Amplified Mycobacterium Tuberculosis Direct Test

For *In Vitro* Diagnostic Use
50 Test Kit

Intended Use

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

The Amplified MTD test is intended for use only with specimens from patients showing signs and symptoms consistent with active pulmonary tuberculosis (TB). MTD is to be used as an adjunctive test for evaluating either AFB smear positive or negative sediments prepared using NALC-NaOH digestion-decontamination of respiratory specimens. Patients who are suspected of having pulmonary TB based on clinical evaluation and who have received no antituberculous therapy, less than 7 days of such therapy, or have not received such therapy in the last 12 months may be evaluated with this test. The MTD test should be performed only in laboratories proficient in the culture and identification of *M. tuberculosis* (Level II and III or extent 3 and 4). The MTD test must be performed in conjunction with mycobacterial culture.

Warnings

A. A negative test does not exclude the possibility of isolating an *M. tuberculosis* complex (MTBC) organism from the specimen.

B. The Positive Predictive Value (PPV) of MTD results for AFB smear negative patients is lower than the PPV of MTD results for AFB smear positive patients. The average prevalence of TB in this population was 27.7% (57/206 patients). The predictive values associated with any diagnostic test are related to the prevalence of disease in a given patient group. Refer to Table 1 in the **Limitations** section for the hypothetical estimates of positive and negative predictive values across varying prevalence rates.

C. The efficacy of this test has not been demonstrated for the direct detection of *M. tuberculosis* rRNA using other clinical specimens (e.g., blood, CSF, tissue, 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.

D. 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.

E. *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 rarely isolated from clinical specimens.

F. Samples may be MTD test negative and *M. tuberculosis* complex culture positive. This condition may be caused by inhibition of the MTD test or the presence of low levels of the *M. tuberculosis* complex organism.

G. Specimens from pediatric patients have not been evaluated with the MTD test.

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

I. Specimens that are grossly bloody should not be tested with the MTD test; blood may cause nonspecific positivity in the MTD test.

J. Sediments must be resuspended in a phosphate buffer concentration of 67 mM. Concentrations substantially above 67 mM may interfere with amplification of the MTD test, decreasing the ability to detect *M. tuberculosis* complex in the specimen. Sediments prepared using Alpha-Tec Systems, Inc. NAC-PAC XPR-plus A. F. B. Processing Buffer have been shown to interfere with amplification.

Precautions

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.*

A. For *In Vitro* Diagnostic Use.

B. The MTD test does not differentiate among members of the *M. tuberculosis* complex, i.e., *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. microti*, *M. canetti,* and *M. pinnipedii*.

C. 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 and Prevention (CDC). 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 mM. Final specimen digestant concentrations greater than 1.5% NaOH may inhibit detection of *M. tuberculosis* complex.

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 test. Preparation of digested and decontaminated sediments, and MTD procedures, should be done using Biosafety Level 2 practices.

G. Use only supplied or specified disposable laboratory ware.

H. Work surfaces, pipettors, and equipment must be decontaminated of rRNA amplicon with a 2.5% to 3.5% (0.35M to 0.5M) sodium hypochlorite solution (1 part of bleach and 1 part of water) as described in the Test Procedure. Work surface may be wiped with water after 15 minutes to remove the solution.

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. Reconstituted Enzyme Reagent (E) contains N-acetyl-L-cysteine and loses activity gradually. Thirty-six (36) *M. tuberculosis* culture positive samples were tested on the day of Enzyme Reagent reconstitution and yielded positive results (> 500,000 RLU). After the Enzyme Reagent had been reconstituted for 30 days, 32 of 36 specimens gave positive results. One sample was negative. The remaining 3 specimens yielded results in the equivocal zone. These samples were positive when re-tested.

L. Separate pipettors must be used for steps that precede amplification and those that follow amplification.

M. After reading reaction tubes in the luminometer, decontaminate and carefully dispose of them as described in the Test Procedure in order to avoid contamination of the laboratory environment with amplicon.

N. 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.

O. Do not cover water bath during incubations, especially when using snap caps. (Condensation from the cover may be a possible source of contamination.)

P. Adequate vortexing after addition of Selection Reagent is necessary to achieve accurate assay results. DO NOT VORTEX LYSATES.

Q. Please see NCCLSMM-3A2, for additional guidance on testing of reagents and controls, inhibition, and general laboratory procedures.

R. 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.

S. To help prevent lab areas from becoming contaminated with amplicon, the laboratory area should be arranged with a uni-directional 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 HPA to qualitatively detect *M. tuberculosis* complex 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*, *M. canetti,* and *M. pinnipedii.* The MTD test can 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 Africa*. M. canetti,* with smooth, round, glossy colonies unusual for a member of the *M. tuberculosis* complex, is rarely described as an etiologic agent of disease. *M. tuberculosis* is by far the most common member of the complex that is responsible for human disease worldwide. Patients at high risk for developing TB include those who are from high burden countries or are immunocompromised. Strains of TB can be resistant to one or more drug. In 2011, the CDC reported that 1.2% of TB cases in the U.S. were resistant to at least two anti-TB drugs. The public health implications of these facts are considerable*.*

Conventional culture methodologies can detect TB growth as early as 1 week, but may take up to 8 weeks. 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.* The CDC has recommended the use of nucleic acid amplification tests, such as the MTD test, for use in all patients suspected of having TB.

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 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 HPA method. 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:

Reagent Name	Volume
MYCOBACTERIUM TUBERCULOSIS AMPLIFICATION TRAY	
Mycobacterium Specimen Dilution Buffer (SDB) *Tris buffered solution containing < 3% detergent*	1 x 2.5 mL
Mycobacterium Tuberculosis Amplification Reagent (A) *Nucleic acids lyophilized in tris buffered solution containing 5% bulking agent*	1 x 3 mL (when reconstituted)
Mycobacterium Amplification Buffer (AB) *Aqueous solution containing preservatives*	1 x 3 mL
Mycobacterium Oil Reagent (O) *Silicone Oil*	1 x 10 mL
Mycobacterium Enzyme Reagent (E) *Reverse transcriptase and RNA polymerase lyophilized in HEPES buffered solution containing < 10% bulking agent and ≥ 15 mM N-acetyl-L-cysteine*	1 x 1.5 mL *(*when reconstituted)
Mycobacterium Enzyme Dilution Buffer (EDB) *Tris buffered solution containing a surfactant and glycerol*	1 x 1.5 mL
MYCOBACTERIUM TUBERCULOSIS HYBRIDIZATION TRAY	
Mycobacterium Tuberculosis Hybridization Reagent (H) *< 100 ng/vial non-infectious DNA probe with a chemiluminescent label lyophilized in succinate buffered solution containing bulking agent and detergent*	1 x 6 mL (when reconstituted)
Mycobacterium Hybridization Buffer (HB) *Succinate buffered solution containing < 4% detergent*	1 x 6 mL
Mycobacterium Selection Reagent (S) *Borate buffered solution containing surfactant*	1 x 15 mL
Mycobacterium Lysing Tubes *Glass Beads, Bulking Agent*	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 up to 1 month at 2°C to 8°C after reconstitution. Once reconstituted, seal stoppered vials with Parafilm® to prevent exposure to air and store in an upright position to prevent spillage.

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

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 7 days (generally less than 24 hours) prior to processing.

Transport:
Transport specimens to the laboratory as soon as possible. Specimens must be shipped in accordance with applicable national and international transportation 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 of members of the *M. tuberculosis* complex using respiratory 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 ≥ 3,000 x *g*. Resuspension fluids other than phosphate buffer (67 mM) or bovine serum albumin should not be used. Final specimen concentrations of NaOH other than 1 to 1.5% should not be used for processing specimens to be tested.

Processed Sediment Storage:
Sediments may be stored at 2°C to 8°C for up to 3 days prior to testing.

Materials

Materials Provided

Cat. No. 301001	50 tests
MYCOBACTERIUM TUBERCULOSIS AMPLIFICATION TRAY	
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	
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	2 x 25 Tubes
Sealing Cards	1 package

Materials Required But Available Separately

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
Positive Cell Controls (e.g., *M. tuberculosis*, ATCC 25177 or ATCC 27294)
Negative Cell Controls (e.g., *M. gordonae*, ATCC 14470, or *M. terrae*, ATCC 15755)
Bleach, 5% to7% (0.7M to 1.0M) sodium hypochlorite solution
Plastic-backed laboratory bench covers

Additional Materials Available From Hologic:

LEADER Luminometer
Ultrasonic Water Bath
Detection Reagent Kit
Water Bath (42° ± 1°C and 60° ± 1°C)
Dry Heat Bath* (42° ± 1°C, 60° ± 1°C, and 95° ± 5°C)
Ultrasonic Water Bath Rack
Test tube racks
Pipette tips with hydrophobic plugs
Extended length pipette tips with hydrophobic plugs
Tubes, polypropylene, 12 x 75 mm
Micropipettes capable of dispensing 25 µL, 50 µL, 100 µL, 200 µL, 300 µL, and 450 µL
Snap top polypropylene caps for 12 x 75 mm tubes
Repeat pipettors

Test Procedure

Controls

1. Suggested Preparation of Cell Controls

   Cells used for the 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. gordonae* (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**.)

   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
   - 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 1 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. These recommended controls may be used for internal quality control or users may develop their own internal quality control material, as defined by NCCLS C24-A3.

* Heating blocks must have wells properly sized for 12 x 75 mm tubes. Use of Dry Heat Bath is recommended.

Additional controls may be tested according to guidelines or requirements of local, state, and/or federal regulations or accrediting organizations. Please refer to NCCLS C24-A3 and to NCCLS MM3A2 for additional guidance on appropriate internal quality control testing practices.

2. Specimen Inhibition Controls

When the AFB smear is positive and the MTD test is negative for untreated patient specimens, there are 3 conditions that might exist:
(1) the specimen is inhibitory, or
(2) the specimen contains *Mycobacterium* other than tuberculosis complex (MOTT), or
(3) the specimen contains a mixture of large numbers of MOTT and a low number of *M. tuberculosis* complex organisms.

When the AFB smear is negative and the MTD test is negative, there are 3 conditions that might exist:
(1) the specimen is inhibitory,
(2) the specimen contains *Mycobacterium* other than tuberculosis complex (MOTT), or
(3) the specimen does not contain *Mycobacterium tuberculosis* or MOTT.

When the AFB smear is negative and the MTD test is negative, testing may also be performed to establish whether the specimen is inhibitory.

To test for specimen inhibition, the following procedure may be performed:
a. Place 50 µL Specimen Dilution Buffer into 2 Mycobacterium Lysing Tubes (seeded and unseeded).
b. Add 50 µL 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 ≥ 30,000, then the specimen does not contain inhibitory substances that prevent amplification. If the RLU value of the seeded tube is below 30,000, the specimen inhibits amplification and another sample should be evaluated. If the repeat testing of the unseeded specimen is positive, random sampling variability may have occurred (i.e., the first aliquot did not contain target for amplification, while the second aliquot did). Contamination may also be a source of such discrepant results. If inhibitors are present, negative results in the unseeded tube cannot be reliably interpreted. The use of this inhibition control procedure was not evaluated during clinical trials.

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 2.5% to 3.5% (0.35M to 0.5M) sodium hypochlorite solution (1 part of bleach and 1 part of water).
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 ≥ 30,000 RLU, the surface is contaminated and should be decontaminated by treating with sodium hypochlorite solution as recommended in the 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 2.5% to 3.5% (0.35M to 0.5M) sodium hypochlorite solution prior to starting. The solution must be in contact with the surface for at least 15 minutes. Work surfaces may be wiped with water to remove the solution. 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

This step should be performed prior to beginning specimen preparation. The pipettors used for this step should be dedicated for use in the Reagent Preparation, Sample Preparation, and Amplification steps. Do not use for adding *reconstituted* Hybridization Reagent or Selection Reagent to specimen tubes.

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.

Reconstitute the vial of Mycobacterium Enzyme Reagent (E) with 1.5 mL Mycobacterium Enzyme Dilution Buffer (EDB). Swirl to mix. Do not vortex. After use, seal stoppered vials tightly and refrigerate for up to 30 days.

Sample Preparation

1. Label a sufficient number of Mycobacterium Lysing Tubes to test the samples and 1 each of 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.

   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.

   b. Specimen:
   Transfer 450 µL decontaminated well-vortexed specimen from its container to the correspondingly labeled Mycobacterium Lysing Tube.

3. Recap the Mycobacterium Lysing Tubes after addition of each sample.

4. Vortex 3 seconds.

Sample Lysis

1. Place rack on ultrasonic water bath. Push the Mycobacterium Lysing Tubes 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. DO NOT ALLOW THE TUBES TO TOUCH THE BOTTOM OR SIDES OF THE ULTRASONIC WATER BATH.

2. Sonicate for 15 minutes but no more than 20 minutes. Samples and controls that have been sonicated are now referred to as "lysates".

Amplification

The pipettors used for this step must be dedicated for use in the Reagent Preparation, Sample Preparation, and Amplification steps (see **Precautions**).

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. Also label amplification tubes for each of the Cell Positive and Negative 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. For repeat testing of lysates, bring stored lysate to room temperature.

4. Incubate the tubes at 95°C for 15 minutes, but no more than 20 minutes, in the dry heat bath.

5. 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.

6. Add 25 µL reconstituted Enzyme Reagent (E) 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.

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 Protection Assay (HPA)

The repeat pipettor used for this step must be dedicated for use in this step only. Perform this step in the dedicated HPA area (see **Precautions**).

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). The *reconstituted* Hybridization Reagent is stable for 1 month at 2°C to 8°C after reconstitution. If the *reconstituted* Hybridization Reagent has been refrigerated, 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* 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* 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 0.5% to 0.7% (0.07M to 0.1M) sodium hypochlorite solution 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 2.5% to 3.5% (0.35M to 0.5M) sodium hypochlorite solution with water for a minimum of 15 minutes. The solution 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 2.5% to 3.5% (0.35M to 0.5M) sodium hypochlorite solution.

Repeat Testing

1. To repeat patient specimen testing (e.g., if controls are unacceptable or initially equivocal results are repeated), bring prepared lysate to room temperature. DO NOT VORTEX LYSATE.

2. Follow **Test Procedure** protocol as outlined, beginning with the Amplification step.

* 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.

Procedural Notes

A. Reagents

1. Enzyme Reagent can be held at room temperature up to 6 different times for no more than 15 minutes at a time. Enzyme Reagent should not be held at room temperature more than 15 minutes on any single occasion.

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.

3. Seal reconstituted stoppered vials with parafilm to prevent exposure to air and store in an upright position to prevent spillage.

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° ± 1°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 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 greater than 30,000 is considered positive.

A. Quality Control Results and Acceptability

The Specimen Processing Negative Control and Specimen Processing Positive Control should produce the following values:

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 or limits established in each laboratory. See **Troubleshooting** section for further information.

Target values or limits for Specimen Processing Cell Controls should be determined in each laboratory using test results for each batch of prepared controls. See CLSI recommended procedures for using target values and limits, C24-A3, Statistical Quality Control for Quantitative Measurement Procedures; Approved Guideline-Third Edition.

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

C. 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 ≥ 500,000 RLU, or the repeat MTD test result is positive at ≥ 30,000 RLU, then report the following:

Report:	*Mycobacterium tuberculosis* complex rRNA detected. AFB smear (positive or negative).
Additional Information:	AFB culture pending. Specimen may contain both MOTT and *M. tuberculosis* or *M. tuberculosis* alone. If the first sputum specimen is smear-positive and Nucleic Acid Amplification (NAA)-positive, the patient can be presumed to have TB without additional NAA testing. If sputum is smear-negative and MTD-positive, additional specimens (not to exceed three) should be tested with MTD. The patient can be presumed to have TB if a subsequent specimen is MTD-positive.

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).
Additional Information:	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 TB is clinically suspected or specimen inhibition is suspected. The clinician must rely on clinical judgment in decisions regarding the need for antituberculous therapy and further diagnostic work-up because negative NAA results do not exclude the possibility of active pulmonary TB. If the first sputum is smear-positive and NAA-negative, a test for inhibitors should be done. If inhibitors are not detected, additional specimens (not to exceed a total of three) should be tested. The patient can be presumed to have NTM if a second sputum specimen is smear-positive, NAA-negative, and has no inhibitors detected. If inhibitors are detected, the NAA test is of no diagnostic help. Additional specimens (not to exceed a total of three) can be tested with NAA. If sputum is smear-negative and MTD-negative, an additional specimen should be tested with MTD. The patient can be presumed not to be infectious if all smear and MTD results are negative.

Limitations

A. Use only for the detection of members of the *M. tuberculosis* complex using sediments prepared following the NALC-NaOH or NaOH (<1.5%) procedures recommended by the CDC. 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). Other specimen types have not been evaluated using MTD.

B. The MTD test does not differentiate among members of the *M. tuberculosis* complex, i.e., *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, M. *microti*, *M. canetti,* and *M. pinnipedii*. Culture recovery is necessary to distinguish *M. bovis* from *M. tuberculosis*. *M. celatum* 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. Culture procedures are also necessary for susceptibility testing.

C. Test results may be affected by specimen collection and transport, specimen sampling variability, laboratory procedural errors, inhibitors, sample misidentification, and transcriptional errors. A negative test does not exclude the possibility of isolating a *M. tuberculosis* complex organism from the specimen. Performance of the MTD is dependent on operator proficiency and adherence to procedural directions. Laboratory procedural errors may cause false positive or false negative results. Testing should be performed by properly trained personnel.

D. Table 1 below demonstrates the predictive values associated with the MTD test for a group of patients suspected of having pulmonary TB based on clinical evaluation. As an example, in a smear negative patient group with a prevalence of 5% the positive and negative predictive values are 84.3% and 98.5% with confidence intervals of 42.1% to 99.6% and 95.6% to 99.8%, respectively. In a smear positive patient group with a prevalence of 20% the positive and negative predictive values are 100% and 99.2% with confidence intervals of 63.1 % to 100% and 88.8% to 100%, respectively. These data provide the physician additional information to aid in the diagnosis of TB. The predictive values associated with any diagnostic test are related to the prevalence of the disease in a given patient group. PPV generally increase as the prevalence of disease in the group increases. NPV generally decrease as the prevalence of disease in the group increases.

E. The average prevalence of TB in the clinical trial for patients suspected of having active pulmonary TB was 27.7% (57/206). MTD performance in this patient group of 206 patients is presented in the following table as PPV and NPV. The predictive values are shown as a function of prevalence using the sensitivity and specificity generated from the clinical trial data. Data are shown by AFB smear status; predictive value ranges are presented for smear negative patients from 1% to 20% prevalence where the expected disease prevalence is lower than that for smear positive patients. The predictive values for smear positive patients are represented at a range of 20% to 80% prevalence. The sensitivity/specificity were 72.0%/99.3% for smear negative patients and 96.9%/100% for smear positive patients, respectively.

Table 1: Hypothetical Predictive Values as a Function of Prevalence

Patient	**Predictive**	**Prevalence**				
Status	**Value**	**20%**	**30%**	**40%**	**60%**	**80%**
Smear Positive	**PPV**	100% (63.1%-100%)	100% (71.5%-100%)	100% (78.2%-100%)	100% (85.2%-100%)	100% (88.4%-100%)
	NPV	99.2% (88.8%-100%)	99.0% (81.7%-99.9%)	98.0% (78.9%-99.9%)	95.5% (79.4%-100%)	88.9% (51.8%-99.7%)
		1%	**5%**	**10%**	**15%**	**20%**
Smear Negative	**PPV**	50.8% (1.3%-98.7%)	84.3% (42.1%-99.6)	91.9% (64.0%-99.8%)	94.7% (74.0%-99.9%)	96.2% (79.6%-99.9%)
	NPV	99.7% (96.7%-100%)	98.5% (95.6%-99.8%)	97.0% (92.6%-98.9%)	95.3% (90.5%-98.1%)	93.4% (88.3%-97.1%)

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= 339)	
	Range	RLU Values
Positive Cell Control	556,000 to > 2,000,000*	> 2,000,000
Negative Cell Control	1300 to 18,800**	**mean** =3500

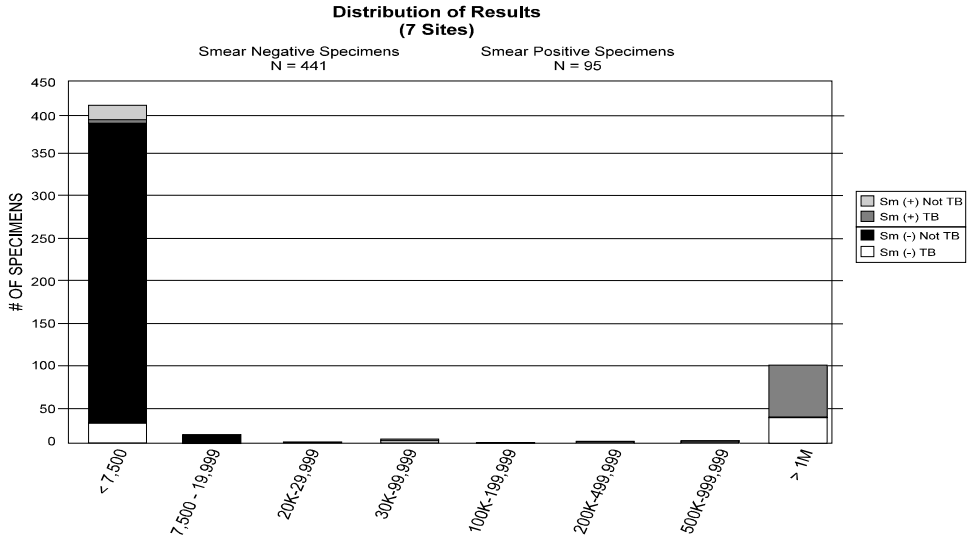
*336/339 positive control results were > 1,000,000 RLU.
**331/339 negative control results were < 7,500 RLU.

B. Range of RLU Values for Clinical Specimens

A frequency distribution of the RLU values for the 536 clinical specimens from the 206 patients, enrolled in the clinical trial whose MTD results are based on testing of fresh specimens are shown below. Results are presented versus patient diagnosis and are further defined based on the smear status of each specimen.

The range of RLU values for the 104 specimens that were MTD test positive from patients diagnosed with TB was 541,882 to > 2,000,000 RLU. Nine (9) clinical specimens from patients diagnosed with TB fell within the equivocal zone on initial testing. Of these equivocal specimens 7 were positive after repeat testing from the reserved lysate.

The range of RLU values for the 389 clinical specimens that were MTD test negative from patients not diagnosed with TB was 1,335 to 25,146 RLU. No results fell within the equivocal zone from these patients.



Patient Diagnosis	RLU							
Sm (+) Not TB	18	0	0	0	0	0	0	0
Sm (+) TB	5	0	0	2	0	2	2	66
Sm (-) Not TB	363	7	1	0	0	0	0	1
Sm (-) TB	28	0	0	3	1	1	2	34

Performance Characteristics

A. Clinical Evaluation

1. Study Design

The MTD test was evaluated at 7 geographically diverse clinical sites: a European national mycobacteriology laboratory, a public health laboratory and 5 large metropolitan hospital centers with TB treatment centers. The trial was performed prospectively by enrolling patients suspected of having active pulmonary TB based on clinical evaluation. Patients enrolled had been on multi-drug anti-TB therapy for 7 days or less within the 3 months prior to study participation. MTD results were compared to the patient's final diagnosis as determined by physician(s) at each site.

Of the 206 patients enrolled in the study whose MTD results were based on testing of fresh specimens, 57 patients were diagnosed with pulmonary TB (of which 4 patients were culture negative and 4 patients had both MTBC and MOTT recovered); 1 patient was diagnosed with having extra-pulmonary TB; and 148 patients were diagnosed as not having TB. The 206 patients that were enrolled in the study contributed 536 specimens that were tested with MTD (a repeat MTD test was also performed from the reserved lysate for each specimen). A patient having one or more positive MTD results was defined as an MTD positive patient.

2. MTD Performance Using Patient Diagnosis as the Endpoint (1st Specimen Analysis)

For the following tables, patient smear status was determined by the smear result from the first specimen collected. Of the 206 patients, the first specimen from 167 patients was smear negative and the first specimen from 39 patients was smear positive. Patients contributed 1 to 6 specimens, with an average of 2.6 specimens per patient. A portion of these 206 patients had no 2nd specimen collected or the 2nd specimen was not available for testing.

Table A.2-a and Table A.2-b provide MTD test results (sensitivity, specificity, positive predictive value, negative predictive value and the corresponding 95% confidence intervals) using patient diagnosis as the endpoint, stratified by smear status. Confidence intervals are presented in brackets. Data are presented for the MTD test results from the first specimen collected from all patients as Table A.2-a; data are presented for the cumulative MTD test results from the first two specimens from those patients that contributed two or more specimens as Table A.2-b. For those patients that contributed at least two specimens, if either or both of those specimens are MTD positive, then the patient is defined as

MTD positive. A patient whose first specimen evaluated as smear positive is defined as a smear positive patient. A patient whose first specimen evaluated as smear negative is defined as a smear negative patient.

Table A.2-a: MTD Performance Using Patient Diagnosis as the Endpoint

1st Specimen
N=206

	Smear Positive Patient	Smear Negative Patient
Sensitivity	87.5% (28/32) [71.0%-96.5%]	64.0% (16/25) [42.5%-82.0%]
Specificity	100% (7/7) [59.0%-100%]	100% (142/142) [97.4%-100%]
PPV	100% (28/28) [87.7%-100%]	100% (16/16) [79.4%-100%]
NPV	63.6% (7/11) [30.8%-89.1%]	94.0% (142/149) [90.6%-98.1%]

Table A.2-b: MTD Performance Using Patient Diagnosis as the Endpoint

1st and 2nd Specimen
N=165

	Smear Positive Patient	Smear Negative Patient
Sensitivity	100% (24/24) [85.8%-100%]	71.4% (15/21) [47.8%-88.7%]
Specificity	100% (6/6) [54.1%-100%]	99.1% (113/114) [95.2%-100%]
PPV	100% (24/24) [85.8%-100%]	93.8% (15/16) [69.8%-99.8%]
NPV	100% (6/6) [54.1%-100%]	95.0% (113/119) [89.3%-98.1%]

3. MTD and Other Laboratory Test Performance Using Patient Diagnosis as the Endpoint

The following tables present MTD test results as well as results collected from the other laboratory methods used in the study for the 206 patients evaluated. Results of each method are presented using patient diagnosis as the endpoint. This is a by patient analysis and all specimens from a patient are utilized in categorizing that patient. The number of specimens per patient ranges from 1 to 6, with an average of 2.6 specimens per patient. A patient contributing one or more positive MTD results is defined as an MTD positive patient. A patient contributing one or more BACTEC (or LJ or 7H10/7H11) culture positive results for MTBC is defined as a BACTEC (or LJ or 7H10/7H11) positive patient. Data are presented overall in Table A.3-a as well as by patient smear status in Table A.3-b and Table A.3-c.

Table A.3-a: MTD and Other Laboratory Test Performance Using Patient Diagnosis as the Endpoint

Test	Sensitivity	Specificity	PPV	NPV	# Patients
MTD	86.0% (49/57) [74.2%-93.7%]	99.3% (148/149) [96.3%-100%]	98.0% (49/50) [89.4%-99.9%]	94.9% (148/156) [90.1%-97.8%]	206
Smear	64.9% (37/57) [51.1%-77.1%]	89.3% (133/149) [83.1%-93.7%]	69.8% (37/53) [55.7%-81.7%]	86.9% (133/153) [80.5%-91.8%]	206
BACTEC	84.2% (48/57) [72.1%-92.5%]	100% (149/149) [97.6%-100%]	100% (48/48) [92.6%-100%]	94.3% (149/158) [89.5%-97.4%]	206
LJ	80.7% (46/57) [68.1%-90.0%]	100% (142/142) [97.4%-100%]	100% (46/46) [92.3%-100%]	92.8% (142/153) [87.5%-96.4%]	199
7H10/7H11	80.0% (44/55) [67.0%-89.6%]	100% (149/149) [97.6%-100%]	100% (44/44) [92.0%-100%]	93.1% (149/160) [88.0%-96.5%]	204

The following tables, Table A.3-b and Table A.3-c, are derived from Table A.3-a and are stratified by the patients' smear status. A patient whose first specimen evaluated as smear positive is defined as a smear positive patient. A patient whose first specimen evaluated as smear negative is defined as a smear negative patient.

Table A.3-b: MTD and Other Laboratory Test Performance Using Patient Diagnosis as the Endpoint Smear Positive Patients

Test	Sensitivity	Specificity	PPV	NPV	# Patients
MTD	96.9% (31/32) [83.8%-99.9%]	100% (7/7) [59.0%-100%]	100% (31/31) [88.8%-100%]	87.5% (7/8) [47.3%-99.7%]	39
BACTEC	96.9% (31/32) [83.8%-99.9%]	100% (7/7) [59.0%-100%]	100% (31/31) [88.8%-100%]	87.5% (7/8) [47.3%-99.7%]	39
LJ	87.5% (28/32) [71.0%-96.5%]	100% (7/7) [59.0%-100%]	100% (28/28) [87.7%-100%]	63.6% (7/11) [30.8%-89.1%]	39
7H10/7H11	96.7% (29/30) [82.8%-99.9%]	100% (7/7) [59.0%-100%]	100% (29/29) [88.1%-100%]	87.5% (7/8) 47.3%-99.7%]	37

Table A.3-c: MTD and Other Laboratory Test Performance Using Patient Diagnosis as the Endpoint Smear Negative Patients

Test	Sensitivity	Specificity	PPV	NPV	# Patients
MTD	72.0% (18/25) [50.6%-87.9%]	99.3% (141/142) [96.1%-100%]	94.7% (18/19) [74.0%-99.9%]	95.3% (141/148) [90.5%-98.1%]	167
BACTEC	68.0% (17/25) [46.5%-85.1%]	100% (142/142) [97.4%-100%]	100% (17/17) [80.5%-100%]	94.7% (142/150) [89.8%-97.7%]	167
LJ	72.0% (18/25) [50.6%-87.9%]	100% (135/135) [97.3%-100%]	100% (18/18) [81.5%-100%]	95.1% (135/142) [90.1%-98.0%]	160
7H10/7H11	60.0% (15/25) [38.7%-78.9%]	100% (142/142) [97.4%-100%]	100% (15/15) [78.2%-100%]	93.4% (142/152) [88.2%-96.8%]	167

Sensitivity was decreased for all tests in smear negative patients, whereas specificity remained high. There were 25 smear negative patients who were diagnosed with TB. MTD correctly identified 72.0% (18/25) of the 25 patients which smear missed completely. The MTD test was comparable to the individual culture media whose sensitivity in smear negative patients ranged from 60.0% to 72.0%.

4. MTD vs. Culture Status

The following Table A.4-a is a by specimen presentation of the MTD result versus the final culture result stratified by that specimen's smear status. An MTBC culture specimen is defined as positive if any of the three culture media are positive for MTBC. An MTBC culture negative specimen is defined as having all culture media negative for MTBC. The number of MTD results that were initially in the equivocal zone are presented in parentheses, e.g., (1 equiv).

Table A.4-a: MTD vs. Culture Status

	AFB Sm (+)		AFB Sm (-)		
	MTD+	MTD-	MTD+	MTD-	Total
MTBC Culture Positive	75 (3 equiv)	3 (1 equiv)	32 (4 equiv)	7 (1 equiv)	117
MTBC Culture Negative from patients with other MTBC culture positive specimens	0	1	1	16	18
MTBC Culture Negative/MOTT recovered	0	27	0	66	93
MTBC Culture Negative/no MOTT recovered	1*	3	3(2)*	301**	308
Total	76	34	36	390	536

* specimen(s) from patients diagnosed with culture negative TB
** includes 7 specimens from patients diagnosed with culture negative TB

B. Precision Studies

Precision panels, consisting of 2 negative samples, 2 low positive samples (≈ 100 CFU/test) and 2 moderately high positive samples (≈ 1000 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 Cell 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.

Table B.1: Precision Studies

	# Observations	% Correct	Range (RLU)	Mean (RLU)
Sample 1: High Positive	108	100%	> 2,000,000	> 2,000,000
Sample 2: Low Positive	108	100%	> 2,000,000	> 2,000,000
Sample 3: Negative	105*	100%	1,484 -13,129	2,605
Positive Cell Control	54	100%	> 2,000,000	> 2,000,000
Negative Cell Control	52**	100%	2,129 -3,525	2,542

* Three observations have been removed from final study results as a result of operator error.
**Two observations have been removed from final study results due to one operator reporting splashing during one run. One observation yielded a positive result; one observation yielded a result in the equivocal zone.

C. Reproducibility

Reproducibility Panel
The Reproducibility Panel consisted of 25 samples with Negative Cell 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 97.5% (78/80) of the positive samples yielded the expected results (two equivocal results were obtained from the low positive sample at one study site).

Clinical Specimen Lysate Reproducibility
Specimens in the clinical study were repeat tested from the reserved specimen lysate. Data below represents the 536 specimens tested with MTD from fresh specimens.

There were 104 specimens from patients diagnosed with TB whose initial test results were > 500,000 RLU. Upon repeat testing, 103 of 104 yielded values greater than 30,000 RLU; 95 RLU values were > 500,000 RLU, and 8 values ranged from 30,208 to 489,836 RLU.

The initial and repeat test result of 1 specimen with > 500,000 RLU was from a patient diagnosed without TB.

There were 9 specimens from patients diagnosed with TB and no specimens from patients not diagnosed with TB that were initially in the equivocal zone. Upon retesting, 7 specimens were greater than 30,000 RLU, of which 4 were greater than 500,000 RLU.

There were 389 specimens with initial test results < 30,000 RLU. Upon repeat testing, 386 yielded values < 30,000 RLU, 1 was in the equivocal zone, and 2 were > 500,000 RLU.

Thirty-three (33) specimens from patients diagnosed with TB had initial MTD results of < 30,000 RLU. Upon repeat testing of the reserved lysate, 30 were < 30,000 RLU, 1 was in the equivocal zone and 2 were > 500,000 RLU.

No specimens were originally in the equivocal zone from patients not diagnosed with TB.

D. Analytical Specificity

Specificity of the MTD test was assessed using bacteria, fungi, and viruses. For bacteria and fungi, specificity testing included 159 strains (150 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 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 the spiked samples 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. Inhibition Testing

There were 57 patients diagnosed with active pulmonary TB in this study. Specimens from 55 patients were available and tested for inhibition. Of these 55 patients, 36 patients contributed smear positive specimens. All specimens from 1 patient were inhibitory, yielding an inhibition rate of 2.8% (1/36). Nineteen (19) patients contributed all smear negative specimens. All specimens from 3 patients were inhibitory, yielding an inhibition rate of 15.8% (3/19).

There were 8 patients who were MTD false negative and specimens from 7 patients were available and tested for inhibition. For 3 of these patients, all specimens contributed were inhibitory to amplification. These patients contributed 1, 3, and 3 specimens, respectively.

F. 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 was positive with >1,000,000 RLU for 1 CFU per test of all 30 strains.

G. Recovery

Mycobacterium tuberculosis rRNA at a concentration equivalent to 5 CFU per test (25 fg) was tested in the presence of approximately 540,000 CFU per test (450 µL) 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 Specimen Processing Negative Controls	• 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 0.5% to 0.7% (0.07M to 0.1M) sodium hypochlorite solution as described in the **Test Procedure** section. Laboratory bench surfaces, dry heat bath, water baths and pipettors must be decontaminated with a 2.5% to 3.5% (0.35M to 0.5M) sodium hypochlorite solution as described in the Test Procedure.
	• Contamination of lab surface or reagents.	
	• Skipped 5 minute cool down step.	
	• 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.
Low Specimen Processing Positive Controls	• 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.	Reconstitute new vial and repeat.
	• Reconstituted Enzyme Reagent (E) activity reduced.	
	• Insufficient mixing after addition of the *reconstituted* Mycobacterium Tuberculosis Hybridization Reagent.	Carefully vortex as specified. (Hybridization Protection Assay (HPA), 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.

Bibliography

1. **American Thoracic Society, Medical Section of the American Lung Association.** 1983. Levels of laboratory services for mycobacterial diseases. Am. Rev. Respir. Dis. **128**:213.

2. **Centers for Disease Control and Prevention.** 2010. Basic TB Facts: Risk Factors. [Online]. Available from: http://www.cdc.gov/tb/topic/basics/risk.htm. Accessed 2012 June 14.

3. **U.S. Department of Health and Human Services, Public Health Service Centers for Disease Control and Prevention, National Institutes of Health.** 2009. Biosafety in Microbiological and Biomedical Laboratories, 5th Edition HHS Publication No. (CDC) 21-1112. Available from: http://www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf. Accessed 2012 June 14.

4. **Centers for Disease Control and Prevention.** Updated guidelines for the use of nucleic acid amplification tests in the diagnosis of tuberculosis. 2009. *MMWR.* 58 (01); 7-10.

5. **Centers for Disease Control and Prevention.** 2011. *Reported Tuberculosis in the United States, 2010*. Atlanta, GA: U.S. Department of Health and Human Services, CDC, (p.140).

6. **Clinical and Laboratory Standards Institute (CLSI).** 2009. Statistical Quality Control for Quantitative Measurements: Principles and Definitions; Approved Guideline-Third Edition. CLSI document C24-A3 (ISBN: 1-56238-613-1) Clinical and Laboratory Standards Institute. Wayne, Pennsylvania,

7. **Clinical and Laboratory Standards Institute (CLSI).** 2006. Molecular diagnostic methods for infectious diseases; approved guideline - Second Edition. CLSI document MM3-A2 (ISBN 1-56238-596-8) Clinical and Laboratory Standards Institute. Wayne, Pennsylvania.

8. **Della-Latta. P. and V. Jonas**. 1999. Inhibitory effect of Alpha-Tec XPR-Plus phosphate buffer on the enhanced Hologic Amplified Mycobacterium Tuberculosis Direct Test. J. Clin. Microbiol. **37:**1234-1235.

9. **Kent, P. T. and G. P. Kubica**, 1985. Public health mycobacteriology: a guide for the level III laboratory. U.S. Department of Public Health and Human Services, Atlanta, GA.

10. **Manual of Clinical Microbiology – 9th Edition.** 2007. ASM Press, Washington, D.C. (ISBN-10:1-55581-371-2 (set)).

11. **Van Soolingen, D**., et al. 1997. A novel pathogenic taxon of the Mycobacterium tuberculosis complex, canetti: characterization of an exceptional isolate from Africa. Intl. J. Syst. Bacteriol. **47:**1236-1245.

IVD

Hologic, Inc.
10210 Genetic Center Drive
San Diego, CA 92121 USA

Customer Support: +1 844 Hologic (+1 844 465 6442)
customersupport@hologic.com

Technical Support: +1 888 484 4747
molecularsupport@hologic.com

For more contact information visit www.hologic.com.

IN0014 Rev. 001
2015-03