

Amplified Mycobacterium Tuberculosis Direct Test

For In Vitro Diagnostic Use (50 Test Kit)
(bioMérieux ref. 39006 / Hologic Cat. No. 301001)

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Intended Use

The Hologic Amplified MTD (Mycobacterium Tuberculosis Direct) Test is a target-amplified nucleic acid probe test for the *in vitro* diagnostic detection of *Mycobacterium tuberculosis* complex rRNA in sediments prepared from sputum (induced or expectorated), bronchial specimens (e.g., bronchoalveolar lavages or bronchial aspirates) or tracheal aspirates.

Warnings

The efficacy of this test has not been demonstrated for the direct detection of *M. tuberculosis* rRNA using other clinical specimens (e.g., blood, urine, or stool). Performance of the MTD test has not been established for sediments processed in a different fashion than described, or stored for different time periods or temperatures than specified in this Package Insert.

Positive sediments must be cultured to determine if *Mycobacterium* other than tuberculosis complex (MOTT) are present in addition to *M. tuberculosis* complex and to perform antimycobacterial susceptibility testing. Culture for AFB should also be performed to determine which subspecies of the *M. tuberculosis* complex (e.g., *M. bovis*) is present.

Although specimens from pediatric patients, HIV positive patients, and patients with MOTT infections were tested during the clinical evaluations, total numbers were insufficient to definitely conclude that there were no statistical performance differences in these specific patient populations.

The MTD test has not been studied for use with specimens from patients being treated with antituberculous agents to determine bacteriologic cure or to monitor response to such therapy.

Specimens that are grossly bloody should not be tested with the MTD test.

Precautions

- A. For In Vitro Diagnostic Use.
- B. The MTD test is specific for, but does not differentiate among, members of the *M. tuberculosis* complex, i.e., *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. microti* and *M. canetti* (13). *M. celatum* and *M. terrae*-like organisms will cross-react if present at concentrations higher than 30 colony forming units (CFU) per test. However, *M. celatum* and *M. terrae*-like organisms are rare clinical isolates.
- C. A negative test does not exclude the possibility of isolating an *M. tuberculosis* complex organism from the specimen. Test results may be affected by specimen collection and transport, specimen sampling variability, laboratory procedural errors, sample misidentification, and transcriptional errors.
- D. Use only for the detection of members of the *M. tuberculosis* complex using sediments prepared following the NALC-NaOH or NaOH procedures recommended by the Centers for Disease Control (CDC)⁷. This test may only be used with concentrated sediments prepared from sputum (induced or expectorated), tracheal aspirates, or bronchial specimens (e.g., bronchoalveolar lavages or bronchial aspirates). Care must be taken when resuspending the sediment in phosphate buffer to ensure that the phosphate concentration is 67 mM⁷.

- E. Avoid contact of Detection Reagents I and II with skin, eyes, and mucous membranes. Wash with water if contact with these reagents occurs. If spills of these reagents occur, dilute with water before wiping dry.
- F. Use universal precautions when performing this test⁴. Preparation of digested and decontaminated *sediments*, and MTD procedures should be done using Biosafety Level 2 practices⁵.
- G. Use only supplied or specified disposable laboratory ware.
- H. Work surfaces, pipettors, and equipment must be decontaminated of RNA amplicon with a 1:1 dilution of household bleach. Work surface may be wiped with water after 15 minutes to remove the bleach.
- I. Positive displacement pipettors or air displacement pipettors with hydrophobically plugged tips must be used when performing this test. When transferring lysate from Lysing Tube to Amplification Tube, extended length hydrophobically plugged tips must be used. A separate disposable tip must be used for each reaction tube. Waving of a pipette tip containing specimen over the rack of tubes should be avoided. Spent pipette tips must be immediately discarded in an appropriate biosafety waste container.
- J. When using repeat pipettors for reagent addition, after the lysate has been added to the tube, avoid touching the tube with the pipette tip in order to minimize the chance of carryover from one tube to another. The reagent stream should be aimed against the interior wall of the test tube to prevent splashing. Careful pipetting is important to avoid carryover contamination.
- K. Separate pipettors must be used for steps that precede amplification and those that follow amplification.
- L. After reading reaction tubes in the luminometer, decontaminate and carefully dispose of them as described in the TEST PROCEDURE and PROCEDURAL NOTES in order to avoid contamination of the laboratory environment with amplicon.
- M. Sealing cards or snap caps should be disposed of in an appropriate biosafety waste container immediately after removing them from reaction tubes. Fresh sealing cards or snap caps should always be used to avoid cross-contamination. These materials should NEVER be reused from a previous step. Sealing cards should be firmly fixed to the top of all reaction tubes.
- N. Do not cover water bath during incubations, especially when using snap caps. (Condensation from the cover may be a possible source of contamination.)
- O. Adequate vortexing after addition of Selection Reagent is necessary to achieve accurate assay results.

- P. A segregated area for the Hybridization Protection Assay (HPA) step is recommended to minimize amplicon contamination in the assay. This dedicated area should be separated from the specimen and reagent preparation and amplification areas.
- Q. To help prevent lab areas from becoming contaminated with amplicon, the laboratory area should be arranged with a unidirectional workflow. For example, proceed from specimen and reagent preparation to amplification and then to HPA areas. Specimens, equipment, and reagents should not be returned to the area where a previous step was performed. Also, personnel should not move back into previous work areas without proper anti-contamination safeguards. It is strongly recommended that the biosafety cabinet used for specimen processing not be used for performing the MTD test.

Summary and Explanation of the Test

The MTD test utilizes Transcription-Mediated Amplification (TMA) and the Hybridization Protection Assay (HPA²) to qualitatively detect *M. tuberculosis* complex ribosomal ribonucleic acid (rRNA). The MTD test will detect rRNA from both cultivable and non-cultivable organisms. Organisms of the *M. tuberculosis* complex include *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. microti* and *M. canetti* (12,13). The MTD test will detect all organisms within the *M. tuberculosis* complex. However, *M. microti* infects only animals, *M. bovis* is uncommonly transmitted from infected animals to humans, and *M. africanum* causes pulmonary disease in humans in tropical Africa¹². *M. tuberculosis* is by far the most common member of the complex that is responsible for human disease worldwide. The CDC has recently reported a rise in the incidence of tuberculosis associated with AIDS, foreign-born cases, and increased transmission in higher risk populations^{6,9}. There has also been a rise in the number of *M. tuberculosis* strains that are resistant to one or more than one antituberculous drugs¹¹. The public health implications of these facts are considerable.

Conventional culture methodologies can detect tuberculosis growth as early as 1 week, but may take up to 8 weeks^{7,10}. Comparatively, the MTD test provides detection of *M. tuberculosis* complex rRNA within 2.5 to 3.5 hours after beginning the test procedure. Thus, while the MTD test cannot ascertain drug susceptibility, it can result in rapid and reliable detection of *M. tuberculosis*. This could lead to more appropriate use of isolation facilities, more appropriate initiation of therapy, and earlier detection and containment of infected contacts³.

Principles of the Procedure

The MTD test is a two-part test in which amplification and detection take place in a single tube. Initially, nucleic acids are released from mycobacterial cells by sonication. Heat is used to denature the nucleic acids and disrupt the secondary structure of the rRNA. The Transcription-Mediated Amplification (TMA) method, using a constant 42°C temperature, then amplifies a specific mycobacterial rRNA target by transcription of DNA intermediates, resulting in multiple copies of mycobacterial RNA amplicon.

M. tuberculosis complex-specific sequences are then detected in the RNA amplicon using the Hybridization Protection Assay (HPA) method². The Mycobacterium Tuberculosis Hybridization Reagent contains a single-stranded DNA probe with a chemiluminescent label. This probe is complementary to *M. tuberculosis* complex-specific sequences. When stable RNA:DNA hybrids are formed between the probe and the specific sequences, hybridized probe is selected and measured in a Leader® luminometer.

Reagents (50 Test Kit)

Note: For information on any hazard and precautionary statements that may be associated with reagents, refer to the Safety Data Sheet Library at www.hologic.com/sds.

Reagents for the MTD test are provided as follows:

MYCOBACTERIUM TUBERCULOSIS AMPLIFICATION TRAY

Reagent Name	Volume
Mycobacterium Specimen Dilution Buffer (SDB) <i>Tris buffered solution containing < 3% detergent</i>	1 x 2.5 mL
Mycobacterium Tuberculosis Amplification Reagent (A) <i>Nucleic acids lyophilized in tris buffered solution containing 5% bulking agent</i>	1 x 3 mL (when reconstituted)
Mycobacterium Amplification Buffer (AB) <i>Aqueous solution containing preservatives</i>	1 x 3 mL
Mycobacterium Oil Reagent (O) <i>Silicone Oil</i>	1 x 10 mL
Mycobacterium Enzyme Reagent (E) <i>Reverse transcriptase and RNA polymerase lyophilized in HEPES buffered solution containing < 10% bulking agent and ≥ 15 mM N-acetyl-L-cysteine</i>	1 x 1.5 mL (when reconstituted)
Mycobacterium Enzyme Dilution Buffer (EDB) <i>Tris buffered solution containing a surfactant and glycerol</i>	1 x 1.5 mL

MYCOBACTERIUM TUBERCULOSIS HYBRIDIZATION TRAY

Reagent Name	Volume
Mycobacterium Tuberculosis Hybridization Reagent (H) <i>< 100 ng/vial non-infectious DNA probe with a chemiluminescent label lyophilized in succinate buffered solution containing bulking agent and detergent</i>	1 x 6 mL (when reconstituted)
Mycobacterium Hybridization Buffer (HB) <i>Succinate buffered solution containing < 4% detergent</i>	1 x 6 mL
Mycobacterium Selection Reagent (S) <i>Borate buffered solution containing surfactant</i>	1 x 15 mL
Mycobacterium Lysing Tubes (LT) <i>Glass Beads, Bulking Agent</i>	2 x 25 Tubes

Storage and Handling Requirements

A. The following liquid or unreconstituted components must be stored at 2°C to 8°C and are stable until the expiration date indicated:

- Mycobacterium Specimen Dilution Buffer (SDB)
- Mycobacterium Tuberculosis Amplification Reagent (A)
- Mycobacterium Amplification Buffer (AB)
- Mycobacterium Enzyme Reagent (E)
- Mycobacterium Enzyme Dilution Buffer (EDB)
- Mycobacterium Tuberculosis Hybridization Reagent (H)

The reconstituted Mycobacterium Tuberculosis Amplification Reagent (A) is stable for 2 months at 2°C to 8°C. The Mycobacterium Tuberculosis Hybridization Reagent (H) and the Mycobacterium Enzyme Reagent (E) are stable for 1 month at 2°C to 8°C after reconstitution, or for 2 months at -20°C or colder if stored as frozen aliquots on the day of initial reconstitution. Frozen aliquots must be used on the day thawed. Frost-free freezers must not be used.

B. The following kit components are stable when stored at 2°C to 25°C until the expiration date indicated.

- Mycobacterium Oil Reagent (O)
- Mycobacterium Hybridization Buffer (HB)
- Mycobacterium Selection Reagent (S)
- Mycobacterium Lysing Tubes (LT)

Specimen Collection, Storage, Transport, and Processing

Specimen Collection and Storage

Specimens must be collected in sterile plastic containers, and stored at 2°C to 8°C until transported or processed. Specimens included in the clinical trial were stored for no more than 4 days (generally less than 24 hours) prior to processing.

Transport

Transport specimens to the laboratory as soon as possible according to appropriate regulations.

Processing (Decontamination and Concentration)

Specimens that are grossly bloody should not be tested with the MTD test. The MTD test is designed to detect rRNA from members of the *M. tuberculosis* complex using sediments prepared from generally accepted current adaptations of the NALC-NaOH or NaOH decontamination protocols described by the CDC using 1% to 1.5% NaOH for 15 to 20 minutes and centrifugation at $\geq 3,000 \times g^7$.

Processed Sediment Storage

Sediments may be stored at 2°C to 8°C for up to 3 days prior to testing. Sediments may also be stored frozen at -20°C or -70°C for up to 6 months. Frost-free freezers must not be used.

Materials

A. Materials Provided

MYCOBACTERIUM TUBERCULOSIS AMPLIFICATION TRAY

(bioMérieux Ref. No. 39006 / Hologic Cat. No. 301001)

Component	50 Tests
Mycobacterium Specimen Dilution Buffer (SDB)	1 x 2.5 mL
Mycobacterium Tuberculosis Amplification Reagent (A)	1 x 3 mL (when reconstituted)
Mycobacterium Amplification Buffer (AB)	1 x 3 mL
Mycobacterium Oil Reagent (O)	1 x 10 mL
Mycobacterium Enzyme Reagent (E)	1 x 1.5 mL (when reconstituted)
Mycobacterium Enzyme Dilution Buffer (EDB)	1 x 1.5 mL

MYCOBACTERIUM TUBERCULOSIS HYBRIDIZATION TRAY

Component	50 Tests
Mycobacterium Tuberculosis Hybridization Reagent (H)	1 x 6 mL (when reconstituted)
Mycobacterium Hybridization Buffer (HB)	1 x 6 mL
Mycobacterium Selection Reagent (S)	1 x 15 mL
Mycobacterium Lysing Tubes (LT)	2 x 25 Tubes
Sealing Cards	1 package

B. Materials Required But Not Provided

- Micropipettes capable of dispensing 25 µL, 50 µL, 100 µL, 200 µL, 300 µL, and 450 µL
- Vortex mixer
- Sterile water (filtered or autoclaved)
- Culture tubes
- Sterile 3 mm glass beads
- Screw cap microcentrifuge tubes
- Amplification Positive Cell Controls (e.g., *M. tuberculosis*, ATCC 25177 or ATCC 27294)
- Amplification Negative Cell Controls (e.g., *M. gordonae*, ATCC 14470, or *M. terrae*, ATCC 15755)
- Household bleach (5.25% hypochlorite solution)
- Plastic-backed laboratory bench covers
- 1000 µL pipette tips with hydrophobic plugs

C. Additional Materials Available From Your Hologic Distributor:

	<u>Cat. No.</u>
Leader 50i Luminometer (<i>bioMérieux ref. 39400</i>)	103100i
Ultrasonic Water Bath (<i>bioMérieux ref. 39409</i>)	901104
Detection Reagent Kit (<i>bioMérieux ref. 39300</i>)	201791
MTD Amplification Controls (<i>bioMérieux ref. 39223</i>)	301043F
Dry Heat Bath ^A (42° ± 1°C, 60° ± 1°C, and 95° ± 5°C) (<i>bioMérieux ref. 39405, 39406, 39407</i>)	105524, 105524F, 105524J
Ultrasonic Water Bath Rack (<i>bioMérieux ref. 39313</i>)	104027
Test tube racks (<i>bioMérieux ref. 39311</i>)	104769
Extended length pipette tips with hydrophobic plugs (1250 µL) (<i>bioMérieux ref. 39315</i>)	104316
Tubes, polypropylene, 12 x 75 mm (<i>bioMérieux ref. 39308</i>)	102440
Snap top polypropylene caps for 12 x 75 mm tubes (<i>bioMérieux ref. 39320</i>)	400713

Test Procedure

Controls

Cells used for the Amplification Positive Cell Control should be a member of the *M. tuberculosis* complex, such as avirulent H37Ra (ATCC 25177) or virulent H37Rv (ATCC 27294). Cells used for the Negative Cell Control should be MOTT, such as *M. goodii* (ATCC 14470) or *M. terrae* (ATCC 15755). Controls must be prepared prior to sample testing.

Cell controls must contain 25 - 150 CFU per 50 µL so that a final concentration of 1 - 10 CFU per assay is achieved. This concentration should be verified by culture. These cell controls will be utilized in the preparation of Specimen Processing Controls. (See Sample Preparation.)

1. Suggested Preparation of Cell Controls
 - a. Place 3 to 5 sterile 3 mm glass beads in a clean culture tube.
 - b. Add 1- 2 mL sterile water. Add several 1 µL loopfuls of growth from the appropriate culture. Cap the tube and vortex repeatedly at high speed.
 - c. Allow the suspension to settle for 15 minutes.
 - d. Transfer the supernatant to a clean culture tube. Adjust turbidity to the equivalent of a #1 McFarland nephelometer standard using a McFarland reference.
 - e. Make a 1:100 dilution of the suspension by placing 100 µL of the #1 McFarland suspension into 10 mL sterile water. Cap and vortex. This is Dilution 1.
 - f. Make a second 1:100 dilution by placing 100 µL of Dilution 1 into 10 mL sterile water. Cap and vortex. This is Dilution 2. This dilution should contain approximately 25 - 150 CFU per 50 µL.

Aliquotting and Storage of Cell Controls

- a. The dilutions must be aliquotted into clean 1.5 mL screw cap microcentrifuge tubes as single use aliquots (500 µL) and stored frozen at -20°C for 6 months or -70°C for one year. Frost-free freezers must not be used.

Testing of the recommended *M. tuberculosis* cell positive control will monitor for substantial reagent failure only. The positive control is designed to monitor effect of reagents used during processing for interference from excess NaOH and phosphate buffer. Procedural variations in timing or temperatures that may affect efficiency of amplification or adequacy of selection time may not be detected using the recommended cell controls. Additional controls may be tested according to guidelines or special requirements.

2. Specimen Inhibition Controls

If the MTD test is negative and the physician strongly suspects that the patient has tuberculosis, it is possible to check for specimen inhibition using the following procedure:

- a. Place 50 µL Specimen Dilution Buffer into 2 Mycobacterium Lysing Tubes (LT) (seeded and unseeded).
- b. Add 50 µL Amplification Positive Cell Control and 450 µL sediment to 1 tube (seeded). Add 450 µL sediment to the second tube (unseeded). Proceed with the testing as usual.

Interpretation

If the RLU value of the seeded tube is $\geq 30,000$, then the sample does not inhibit amplification and there apparently was no target available for amplification. If the RLU value of the seeded tube is below 30,000, then the sample inhibits amplification and another sample should be evaluated. If the repeat testing of the unseeded specimen is positive, the MTD test result may be reported as positive. The most likely explanation for this type of result is random sampling variability; i.e., that the first aliquot did not contain target for amplification, while the second aliquot did. The RLU value of the unseeded tube may be either positive or negative because the aliquot of sediment may or may not contain *M. tuberculosis* rRNA.

3. Laboratory Contamination Monitoring Control

To monitor for laboratory contamination with amplicon or *M. tuberculosis* cells, the following procedure can be performed:

- a. Place 1 mL of sterile water in a clean tube. Wet a sterile polyester or dacron swab with sterile water.
- b. Wipe area of bench or equipment to be tested.
- c. Place the swab in the water and swirl gently. Remove the swab while expressing it along the side of the tube. Discard the swab into a container containing a 1:1 dilution of household bleach.
- d. Add 25 µL of the water containing the expressed swab material into an Amplification Tube containing 50 µL Amplification Reagent and 200 µL Oil Reagent.
- e. Follow the TEST PROCEDURE for amplification and detection.

Interpretation

If the results are $\geq 30,000$ RLU, the surface is contaminated and should be decontaminated by treating with a bleach as recommended in TEST PROCEDURE, Equipment Preparation. If contamination of the water bath is suspected, 25 µL of water bath water can be amplified as described for the expressed swab material providing no antimicrobials are used in the water bath.

Equipment Preparation

1. For optimal transfer of sonic energy in an ultrasonic water bath, water must be thoroughly degassed according to the following procedure prior to each run:
 - a. Add enough ambient temperature tap water to fill the ultrasonic water bath to within 1/2 inch of the top of the tank.
 - b. Run the ultrasonic water bath for 15 minutes to thoroughly degas the water.
2. Adjust 1 dry heat bath to 95°C, 1 dry heat bath or water bath to 60°C and another dry heat bath or water bath to 42° ± 1°C.
3. Wipe down work surfaces, equipment, and pipettors with a 1:1 dilution of household bleach prior to starting. Bleach must be in contact with the surface for at least 15 minutes. Work surfaces may be wiped with water to remove the bleach. Cover the surface on which the test will be performed with plastic-backed laboratory bench covers.
4. Prepare the Leader Luminometer for operation. Make sure there are sufficient volumes of Detection Reagents I and II to complete the tests and ensure that the reagent lines are primed. Refer to the Instrument Operator's Manual for further instructions on loading of Detection Reagents. (Detection Reagents are sold separately).

Reagent Preparation

Reconstitute the vial (50 tests) of lyophilized *Mycobacterium Tuberculosis* Amplification Reagent (A) with 3.0 mL *Mycobacterium* Amplification Buffer (AB). Vortex until the solution is mixed. Let

reconstituted reagent sit at room temperature until clear. The reconstituted Mycobacterium Tuberculosis Amplification Reagent may be stored at 2°C to 8°C for 2 months. The reconstituted Mycobacterium Tuberculosis Amplification Reagent should be allowed to come to room temperature before use.

Sample Preparation

1. Label a sufficient number of Mycobacterium Lysing Tubes (LT) to test the samples and 1 each of either the Amplification Cell Positive and Negative or the Specimen Processing Positive and Negative Controls. Remove and retain the caps.
2. Pipette 50 µL Mycobacterium Specimen Dilution Buffer (SDB) into all Mycobacterium Lysing Tubes (LT). Follow the directions in A or B below for controls and in C for specimens.
 - a. Specimen Processing Controls:

For each control, add 1 mL of the NALC/NaOH solution and 3 mL of phosphate buffer used to process sputum with 1 mL of sterile water to a sample processing tube.

 - i. Vortex to mix.
 - ii. Transfer 450 µL of the NALC/NaOH/phosphate buffer solution and 50 µL Cell Control dilution to the correspondingly labeled Mycobacterium Lysing Tube (LT).
 - b. If Amplification Cell Controls are being used, transfer 450 µL Amplification Cell Control from its container to the correspondingly labeled Mycobacterium Lysing Tube (LT).
 - c. Specimen: Transfer 450 µL decontaminated well-vortexed specimen from its container to the correspondingly labeled Mycobacterium Lysing Tube (LT).
3. Recap the Mycobacterium Lysing Tubes (LT) after addition of each sample.
4. Vortex 3 seconds.

Sample Lysis

1. Push the Mycobacterium Lysing Tubes (LT) through the ultrasonic water bath rack so that the reaction mixture in the bottom of the tube is submerged but the caps are above water. Place ultrasonic water bath rack on ultrasonic water bath. **DO NOT ALLOW THE TUBES TO TOUCH THE BOTTOM OR SIDES OF THE ULTRASONIC WATER BATH.**
2. Place rack on ultrasonic water bath for 15 minutes but no more than 20 minutes. Samples and controls that have been sonicated are now referred to as "lysates." **DO NOT VORTEX LYSATES.**

Amplification

1. Label amplification tubes (12 x 75 mm polypropylene tubes) near the top of the tube with numbers that correspond to those used on the Mycobacterium Lysing Tubes (LT). Also label amplification tubes for each of the Amplification Cell Positive and Negative Controls. If RNA controls are being used, label amplification tubes to correspond to Negative and Positive Controls.

2. Add 50 µL *reconstituted* Mycobacterium Tuberculosis Amplification Reagent to the bottom of each amplification tube using a repeat pipettor. Add 200 µL Mycobacterium Oil Reagent (O) to each amplification tube using a repeat pipettor.
3. DO NOT VORTEX LYSATE. Transfer 25 µL lysate to the bottom of the appropriately labeled amplification tube using a separate extended length hydrophobically plugged pipette tip for each transfer. Remaining lysate may be stored at 2°C to 8°C for up to 7 days or stored frozen at -20°C or below for up to 1 month. Frost-free freezers must not be used. If additional testing is to be performed on patient specimen lysates, bring stored lysate to room temperature. DO NOT VORTEX LYSATE.
4. Incubate the tubes at 95°C for 15 minutes, but no more than 20 minutes, in the dry heat bath.
5. Prepare the enzyme mix by adding 1.5 mL Mycobacterium Enzyme Dilution Buffer (EDB) to the lyophilized Mycobacterium Enzyme Reagent (E). Swirl to mix. Do not vortex. The *reconstituted* Enzyme Reagent is stable for 1 month at 2°C to 8°C or for 2 months at -20°C or colder if stored as frozen aliquots^B on the day of initial reconstitution. If testing with frozen enzyme aliquots, first bring frozen aliquot to room temperature; do not thaw aliquots at elevated temperatures. To mix aliquot after thawing, gently aspirate and expel mixture with repeat pipettor before adding to amplification tubes.
6. Transfer the tubes to the 42° ± 1°C dry heat bath or water bath and allow them to cool for 5 minutes. DO NOT ALLOW THE TUBES TO COOL AT ROOM TEMPERATURE. DO NOT COVER THE WATER BATH.
7. Add 25 µL enzyme mix to each amplification tube using a repeat pipettor while tubes are at 42° ± 1°C. Shake to mix. Incubate at 42°C for 30 minutes, but no more than 60 minutes. Sealing cards or snap caps should be used during this incubation step. DO NOT COVER THE WATER BATH.
8. Tubes may be covered and placed at 2°C to 8°C for up to 2 hours or at -20°C overnight after the 30 minute incubation. If stored at -20°C overnight, tubes must be completely thawed at room temperature or no greater than 60°C prior to the Hybridization step. If held overnight, snap caps rather than sealing cards should be used.

Hybridization

1. Reconstitute lyophilized Mycobacterium Tuberculosis Hybridization Reagent (H) with 6 mL Mycobacterium Hybridization Buffer (HB). Mycobacterium Tuberculosis Hybridization Reagent (H) and Mycobacterium Hybridization Buffer (HB) must be at room temperature prior to reconstitution. If Mycobacterium Hybridization Buffer (HB) has been refrigerated, warm at 60°C while swirling gently to ensure that all the components are in solution. Vortex until the solution is clear (this could take up to 1 minute) to ensure that all components are in solution. The *reconstituted* Hybridization Reagent is stable for 1 month at 2°C to 8°C or for two months at -20°C or colder if stored as frozen aliquots^C on the day of initial reconstitution. If the *reconstituted* Hybridization Reagent has been refrigerated or frozen, warm at 60°C while swirling gently to ensure that all components are in solution.
2. Add 100 µL *reconstituted* Hybridization Reagent to each tube using a repeat pipettor. Cover tubes with sealing cards or snap caps. Vortex 3 times for **at least** 1 full second each

time^D at medium speed. To achieve proper mixing in reaction tube(s), maintain tubes in an upright position and allow reaction mixture to reach upper half of tube wall throughout vortexing procedure. (To avoid possible contamination do not allow reaction mixture to come in contact with sealing cards or caps.) After adequate vortexing, the reaction mixture should be uniformly yellow.

3. Incubate at 60°C for 15 minutes, but no more than 20 minutes, in a dry heat bath or water bath.

Selection

1. Mycobacterium Selection Reagent (S) must be at room temperature prior to starting the test. Remove tubes from the 60°C water bath or dry heat bath and add 300 µL Mycobacterium Selection Reagent (S) using a repeat pipettor. Cover tubes with sealing cards or snap caps. Vortex 3 times for **at least 1** full second each time^D at medium speed. To achieve proper mixing in reaction tube(s), maintain tubes in an upright position and allow reaction mixture to reach upper half of tube wall throughout vortexing procedure. (To avoid possible contamination do not allow reaction mixture to come in contact with sealing cards or caps.) After adequate vortexing the reaction mixture should be uniformly pink.
2. Incubate tubes at 60°C for 15 minutes, but no more than 16 minutes, in a dry heat bath or water bath.
3. Remove tubes from the water bath or dry heat bath. Cool tubes at room temperature for at least 5 minutes but not more than 1 hour. Remove sealing cards or caps just prior to detection.

Detection

1. Select the appropriate protocol from the menu of the luminometer software. Use a 2 second read time.
2. Using a damp tissue or lint-free paper towel, wipe each tube to ensure that no residue is present on the outside of the tube, and insert the tube into the luminometer according to the instrument directions. Tubes must be read within 1 hour of Selection Step 3.
3. When the analysis is complete, remove the tube(s) from the luminometer.
4. After reading the reaction tubes, carefully fill them to the top with a 1:9 dilution of household bleach using a squirt bottle. Allow tubes to sit with solution for a minimum of 1 hour before discarding. This will help to prevent contamination of the laboratory environment with amplicon.
5. Test tube racks, including racks used for the specimens and tests, should be decontaminated by complete immersion in a 1:1 dilution of household bleach for a minimum of 15 minutes. The bleach should then be rinsed off with water and the racks should be wiped dry or allowed to air dry.
6. Decontaminate the laboratory surfaces and equipment using a 1:1 dilution of household bleach.

Repeat Testing

1. If additional testing is to be performed on patient specimen lysates, bring prepared lysate to room temperature. DO NOT VORTEX LYSATE.
2. Follow TEST PROCEDURE protocol as outlined, beginning with the Amplification step.

Procedural Notes

A. Reagents

1. Enzyme Reagent should not be held at room temperature for more than 15 minutes after it is reconstituted.
2. Mycobacterium Hybridization Buffer (HB) may precipitate. Warming and mixing the Mycobacterium Hybridization Buffer (HB) or *reconstituted* Hybridization Reagent at 60°C will dissolve the precipitate.

B. Temperature

1. The amplification, hybridization and selection reactions are temperature dependent; ensure that the water bath or dry heat bath is maintained within the specified temperature range.
2. The tubes must be cooled at 42°C for 5 minutes before addition of enzyme mix for optimal amplification performance.
3. The temperature is critical for the amplification ($42^{\circ} \pm 1^{\circ}\text{C}$).

C. Time

It is critical that the time limits specified in the TEST PROCEDURE be followed.

D. Water Bath

1. The level of water in the water bath should be maintained to ensure that the entire liquid reagent volume in the reaction tubes is submerged, but the level must not be so high that water gets into the tubes.
2. During the Amplification step, water bath covers should not be used to ensure that condensate cannot drip into or onto the tubes.

E. Vortexing

It is important to have a homogeneous mixture during the Hybridization and Selection steps, specifically after the addition of the *reconstituted* Mycobacterium Tuberculosis Hybridization Reagent (H) (the reaction mixture will be uniformly yellow) and Mycobacterium Selection Reagent (S) (the reaction mixture will be uniformly pink).

Vortexing is the manipulation of a solution to produce a uniform suspension. When the reagents are placed into a test tube and supplied with an external energy source, a rapid rotation of the solution about the tube axis is produced. The output of this rapid rotation is the

production of a uniform test suspension. During vortexing the tubes should be held in an upright, vertical position and supported by the top portion of the tube to ensure that adequate vortexing is achieved. If an adequate vortexing motion is achieved, the suspension rotates in a circular motion at a rate capable of lifting the solution to a height within the upper half of the tube. During the Hybridization and Selection steps, this manipulation is applied sequentially 3 times and the vortex maintained for **at least** 1 full second each time.

Test Interpretation

The specimen result when tested using the Amplified Mycobacterium Tuberculosis Direct (MTD) Test is interpreted based on an initial negative result (< 30,000 RLU), an initial positive result (≥ 500,000 RLU), or an initial equivocal result (30,000 to 499,999 RLU). The MTD test should be repeated from the reserved lysate when an initial test result is equivocal. A repeat result from the lysate ≥ 30,000 is considered positive.

A. Quality Control Results and Acceptability

Controls should produce the following values:

- Amplification Cell Negative Control < 20,000 RLU
- Amplification Cell Positive Control ≥ 500,000 RLU
- Specimen Processing Negative Control < 20,000 RLU
- Specimen Processing Positive Control ≥ 1,000,000 RLU

Patient test results must not be reported if the MTD test control values do not meet the criteria above. See TROUBLESHOOTING section for further information.

Target values for controls should be determined in each laboratory using test results for each batch of prepared controls.

B. Patient Test Results

If the controls do not yield the expected results, test results on patient specimens in the same run must not be reported.

Results:

- ≥ 500,000 RLU positive for *M. tuberculosis* complex rRNA
- < 30,000 RLU negative for *M. tuberculosis* complex rRNA
- 30,000 to 499,999 RLU probable *M. tuberculosis* complex rRNA positive; repeat to verify results:
 - Repeat ≥ 30,000 RLU positive for *M. tuberculosis* complex rRNA
 - Repeat < 30,000 RLU negative for *M. tuberculosis* complex rRNA

Reporting of Results

Results from the MTD test should be interpreted in conjunction with other laboratory and clinical data available to the clinician. Based upon the degree of clinical suspicion, testing of an additional specimen should be considered.

If the initial MTD test result is positive at $\geq 500,000$ RLU, or the repeat MTD test result is positive at $\geq 30,000$ RLU, then report the following:

Report:	Mycobacterium tuberculosis complex rRNA detected. AFB smear (positive or negative).
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Additional Information: AFB culture pending. Specimen may contain both MOTT and *M. tuberculosis* or *M. tuberculosis* alone. This test should not be the sole basis for diagnosing tuberculosis. The positive predictive value for a smear negative patient is lower than for a smear positive patient. This is particularly important in test populations where the prevalence of tuberculosis is low and the positive predictive values of diagnostic methods are correspondingly reduced.

If the initial or the repeat MTD test result is negative at $<30,000$ RLU, then report the following:

Report:	No Mycobacterium tuberculosis complex rRNA detected. AFB Smear (positive or negative).
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Additional Information: No Mycobacterium tuberculosis complex rRNA detected. AFB culture pending. Specimen may not contain *M. tuberculosis*, the result may be falsely negative due to low numbers of *M. tuberculosis* in the presence or absence of MOTT, or the result may be falsely negative due to assay interference by specimen inhibitors. Testing of another patient specimen is recommended if active tuberculosis is clinically suspected or specimen inhibition is suspected.

Limitations

Use only for the detection of members of the *M. tuberculosis* complex using sediments prepared following the NALC-NaOH or NaOH procedures recommended by the CDC⁷. This test may only be used with sediments prepared from sputum (induced or expectorated), tracheal aspirates, or bronchial specimens (e.g., bronchoalveolar lavages and bronchial aspirates).

The MTD test is specific for, but does not differentiate among, members of the *M. tuberculosis* complex, i.e., *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. microti* and *M. canetti* and *M. terrae*-like organisms will cross-react if present at concentrations higher than 30 CFU per test. However, *M. celatum* and *M. terrae*-like organisms are rare clinical isolates.

Test results may be affected by specimen collection and transport, specimen sampling variability, laboratory procedural errors, sample misidentification, and transcriptional errors. A negative test does not exclude the possibility of isolating an *M. tuberculosis* complex organism from the specimen.

Expected Values

A. Range of Control Values Observed in the Clinical Studies

The RLU range for the controls observed in a 7 site clinical study was:

RLU (N=704)		
	Range	Mean
Amplification Cell Positive Control	556,245 to >2,000,000	>2,000,000
Amplification Cell Negative Control	904 to 18,754	3,041

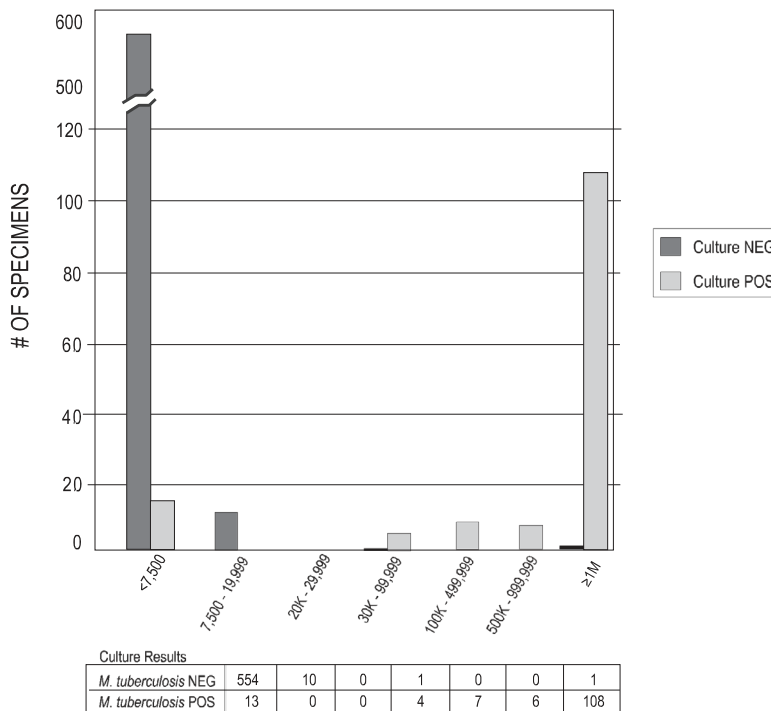
B. Range of RLU Values for Clinical Specimens

The range of RLU values for the 127 specimens that were MTD test positive was 35,777 to > 2,000,000 RLU.

For the 577 MTD test negative specimens, the range of values was 573 to 19,176 RLU.

The frequency distribution of the RLU values for all these specimens after discrepant resolution is shown below: Discrepant resolution is based on the presence of other culture positive specimens from the same patient and/or attending physician's final diagnosis.

Distribution of Results From Untreated Specimens (7 Sites)
N=704



Performance Characteristics

A. Clinical Evaluation

The original MTD test was evaluated in studies at 6 sites comparing AFB smear results to mycobacterial culture results in 6,079 specimens from 2,609 patients. Of these, 4,000 specimens were collected from 1,898 patients not on antituberculous therapy. The 6 study sites were geographically diverse: 5 were large metropolitan hospital centers with tuberculosis treatment centers and one was a state public health laboratory.

The current MTD test format was evaluated in a separate study at 7 sites comparing the MTD test results to mycobacterial culture results. The 7 study sites were geographically diverse; 6 sites were large metropolitan hospital centers with tuberculosis treatment centers and one was also a national mycobacteriology laboratory.

The seventh site was a public health laboratory. Specimens from patients not on therapy were tested. Of these, 132 were culture positive for *M. tuberculosis* complex. MTD detected 119 of these culture positive specimens; 7 specimens grew MOTT in addition to *M. tuberculosis*.

SPECIMENS FROM UNTREATED PATIENTS MTD vs. Culture (N=704)

BY PATIENT (Before Resolution Culture)			BY PATIENT (After Resolution Culture)		
	+	-		+	-
MTD +	54	4	MTD +	56	2
MTD -	4	221	MTD -	4	221

BY SPECIMEN (Before Resolution Culture)			BY SPECIMEN (After Resolution Culture)		
	+	-		+	-
MTD +	119	8	MTD +	125	2
MTD -	13	564	MTD -	13	564

Patients with suspected active pulmonary tuberculosis and not on therapy were enrolled in this study. The overall prevalence of patients that were culture positive for *M. tuberculosis* was 20.5%.

The overall MTD test sensitivity by patient was 93.3% and specificity by patient was 99.1% compared to culture results. The overall MTD test sensitivity by specimen was 90.6% and specificity by specimen was 99.6% compared to culture results. Results from the 7 sites for sensitivity, specificity, Positive Predictive Value (PPV), and Negative Predictive Value (NPV) are shown in the following table, along with the 95% confidence intervals for the performance estimates. All data are shown subsequent to discrepant resolution based on the presence of other culture positive specimens from the same patient and/or attending physician's final diagnosis.

By Patient:

	Overall Percentage	Number vs Total	95% Confidence Interval
Sensitivity	93.3%	56/60	83.8 – 98.2%
Specificity	99.1%	221/223	96.8 – 99.9%
PPV	96.6%	56/58	88.1 – 99.6%
NPV	98.2%	221/225	95.5 – 99.5%

By Specimen:

	Overall Percentage	Number vs Total	95% Confidence Interval
Sensitivity	90.6%	125/138	84.4 – 94.9%
Specificity	99.6%	564/566	98.7 – 100%
PPV	98.4%	125/127	94.4 – 99.8%
NPV	97.7%	564/577	96.2 – 98.8%

Of the 564 specimens that were culture and MTD negative for *M. tuberculosis* complex, 114 specimens grew MOTT on culture, 64 were from patients with other cultures positive for MOTT, and 169 were from patients with negative mycobacterial cultures.

B. Precision Studies

Precision panels, consisting of 2 negative samples, 2 low positive samples (^a100 CFU/test) and 2 moderately high positive samples (^a1000 CFU/test) were tested at 3 sites. The positive samples were prepared by spiking a contrived moderately inhibitory sediment pool with known amounts of *M. tuberculosis*. The samples were tested in triplicate twice a day for 3 days at the 3 sites. Positive and negative amplification controls were included in each run.

Because there was no significant site-to-site or day-to-day variability observed, the data from all 3 sites were combined and are presented below. The RLU values measured are limited by the luminometer photomultiplier tube. Therefore, values greater than 2,000,000 RLU are truncated. No standard deviation or % CV values are given.

Precision Studies				
	# Observations	% Correct	Range (RLU)	Mean (RLU)
Sample 1 High Positive	108	100%	154,103 - >2,000,000	>2,000,000
Sample 2 Low Positive	108	99.1%	16,324 - >2,000,000*	>2,000,000
Sample 3 Negative	108	100%	2,004 - 5,693	2,689
Positive Cell Control	54	100%	>2,000,000	>2,000,000
Negative Cell Control	54	100%	2,272 - 4,241	2,944

*One observation negative.

C. Reproducibility

The reproducibility panel consisted of 25 samples with Amplification Negative Controls interspersed between each sample for a total of 50 samples. The Reproducibility Panel was tested at 4 sites. Overall, 100% (120/120) of the negative samples yielded the expected results and 98.8 % (79/80) of the positive samples yielded the expected results.

D. Analytical Specificity

Specificity of the MTD test was assessed using bacteria, fungi, and viruses. For bacteria and fungi, specificity testing included 160 strains (151 species from 62 genera) of closely related mycobacteria, other organisms causing respiratory disease, and normal respiratory flora or organisms representing a cross-section of phylogeny. Type strains were obtained from the American Type Culture Collection (ATCC), and 5 isolates were obtained from clinical laboratories. Lysates prepared from actively growing cultures (or rRNA in 3 cases) were evaluated in the MTD test according to the TEST PROCEDURE. Approximately 5×10^7 CFU per reaction were tested. Only strains of the *M. tuberculosis* complex yielded positive results, with the exception of *M. celatum* and *M. terrae*-like strains.

At concentrations higher than 30 CFU per test, *M. celatum* and some *M. terrae*-like strains will yield positive MTD test results. At a level of 30 CFU per test, *M. celatum* yielded 26,772 RLU and *M. terrae*-like ranged from 19,470 to 49,976 RLU.

E. Limits of Detection

Thirty (30) strains of *M. tuberculosis* from a wide geographic distribution, including representative drug-resistant and drug-sensitive strains, were detected with the MTD test. The MTD test detected 1 CFU per test of all 30 strains.

F. Recovery

Twenty-five (25) fg *Mycobacterium tuberculosis* rRNA (equivalent to 5 CFU per test) were tested in the presence of approximately 540,000 CFU per test (450 μ L) of the following relevant non-target organisms: *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Mycobacterium gordonae*, *M. avium*, *M. kansasii*, *M. terrae*, *Nocardia asteroides*, *N. otitidis-caviarum*, *Corynebacterium pseudotuberculosis*, *C. diphtheriae*, *Gordona sputi*, and *Rhodococcus bronchialis*. All test results were positive for *M. tuberculosis* rRNA in the presence of these non-target organisms.

Troubleshooting

Observation	Possible Causes	Recommended Actions
Elevated Amplification Cell Negative Control or Specimen Processing Negative Control (≥ 20.000 RLU)	Insufficient mixing or volume added after addition of the Mycobacterium Selection Reagent (S).	Achieve complete mixing. Ensure correct volume is added. Visually verify a uniformly pink solution after vortexing.
	Insufficient care taken during set up of the reactions and the resultant amplification of contaminating materials introduced at that time.	Exercise extreme care when pipetting. The spent reaction tubes must be decontaminated with a 1:9 dilution of household bleach as described in the TEST PROCEDURE section. Laboratory bench surfaces, dry heat bath, water baths and pipettors must be decontaminated with a 1:1 dilution of household bleach as described in the TEST PROCEDURE.
	Skipped 5 minute cool down step.	
	Contamination of lab surface or reagents.	
Low Amplification Cell Positive Control or Specimen Processing Positive Control (< 500.000 RLU)	Failure to wipe tubes prior to reading in the luminometer.	Tubes must be wiped with a damp tissue or lint-free paper towel prior to reading in the luminometer.
	Performed the amplification step outside the recommended temperature range.	Check water bath and/or dry heat bath temperature and adjust as necessary to achieve the temperature ranges specified in procedure.
	Added Amplification Reagent to the side instead of to the bottom of the tube.	
	Insufficient mixing after addition of the reconstituted Mycobacterium Tuberculosis Hybridization Reagent.	Carefully vortex as specified. (See Hybridization, Step 2.) Visually verify solution is yellow after vortexing.
	Added too much Selection Reagent.	Check pipettor volume setting.
	Allowed the Selection step to go over the recommended time limit.	Carefully time the 60°C incubation in the Selection step to be 15 minutes.
	Allowed the tubes to cool down below 42°C after the 95°C incubation.	Transfer tubes directly from the 95°C dry heat bath to the 42°C water bath/dry heat bath.
Detection Reagent lines clogged.	Perform warm water flushes as described in the Instrument Operator's Manual.	

Notes

- A. Heating blocks must have wells properly sized for 12 x 75 mm tubes. Use of Dry Heat Bath is recommended.
- B. Screw-capped microcentrifuge tubes are recommended for storage of frozen aliquots. Individual frozen aliquots may be frozen and thawed for use no more than once. Frost-free freezers must not be used.
- C. 5 mL cryovials are recommended for storage of frozen aliquots. Individual frozen aliquots may be frozen and thawed for use no more than once. Frost-free freezers must not be used.
- D. Due to vortexing equipment differences and set speed variations, a longer vortex time may be required depending on individual vortexing equipment. Adjust vortexer speed and follow vortex handling procedures as described under PROCEDURAL NOTES, Section E, to allow reaction mixture to reach and maintain a height within the upper half of the tube. Adequate vortexing as described is necessary to achieve accurate assay results. Times may be increased up to a total of 15 seconds without affecting assay results.

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