbdbio.com
Email: info@pbdbio.com

1 INTENDED USE

PBD Biotech's Actiphage® TB Blood Test is a bacteriophage assay developed for the detection of viable mycobacteria, belonging to the *Mycobacterium tuberculosis* Complex (MTBC), in blood and this procedure describes this application for human blood samples.

2 PRINCIPLE OF ACTIPHAGE® TB BLOOD TEST

M. tuberculosis is a facultative intracellular pathogen that thrives inside the host's macrophages and other cell types and thus, as part of the blood sample preparation protocol, white blood cells (WBCs) need to be isolated and lysed, releasing the mycobacteria and facilitating access for phage infection. The lysed WBCs and contents are then added to sample tubes together with a lysis reagent (Actiphage® Reagent). If viable mycobacterial cells are present, they will be infected by the mycobacteria-specific bacteriophage. The phage replicate intracellularly until new progeny phage are produced which perforate the bacterial wall allowing their release. Following filtration to remove any whole bacteria, genetic material released from the lysed mycobacteria is collected and purified. Finally, mycobacteria are identified using a MTBC specific qPCR.

3 PRODUCT CONTENTS

Each test contains sufficient materials for 100 reactions. The shelf life of each component is indicated on the respective label.

The test must be stored at 2 °C – 8 °C on receipt but is shipped between 10 °C and 24 °C (ambient temperature).

3.1 ITEMS PROVIDED

- 1 x Actiphage® Medium sachet (PBD-PC-011)
- 2 x Actiphage® Growth Supplement bottles (PBD-PC-012)
- 5 x lyophilised Actiphage® Reagent vials (PBD-PC-010)
- 1 x lyophilised Actiphage® BCG Control vial (PBD-PC-013)
- 110 x filter tubes (PBD-PN-002)
- 3 x HetaSep™ solution bottles (PBD-PN-001)
- 1 x Actiphage® TB PCR Test containing: (PBD-PC-009)
  - 4 x TB Lyophilized PCR Beads
  - 4 x PBD Resuspension Buffer x2

- 4 x Negative Control (NTC - water)
- 4 x Internal Amplification Control (IAC)

3.2 ITEMS REQUIRED BUT NOT PROVIDED

3.2.1 Reagents

- Sterile Phosphate Buffered Saline (PBS) for isolation of WBCs.
- Sterile distilled water for lysis of WBCs.
- DNA purification Kit
- Genomic DNA from *M. tuberculosis* to use as a PCR Positive Template Control (PTC) (Optional).

3.2.2 Accessories and Equipment

General Equipment:

- Microbiology laboratory consistent with local regulations for working with hazard group 3 pathogens.
- 'Pre-PCR Mix' and 'DNA preparation' dedicated laboratory areas
- Class II biosafety cabinet (or containment facilities compliant with local regulations for potentially infectious agents).
- Fridge (2 °C – 8 °C)
- Freezer (-20 °C – -18° C)
- 37 °C incubator
- 45 °C heat-block or water bath
- Autoclave capable of reaching 121 °C (15 psi).
- Pipettes capable of dispensing 1 mL, 200 µl, 100 µL, 20 µL, 10 µL and 1 µL.
- Electronic pipette
- Centrifuge rotor suitable for 15 mL or 50 mL conical tubes and swing out rotor capable of generating at least 120 x g.
  NOTE: Samples must only be centrifuged in equipment with biocontainment lids.
- Microcentrifuge suitable for 2 mL microcentrifuge tubes and capable of generating at least 16,000 x g.
- Vortex mixer
- Quantitative PCR (qPCR) Instrument
- Racks capable of holding 2 mL, 15 mL, and 50 mL tubes.
- 500 mL autoclavable clear bottles
- Howie style side fastening laboratory coat.

- Safety glasses

General Disposables:

- Autoclavable containers for discarding of waste (various sizes).
- Disposable gloves
- Sterile disposable 1.5 mL microcentrifuge tubes capable of withstanding at least 16,000 x g.
- Sterile filtered pipette tips for all listed pipette volumes
- 10 mL graduated pipettes
- Sterile 15 mL and 50 mL conical polypropylene screw cap centrifuge tubes
- qPCR plates and adhesive seals
- Suitable mycobactericidal disinfectant

3.3 STORAGE, RECONSTITUTION, SHELF-LIFE, AND RE-USE OF TEST COMPONENTS

Prior to use, the test components should be stored at 2 °C – 8 °C. Ensure the components to be used are removed from the fridge and brought to room temperature (RT; 18 °C – 25 °C) prior to starting the assay. The components should not be used beyond the expiry date shown on the respective label. To ensure optimal performance, it is important that all test components are reconstituted as directed below and that any unused test components are stored according to the following instructions.

3.3.1 Actiphage® Medium base

To prepare Actiphage® Medium base, in a suitable autoclavable container, mix the contents of one Actiphage® Medium base sachet (2.35 g) with 450 mL purified water. Gently mix to dissolve and autoclave at 121 °C for 10 minutes.

Once reconstituted and autoclaved, the Actiphage® Medium base can be stored for up to four weeks at RT before use. Actiphage® Medium base should not be used if there are any visual signs of contamination (turbidity).

3.3.2 Actiphage® Media Plus

Working aseptically, Actiphage® Media Plus is made by adding both bottles of Actiphage® Growth Supplement to 450 mL of Actiphage® Medium base. Ensure the Actiphage® Medium base is at RT before addition of Actiphage® Growth Supplement. Aliquot the Actiphage® Media Plus into sterile 50 mL conical tubes.

Actiphage® Media Plus is used to reconstitute the lyophilised Actiphage® Reagent (see section 3.3.3) and Actiphage® BCG Positive Control vials (see section 3.3.4).

Actiphage® Media Plus can be stored for up to four weeks at 2 °C – 8 °C before use, but no longer than the expiry date. Use a fresh aliquot of Actiphage® Media Plus each time the Actiphage® TB Blood test is carried out and discard any leftover media. Actiphage® Media Plus should not be used if there are any visual signs of contamination (turbidity).

3.3.3 Actiphage® Reagent

Working aseptically, Actiphage® Reagent is prepared by adding 2.3 mL of Actiphage® Media Plus to the lyophilised Actiphage® Reagent vial and mixing gently to re-hydrate the freeze-dried bacteriophage.

One vial of Actiphage® Reagent is sufficient for 20 reactions.

3.3.4 Actiphage® BCG Positive Control

Actiphage® BCG Positive Control contains approx. 10^4-10^5 cells of attenuated *M. bovis* BCG. To reconstitute the Actiphage® BCG Positive Control, add 1 mL of Actiphage® Media Plus to the lyophilised Actiphage® BCG Positive Control vial and mix gently to re-hydrate the freeze-dried mycobacteria. Incubate the reconstituted Actiphage® BCG Positive Control without shaking at 37°C overnight (approx. 18 hours) to allow BCG cells to recover prior to starting the assay.

One vial of Actiphage® BCG Positive Control is sufficient for running all reactions included in the Actiphage® TB Blood Test.

Once reconstituted, Actiphage® BCG Positive Control can be stored for up to two weeks at 2 °C – 8 °C before use, but no longer than the expiry date.

3.3.5 Filter tubes

Filter tubes supplied with the test are for single use only. The test contains sufficient filter tubes for 100 reactions, plus 10 extra tubes for preparation of five Actiphage® BCG Positive Control samples. Only tubes supplied with the test should be used for the assay procedure.

3.3.6 HetaSep™ solution

The Actiphage® TB Blood Test includes HetaSep™ solution for depletion of red blood cells (RBCs) from fresh blood samples and isolation of nucleated cells – WBCs.

The test includes enough HetaSep™ solution to process up to 100 samples of 3 mL of whole blood collected in sodium heparin anticoagulant.

HetaSep™ solution can be stored at RT or 2 °C – 8 °C and is stable until the expiry date. Ensure the solution warms up to RT and then invert the bottle to mix the contents prior to use.

3.3.7 Actiphage® TB PCR Test

Reconstitute the TB lyophilized PCR bead by adding 280 µL of PBD Resuspension Buffer x2.

One TB lyophilized PCR bead vial is sufficient for 25 reactions.

Resuspended PCR reagents are stable at 2 °C – 8 °C for three weeks, but no longer than the expiry date.

4 ACTIPHAGE® TB BLOOD TEST PROCEDURE

4.1 BLOOD SAMPLE PREPARATION

Blood samples (3 – 10 mL) MUST be collected using sodium heparin as the anticoagulant.

If blood samples are to be stored prior to testing, they **MUST be kept between 10 °C and 24 °C for no longer than 48 hours from collection**. **Refrigerated storage of whole blood samples prior to processing is NOT recommended**. Longer storage, or refrigeration of samples is likely to result in failure to achieve effective recovery of mycobacteria.

PBD Biotech recommend that blood samples are handled in a class II biosafety cabinet or in compliance with local regulations for handling potentially infectious agents.

PBD Biotech recommend using HetaSep™ solution (Stemcell Technologies) to aggregate erythrocytes and quickly separate nucleated cells from RBCs in whole blood samples.

4.1.1 WBC isolation using HetaSep™

1. Gently mix 1-part HetaSep™ solution to 5-parts of blood in a 15 mL centrifuge tube (e.g., 600 µL of HetaSep™ with 3 mL of blood).

2. Centrifuge sample at 90 x g for 2 minutes at RT with brake off.
3. Let sample stand on the bench for 10 minutes at RT to allow further sedimentation of the RBCs and improve recovery of the nucleated cells.
4. Harvest plasma layer **carefully** with a 1 mL pipette, avoiding the plasma interface, taking as little RBC carryover as possible to reduce any downstream inhibition. If necessary or if mixing has occurred leave sample standing for 30 minutes before harvesting plasma.
5. Place harvested plasma into 5 mL (at least 4-fold volume) of sterile PBS in a clean 15 mL centrifuge tube.
6. Centrifuge sample at 120 x g for 10 minutes at RT with the brake off.
7. Carefully remove the supernatant.

NOTE: Safe stopping point. If sample is to be stored overnight, continue to step 8. If processing the sample on the same day, go to step 11.

8. Resuspend pellet in 1 mL of Actiphage® Media Plus by pipetting gently until a uniform suspension is formed and leave overnight at RT.
9. After overnight incubation, centrifuge sample at 120 x g for 10 minutes at RT.
10. Carefully remove the supernatant.
11. Resuspend the pellet in 1 mL of sterile distilled water and mix well by pipetting.
12. Place into to a clean 1.5 mL microcentrifuge tube.
13. Incubate sample for 20 minutes at RT to lyse the WBCs.
14. Centrifuge sample at 13,000 x g for 3 minutes at RT to collect released mycobacteria.
15. Remove the supernatant.
16. Resuspend in 100 µL of Actiphage® reagent by pipetting gently until a uniform suspension is formed.
17. Sample processing will carry on in section 4.4.

NOTE: Detailed directions for use of HetaSep™ solution can be found on the Product Information Sheet available at https://www.stemcell.com

4.2 ACTIPHAGE® BCG POSITIVE CONTROL PREPARATION

Extracellular DNA (eDNA) is an important component of the bacterial extracellular matrix and plays a fundamental structural role by promoting attachment to surfaces and bacterial

aggregation. Preparation of Actiphage® BCG Positive Control is required to remove the background eDNA that could mask the mycobacterial DNA released due to the action of the bacteriophage lytic cycle.

1. Pre-heat a heating block or water bath to 45 ^{o}C.
2. After overnight incubation at 37^{o}C, collect the reconstituted Actiphage® BCG Positive Control vial and mix well by gently pipetting up and down five times.
3. Transfer 100 µL of Actiphage® BCG Positive Control into a 1.5 mL microcentrifuge tube and incubate at 45 ^{o}C for 30 minutes.
4. Prepare a 1/10 dilution by adding 900 µL of Actiphage® Media Plus into the heated Actiphage® BCG Positive Control and mix well by gently pipetting up and down five times.
5. Add 100 µL of diluted Actiphage® BCG Positive Control onto the filter column of the **two** separate labelled filter tubes.

   NOTE: Prepare Actiphage® BCG Positive Control in duplicate. These two control samples will be used in section 4.3 to prepare the Actiphage® Reagent Positive Control (+ phage) and Actiphage® Media Plus positive control (- phage) to evaluate the phage activity.
6. Centrifuge at 400 x g in a microcentrifuge for 5 minutes at RT. If there is residual liquid left on the filter column, repeat this step.
7. Dispose of the filtrate containing the background eDNA of the Actiphage® BCG Positive Control.
8. Wash BCG cells by adding 500 µL of Actiphage® Media Plus to the filter tube column.
9. Centrifuge at 400 x g for 5 minutes at RT. If there is residual liquid left on the filter column, repeat this step.
10. Discard the filtrate.
11. Repeat wash steps 8 -10 twice.
12. Collect two new filter tubes and remove the unused filter columns (discard). Place the filter columns containing BCG cells into the new filter tubes.

4.3 PREPARATION OF ACTIPHAGE® TEST CONTROLS

Negative and positive phage activity controls must be included on every occasion the Actiphage® TB Blood test is carried out. The positive phage activity controls evaluate the performance of the phage lytic ability against the Actiphage® BCG Positive Control.

Prepare each control as described below and label accordingly.

- **Actiphage® Reagent Positive Control**: add 100 µL of reconstituted Actiphage® Reagent to the filter tube column containing the Actiphage® BCG Positive Control previously prepared (see section 4.2). Resuspend BCG cells by pipetting up and down five times.
- **Actiphage® Media Plus Positive Control**: add 100 µL of Actiphage® Media Plus to the filter tube column containing the Actiphage® BCG Positive Control previously prepared (see section 4.2). Resuspend BCG cells by pipetting up and down five times.
- **Actiphage® Reagent Negative Control**: add 100 µL of reconstituted Actiphage® Reagent to a new filter tube.
- **Optional: Actiphage® Media Plus Negative Control**: add 100 µL of Actiphage® Media Plus to a new filter tube.

Table 1. Preparation of Actiphage® test controls

Control	Actiphage BCG Positive Control	Actiphage Reagent
Actiphage® Reagent Positive Control	✓	✓
Actiphage® Media Plus Positive Control	✓	X
Actiphage® Reagent Negative Control	X	✓
Actiphage® Media Plus Negative Control (Optional)	X	X

4.4 ACTIPHAGE® TEST

1. Transfer the sample resuspended in Actiphage® Reagent (section 4.1.1) to pre-labelled filter tube.
2. Treat the positive and negative controls prepared in section 4.3 in the same way as the test samples described below.
3. Incubate the sample and Actiphage® controls for 3 hours 30 minutes at 37 °C.

4. Centrifuge the sample at 13,000 x g in a microcentrifuge for 3 minutes at RT. If all the liquid from the upper chamber does not pass through the filter tube column, this is usually due to cell debris blocking the filter.

   NOTE: In this case, the filter column can be rotated through 180° and the centrifugation step repeated.

5. After centrifugation, remove the filter column of the filter tube and collect the flow-through in the collection tube which contains the released mycobacterial genomic DNA.

6. The DNA can either be used directly for molecular analysis or can be stored frozen at -20 °C until required.

NOTE: Safe stopping point.

The output from the Actiphage® test is mycobacterial genomic DNA, which can be used as a target for molecular diagnostic analyses. Each DNA sample should be sufficient for at least two nucleic acid amplification tests (5 µl each test).

The flow-through must be cleaned and concentrated to a final volume of 12 µL eluted in nuclease free water, using an appropriate DNA purification system. We recommend using the Monarch® PCR & DNA Cleanup Kit (New England BioLabs) or the DNA Clean-up and Concentrator (Zymo Research) following the manufacturer's instructions.

4.5 MOLECULAR ANALYSIS

The Actiphage® TB PCR Test contains a lyophilized PCR bead allowing the detection in the same reaction well of:

- Mycobacteria belonging to MTBC by targeting the insertion sequence IS6110 with hydrolysis probe FAM labelling, with a limit of detection of one genomic copy.
- An Exogenous internal amplification control IAC DNA, with hydrolysis probe Cy5 labelling, to confirm absence of PCR inhibitors.

This PCR test has been validated on a Cobas® z 480 Analyser 96-well thermal block cycler unit (Roche Diagnostics) with halogen lamps using FAM (465-510) and Cy5 (610-670) detection channels. Software version: LCS480 1.5.1.62 SP3 - UDF 2.1.0.26.

Our PCR lyophilized bead contains Uracil N-glycosylase (UNG) to eliminate potential post-PCR carryover contamination associated with routine molecular testing.

1. Establish qPCR plate setup defining each sample position including Actiphage® positive and negative controls and NTC.

2. In a 'Pre-PCR Mix' dedicated area, prepare a Master Mix by mixing 10 µL of resuspended PCR bead solution and 5 µL of IAC per reaction.
3. Transfer 15 µL of Master Mix into each well of interest.
4. In the 'DNA Preparation' dedicated area, add 5 µL of each DNA sample in the corresponding well.
5. Add 5 µL of nuclease-free water in the NTC well.
6. Seal the plate and spin down before placing it in the thermocycler.
7. Run the following qPCR program settings.

Table 2. qPCR program settings.

Cycles	Time	Temperature
UNG*	2 minutes	25 °C
1 cycle start	5 minutes	95 °C
40 cycles	10 seconds	95 °C
	1 minute	60 °C

*Activation step for Uracil N-glycosylate

NOTE: An optional PCR Negative Control (NC) can be included by mixing 15 µL of resuspended PCR bead and 5 µL of NTC (water). An optional PCR Positive Template Control (PTC) can be included by adding 15 µL of Master Mix and 5 µL of quantitative genomic DNA from *M. tuberculosis*.

5 ACCEPTANCE CRITERIA

5.1 PCR Controls

The threshold cycle (Ct or Cp) is the intersection between the amplification curve and the threshold line. It allows the relative measurement of the concentration of the target in the PCR reaction when a reference sample is analysed in the same run. To analyse and interpret the signals obtained by qPCR, the threshold must be set up. On the Cobas z 480 Analyser the FAM and Cy5 channels are individually analysed using "AbsQuant/Fit points" method using the default settings (Background 2-6, Auto baseline and Auto threshold calling).

The qPCR results are validated if the controls (NTC, IAC, NC, PTC) present valid results (see table 3).

To validate the assay, the PCR negative control sample (NTC and NC) must not exhibit a qPCR signal in the relevant target channel (FAM).

IAC is a reaction-independent control designed to co-amplify the genomic region other than our target, using the same reagents (except probe), same annealing temperature and reaction conditions.

To validate the assay from an IAC perspective, any samples with IAC (Cy5) Ct values ≥ 35 should be considered invalid and repeated.

Table 3. Acceptance criteria for PCR controls.

Control	Ct (FAM)	Ct (Cy5)	Interpretation
NTC - Negative Template control	≥ 40 <40		Plate Pass Plate Fail
IAC - Internal Amplification Control		< 35 ≥ 35	Sample Pass Sample Fail
NC - Negative Control (Optional)	≥ 40 <40		Plate Pass Plate Fail
PTC - Positive Amplification Control (Optional)	≥ 40 <40		Plate Fail Plate Pass

5.2 Actiphage® Controls

To confirm phage-mediated lysis of target cells by the Actiphage® Reagent, a positive PCR result should be generated with the Actiphage® positive control samples, and no target DNA should be detected in the negative control samples.

To validate the assay, a shift of at least +2 ΔCt (FAM channel) must be observed between the Actiphage® Reagent Positive Control and the Actiphage® Media Plus Positive Control.

Full acceptance criteria for the Actiphage® assay controls are summarized in the table below.

Table 4. Acceptance criteria for Actiphage® controls table.

Control	Ct (FAM)	ΔCt (FAM)	Interpretation
Actiphage® Reagent Positive Control (A)		B - A ≥ 2	Plate Pass
Actiphage® Media Plus Positive Control (B)		B - A ≤ 2	Plate Fail
Actiphage® Reagent Negative Control	≥ 40		Plate Pass
	< 40		Plate Fail
Actiphage® Media Plus Negative Control	≥ 40		Plate Pass
	< 40		Plate Fail

5.3 Test samples

Once the PCR and Actiphage controls are validated as described in section 5.1 and 5.2, sample results can be interpretated according to the table below.

Table 5. Interpretation of test samples results.

Targets		Interpretation
MTBC (FAM)	**IAC (Cy5)**	
Negative	Positive	Negative
Positive	Positive	Positive
Positive	Negative	Invalid - Repeat analysis Presence of inhibitors, competition with the main target or omission of IAC addition.
Negative	Negative	Invalid - Repeat analysis DNA omission, degradation or not in contact with Master Mix. Presence of inhibitors or omission of IAC addition.

Negative: FAM Ct ≥ 40 or Cy5 Ct ≥ 35.
Positive: FAM Ct < 40 or Cy5 Ct < 35.

6 LIMITATIONS

Detection of mycobacteria by the Actiphage® TB Blood Test is dependent on the quantity and quality of the sample collected, its storage prior to processing, and the number of live organisms present.

The Actiphage® TB Blood Test is dependent on the level of mycobacteria infected WBCs present in the circulating blood and therefore, in the early stages of infection where very low-level bacteraemia may occur it is possible to achieve a false negative result.

A false positive result will only occur if there is cross-contamination of the sample with mycobacterial DNA from the target organism.

7 PRECAUTIONS

7.1 SAFETY PRECAUTIONS

- Suitable safety precautions must be always used when handling clinical samples, including working in an appropriate biosafety cabinet, and using personal protective equipment. Local guidelines must be followed when working with pathogenic material, and for disposal of pathogenic biological waste, and in the event of laboratory accidents.
- The procedure involves working with *M. tuberculosis* and this work must be carried out in a facility suitable for handling this organism.
- All chemicals and biological materials are potentially hazardous. Samples are potentially infectious and must be treated as biohazardous materials.
- Any remaining sample material that generates a positive result using the Actiphage® TB Blood Test should be treated as containing viable cells of the organism detected.

7.2 TECHNICAL PRECAUTIONS

- For use only by personnel competent in aseptic technique and experienced in working with pathogenic *Mycobacterium spp*.
- If the Actiphage® TB Blood Test or any included component appear damaged upon arrival, do not use and contact PBD Biotech.
- Test reagents must not be used beyond the expiry date printed on the label.
- Do not substitute the reagents in the test or mix between batches or lot numbers, as this may affect performance.

- Do not freeze any test component or reconstituted reagent.
- The reagents are provided at defined working concentrations. Assay performance may be compromised if reagents are modified or not used or stored under recommended conditions, as detailed in section 3.3.
- Reconstitute sufficient reagents required to perform the test. Do not return excess reagents to bottles after use.
- All reagents must be brought to RT before use.
- Use aseptic techniques throughout the reagent preparation and assay procedure. All pipettes, pipette tips, glassware and plastic-ware used must be sterile.
- Care must be taken to treat all samples and control reactions in the same manner, according to sections 4.4 and 4.5 detailed in these instructions for use. Omission of, or deviation from, any step could lead to inaccurate results.
- 'Pre-PCR Mix' and 'DNA preparation' need to be carried out in two physically separated areas to reduce the chance of contamination.
- Use individual, dedicated pipets for setting up reaction mixes and adding positive control reagents.
- Use extreme caution to prevent contamination of the controls and reaction mix reagents with the synthetic materials that are contained in the IAC reagent.
- Do not use qPCR reaction volumes (reaction mix plus sample) of less than 20 µL.
- Do not open the PCR plate after the qPCR run has finished.
- Refer to the Cobas® z 480 Analyser user manual (available at www.diagnostics.roche.com) for additional qPCR instrument warnings, precautions, and procedures.
- Caution must be observed to ensure correct sample testing with emphasis on wrong sample entry, loading error, and pipetting.
- To prevent cross-contamination between samples leading to false positive results, it is mandatory to use disposable materials for single use and to avoid direct contact between samples.
- Disposal of test components and any consumables that have been in contact with these reagents, should be carried out in accordance with local regulations for the disposal of microbiological waste.
- Any instrumentation, or connecting parts, including (but not limited to) qPCR

instruments, centrifuges and pipettes must be calibrated, maintained, and cleaned as directed by the manufacturer.

- The operator should seek assistance directly from PBD Biotech if they do not understand anything in these instructions or have problems running the test.

8 MATERIAL SAFETY DATA SHEETS

A material safety data sheet (MSDS) for this test is available from PBD Biotech, upon request.