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MGIT™ Procedure Manual

For BACTEC™ MGIT 960™ TB System
(Also applicable for Manual MGIT)

Mycobacteria Growth Indicator Tube (MGIT)
Culture and Drug Susceptibility Demonstration Projects

by

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TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

Section V: Appendices

PREFACE

The purpose of this procedure manual is to provide additional procedures and comprehensive instructions which may not be included in the package inserts of the BACTEC™ MGIT™ 960 System. These measures and instructions should help in starting a new liquid culture system in a laboratory, especially in developing countries. This procedure manual provides guidelines for the BBL® MGIT™ System, which is a manual system, and for BACTEC MGIT 960 TB System, which is an automatic instrument system. Because contamination of liquid media is a concern for new users, special emphasis is given and guidelines for contamination control have been provided in different sections of the manual.

Every laboratory using the Mycobacteria Growth Indicator Tube (MGIT™) System should keep this manual readily available and should use this document as a reference for mycobacteriology procedures, particularly for the MGIT System. Optimal performance is achievable only if these procedures are strictly followed.

Further changes in the procedure or products, if needed, will be communicated with replacement pages which should be inserted into your copy of this manual. For further procedural details used conventionally in mycobacteriology laboratories, please refer to Clinical Microbiology Procedure Handbook (Section 7) and Public Health Mycobacteriology Level III Guide, CDC Handbook.[1, 2]

Section I: Principle of Procedure

A. Introduction

Demonstration of acid-fast bacilli (AFB) in a smear made from a clinical specimen provides a preliminary diagnosis of mycobacterial disease, while the isolation of mycobacteria on culture provides a definite diagnosis of tuberculosis or disease due to mycobacteria other than *M. tuberculosis* (MOTT bacilli) or non-tuberculous mycobacteria (NTM). As much as 50-60% of AFB culture-positive clinical specimens may fail to reveal AFB on smear made from the specimen. As a consequence, culture techniques play a key role in the diagnosis of mycobacterial disease.

Egg-based media, such as Lowenstein-Jensen (LJ) or Ogawa have been used for cultivation of mycobacteria for several decades. In 1958, Middlebrook and Cohn described an agar-based medium to permit more rapid detection of mycobacterial growth.[3] However, it still required an average of 3-4 weeks to recover mycobacteria from clinical specimens.

In 1969, Deland and Wagner developed a technique for semi-automated detection of the metabolism of bacteria by measuring the $^{14}CO_2$ liberated during the growth and decarboxylation of ^{14}C-labeled substrate incorporated in the growth medium.[4] This radiometric technique was widely used for blood culture using the BACTEC 460 instrument. In 1980, this technique was introduced commercially for mycobacterial recovery from clinical specimens and drug susceptibility testing. A large number of clinical trials were carried out to compare the radiometric BACTEC 460 TB System with solid media for primary isolation and drug susceptibility testing. Several evaluations of the BACTEC 460 TB System published between 1980 and 1985 demonstrated excellent results with significant time savings,[5,6,7,8.9,10] especially from smear negative specimens.[6]

The BACTEC 460 TB System has been reported to yield 15-20% increased culture positivity of clinical specimens as compared to conventional solid media such as LJ medium, with an average time-to-detection of positive growth from 8 to 14 days as compared to 3 to 5 weeks on solid media. The introduction of the BACTEC 460 TB System revolutionized laboratory testing for mycobacteria and has established itself as the gold standard for culture and susceptibility testing.

The high efficiency of the BACTEC TB System is due to the use of liquid medium. Moreover, a growth enhancing substance is added to the medium to further reduce the detection time.[11] Since the introduction of the BACTEC 460 TB System, it has been established that liquid medium is far superior to solid media for recovery, time-to-detection and drug susceptibility testing. Certain species of mycobacteria are reported to grow in liquid medium only, thus failing to be detected on solid media.[12] In 1993, the Centers for Disease Control and Prevention (CDC) recommended that every clinical laboratory must use a liquid medium to isolate mycobacteria in conjunction with solid media.[13] A follow-up survey indicated an increasing trend of using liquid medium for achieving rapid and maximum recovery of mycobacteria from clinical specimens.[14]

Liquid media is more prone to contamination with bacteria that are commonly present as normal flora in certain types of clinical specimens and sometimes survive the decontamination process. Thus, addition of antimicrobials is needed to suppress contamination in liquid media. With the BACTEC 460 TB System, an antimicrobial mixture called PANTA™ (Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim, Azlocillin) is used for this purpose and reduces the contamination rate close to that generally experienced with solid media.[15] Some PANTA formulation is also used in newer liquid media that have been developed in recent years.

One of the disadvantages of the BACTEC 460 TB System is the use of ^{14}C-Labeled radioactive substrate. Because of the strict regulations of handling and waste disposal of radioactive material, it became necessary to develop a non-radiometric technique for mycobacterial culture and susceptibility testing. Becton, Dickinson and Company (BD) developed a new system called Mycobacteria Growth Indicator Tube (MGIT™), which is non-radiometric and offers the same rapid, sensitive and reliable methods of testing as the BACTEC 460 TB System. BBL MGIT™ System is the manual system while BACTEC MGIT 960 (MGIT 960) is the fully automatic system for detection of mycobacterial growth and drug susceptibility testing of *M. tuberculosis.* Numerous studies have been carried out using MGIT System for primary isolation of mycobacteria as compared with LJ and Ogawa media[16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26] and with BACTEC 460 TB.[27, 28, 29, 30, 31, 32, 33, 34, 35, 24, 36, 25, 37, 38, 39, 40] Similarly, testing for drug susceptibility by MGIT has been thoroughly evaluated.[41, 42, 43, 44, 45, 46, 4748, 49, 33, 50, 51, 52] MGIT susceptibility testing for PZA produces results similar to the BACTEC 460 TB system.[53, 54] Some investigators have also evaluated MGIT 960 for second-line and *M. avium* complex drug susceptibility testing.[55, 56] Better performance of MGIT, as compared with other commercially available TB liquid culture and molecular amplification systems, has also been reported.[57, 58, 59, 60]

B. Principle of the BACTEC™ MGIT™ 960 System

1. MGIT medium

The MGIT (Mycobacteria Growth Indicator Tube) consists of liquid broth medium that is known to yield better recovery and faster growth of mycobacteria. The MGIT contains 7.0 ml of modified Middlebrook 7H9 broth base. This medium is terminally sterilized by autoclaving. An enrichment, MGIT OADC (Oleic acid, Albumin, Dextrose and Catalase) or MGIT 960 Growth Supplement, is added to make the medium complete. This Growth Supplement is essential for growth of many mycobacteria, especially those belonging to *M. tuberculosis* complex. Addition of the MGIT PANTA is necessary to suppress contamination.

2. Principle of detection and drug susceptibility testing

In addition to Middlebrook 7H9 liquid media, the MGIT tube contains an oxygen-quenched fluorochrome, tris 4, 7-diphenyl-1, 10-phenonthroline ruthenium chloride pentahydrate, embedded in silicone at the bottom of the tube. During bacterial growth within the tube, the free oxygen is utilized and is replaced with carbon dioxide. With depletion of free oxygen, the fluorochrome is no longer inhibited, resulting in fluorescence within the MGIT tube when visualized under UV light. The intensity of fluorescence is directly proportional to the extent of oxygen depletion.

MGIT tubes may be incubated at 37ºC and read manually under a UV light or entered into a MGIT 960 instrument where they are incubated and monitored for increasing fluorescence every 60 minutes. Growth of bacteria as well as mycobacteria increases the fluorescence. In case of *M. tuberculosis*, at the time of positivity, there are approximately $10^5 – 10^6$ colony-forming units (CFU) per ml of medium. The instrument declares a tube negative if it remains negative for six weeks (42 days). The detection of growth can also be visually observed by the presence of a non-homogeneous light turbidity or small granular/flaky appearance in the medium. Growth of some NTM (most commonly rapid growers) results in light turbidity, while contaminating bacteria generally produce heavy turbidity.

Drug susceptibility testing can be performed based on the same principle. Two MGIT tubes are inoculated with the test culture. A known concentration of a test drug is added to one of the MGIT tubes, and growth is compared with the MGIT tube without the drug (growth control). If the test drug is active against the isolated mycobacteria, it will inhibit the growth and thus there will be suppression of fluorescence, while the growth control will grow uninhibited and will have increasing fluorescence. Growth is monitored by the BACTEC 960 instrument which automatically interprets results as susceptible or resistant.

Section II: Procedure for primary isolation

A. Introduction

Mycobacteria Growth Indicator Tube (BBL MGIT) contains modified Middlebrook 7H9 broth base. When supplemented with MGIT Growth Supplement and PANTA, it provides an optimum medium for growth of a majority of mycobacterial species. All types of specimens, pulmonary as well as extra-pulmonary (except blood), can be inoculated into MGIT for primary isolation of mycobacteria. Urine specimens have not been evaluated by BD but other investigators have reported successful isolation of mycobacteria from urine specimens. Mucoid specimens are expected to contain contaminating bacteria as normal flora and must be digested (liquefaction) and decontaminated before inoculation. On the other hand, aseptically collected body fluids or tissue biopsies do not need to be decontaminated. However, since it is difficult to maintain sterile conditions throughout the collection of specimens, it is recommended that all specimens be decontaminated. Aseptically collected specimens need only light decontamination. Clinical specimens collected in large volumes (of more than 10 ml) require centrifugation before decontamination to reduce the overall volume and to concentrate mycobacteria present in the specimens into a smaller volume. After decontamination, the specimen should be centrifuged again and the sediment used for preparation of smear and inoculation for culture.

B. Important Safety Precautions

Perform all procedures, such as processing of specimens, smear preparation, inoculum preparation, making dilutions, inoculation of media, and subculturing in a suitable biological safety cabinet in a room dedicated for mycobacterial work. The CDC has recommended a Biosafety Level (BSL) 2 laboratory with negative air pressure and with an appropriate ventilation system for mycobacterial work.[2] More recently, the CDC has recommended that work involving manipulation of TB cultures, such as DST, be done in a BSL 3 laboratory.[61] However, this work may be done in a BSL 2 laboratory providing the exhaust air from the laboratory is discharged to the outdoors, the ventilation is balanced to provide directional airflow into the room, access to the room is restricted when work is in progress, and the practices and equipment recommended for BSL-3 are followed. This includes use of proper protective gowns, gloves and respirator masks (approved by OSHA) while handling specimens and mycobacterial cultures. International Safety Standards, along with the local specifications, may also be followed.

Use an appropriate mycobacterial disinfectant such as Amphyl® for cleaning the work area. The CDC states: “With so many disinfectants available, it is important to consult the product brochures to make certain the disinfectant is bactericidal for mycobacteria.”[2]

Prior to use, examine all MGIT tubes for evidence of damage. Do not use any tube that is cracked or has other defects. Do not use a tube if the medium is discolored, cloudy or appears to be contaminated. Comprehensive reviews of laboratory safety procedures may be found in recognized publications of the Centers for Disease Control,[2, 61] the American Society for Microbiology[1] or other International and National guidelines.

C. Specimen Handling

1. Collection

Specimens should be collected in clean, preferably sterile containers with a tight-fitted lid or cap. At least two morning specimens collected on separate days should be processed for each new case. For patients with respiratory symptoms, the specimens should be expectorated sputum and not saliva, with a volume of about 2-10 ml each.

2. Transportation

Specimens should be transported to the laboratory as quickly as possible. Delays in transportation, especially in hot weather, result in an increase in contaminating bacteria that result in higher contamination rate of the medium. Specimens should be transported in a container, such as an ice box, in which temperature is maintained as low as possible. This is especially important in countries with high ambient temperatures.

3. Storage

Upon receipt, the specimens should be refrigerated and processed as soon as possible.

D. Digestion, Decontamination and Concentration

Numerous procedures for digestion and decontamination have been in use throughout the world. Some procedures are known to be compatible with egg-based media only and may not be used with any other medium not containing egg yolk. These procedures include Zephiran-Trisodium Phosphate (Z-TSP), Sodium Lauryl Sulphate, Cetylpyridinium chloride (CPC) or other quaternary ammonium compounds.

It is extremely important to follow the standard procedure for decontamination recommended for MGIT in order to obtain optimal results. Detection of growth in MGIT is based on an oxygen sensor system, and high concentration of N-Acetyl L-Cysteine (NALC) or sodium hydroxide (NaOH) may result in false fluorescence. Processing of specimens may vary according to their type. The following is a general outline of procedures for different types of clinical specimens.[1, 2]

1. Sputum

Proper sputum collection is extremely critical for best results, and early morning specimens are preferred. The specimen should be expectorated sputum and not saliva that often would not yield correct results. A specimen should be between 2-10 ml in volume. Ideally, from a new patient, three specimens should be collected on consecutive days and should be processed separately. WHO recommends two morning specimens and a third spot specimen when a patient visits the clinic. Pooled specimens are not recommended.

a. NaOH-NALC procedure

This is the standard recommended procedure to be used with MGIT, which is also recommended by CDC.[2] In this procedure, the initial concentration of NaOH is 4%. This 4% NaOH solution is mixed with an equal quantity of sodium citrate solution (2.9%) to make a working solution (NaOH concentration in this solution is 2%). When an equal quantity of NaOH-NALC-citrate and sputum are mixed, the final concentration of NaOH in the specimen is 1%. (For the procedure for the preparation of NaOH-NALC, see Appendix B). Commercially prepared NaOH-NALC-sodium citrate solution and phosphate buffer are also available (BD MycoPrep). Use of MycoPrep would minimize the use of non-standard, digestion solutions (Cat. No. 240862, 240863).

Materials and Methods

Materials Required:

- Disposable 50 ml plastic tubes (Falcon tubes)
- Sterile NaOH-NALC-sodium citrate solution, preferably, MycoPrep
- Phosphate buffer pH 6.8 (0.067M). Commercially prepared (MycoPrep) or lab prepared and sterilized
- Centrifuge with a minimum 3000-3500x g force and safety shield (refrigerated centrifuge is preferred)
- Vortex mixer, shaker
- Timer
- Pipettes/transfer pipettes or a pipettor with cotton plugged pipette tips

- If specimen is not collected in a 50 ml centrifuge tube, transfer it to a 50 ml centrifuge tube with a screw cap.

- Add NaOH-NALC-sodium citrate solution in a volume equal to the quantity of specimen. Tighten the cap.

- Vortex lightly or hand mix for about 15-30 seconds. Invert the tube so the whole tube is exposed to the NaOH-NALC solution.

- Wait 15-20 minutes (up to 25 minutes maximum) after adding the NaOH-NALC solution. Vortex lightly or hand mix/invert every 5-10 minutes or put the tubes on a shaker and shake lightly during the whole time.

- Make sure the specimen is completely liquefied. If still mucoid, add a small quantity of NALC powder (30-35 grams) directly to the specimen tube. Mix well.

- At the end of 15-20 minutes, add phosphate buffer (pH 6.8) up to the top ring on the centrifuge tube (plastic tube has a ring for 50 ml mark). Mix well (lightly vortex or invert several times). Addition of sterile water is not a suitable alternative for the phosphate buffer.

- Centrifuge the specimen at a speed of 3000 g or more for 15-20 minutes. Use of refrigerated centrifugation at a higher speed is known to increase recovery of mycobacteria.[2, 62]

- After centrifugation, allow tubes to sit for 5 minutes to allow aerosols to settle. Then carefully decant the supernatant into a suitable container containing a mycobactericidal disinfectant. Make sure the sediment is not lost during decanting of the supernatant fluid. Add a small quantity (1-2 ml) phosphate buffer (pH 6.8) and resuspend the sediment with the help of a pipette or vortex mixer.

- Use the resuspended pellet for making smears and for inoculation of MGIT tubes and other media.

b. Other procedures

These methods are routinely used in laboratories for <u>other culture systems</u>.

- **NaOH Method**: Sodium hydroxide (NaOH) alone (Petroff's Method) is used with the starting concentration of 3-4% NaOH. A higher concentration of NaOH could be toxic to mycobacteria and could affect the oxygen sensor adversely. The procedure is the same as the one for NaOH-NALC. Add buffer after 20 minutes of decontamination and after centrifugation to reduce the pH.

- **Oxalic Acid Method**: Conventionally, oxalic acid (5% aqueous solution) is used only for those specimens which have a persistent *Pseudomonas* contamination problem. Neutralization of the specimen with an alkali after digestion and decontamination is preferred.[2] This method has not been validated for MGIT.

- **Sulfuric Acid Method**: This method is not commonly used and has not been validated for MGIT. It is recommended only for urine specimens or those specimens which cannot be processed by NaOH or NaOH-NALC method due to persistent problems with bacteria contamination.

- **Zephirain-Trisodium Phosphate Method** (Z-TSP): This method is compatible with egg-based media only and does not work with any other solid or liquid media.

DO NOT USE THIS METHOD FOR MGIT!

- **Cetylpyridinium Chloride (CPC) Method**: A mixture of 1.0% CPC and 2.0% NaCl is used as a transport medium as well as decontamination reagent. This is a slow acting decontamination reagent. This method is not compatible with a non egg-based medium.

DO NOT USE THIS METHOD FOR MGIT!

- **Benzalkonium Chloride and Lauryl Sulfate Methods:** These methods are not compatible with non-egg-based media and should not be used with the MGIT system.

c. Important points

- NaOH is bactericidal for contaminating bacteria. It is also harmful for mycobacteria but to a much lesser extent. NaOH also helps in liquefying the specimen.

- NALC only liquefies the specimen and has no decontamination properties.

- The final pH of the specimen concentrate greatly affects the recovery and time-to-detection of mycobacteria.

- High pH will lower the positivity rate and increase the time-to-detection of positive culture.

- High pH may also cause transient false fluorescence.

- Keep the pH as close to neutral as possible. It is not necessary to neutralize the processed specimen, especially with the NaOH-NALC method. Some laboratories routinely neutralize the processed specimen. The neutralization step needs to be controlled very carefully.

- With NaOH-NALC digestion, do not agitate the tube vigorously. Extensive aeration causes oxidation of NALC and makes it ineffective.

- If the specimen has some blood mixed with it, do not use NaOH-NALC method because NALC does not work in the presence of blood. Use the NaOH method[2] instead.

- Mycobacteria, being hydrophobic, are hard to centrifuge down. Lower centrifugation speed (g-force) would not sediment mycobacteria very well and some bacteria would be lost during decanting the supernatant, which will affect the positivity rate. Higher centrifugation speeds and longer time (maximum 25 minutes) result in a better concentration of mycobacteria, which positively affects smear and culture positivity.[62]

- Temperature increase during centrifugation increases the killing effect on mycobacteria which will decrease the positivity rate and increase time-to-detection.[2]

- A refrigerated centrifuge with at least 3000x g force is ideal. If a refrigerated centrifuge is not available, avoid temperature build-up, especially if the room temperature is high. Add refrigerated (chilled) phosphate buffer before centrifugation which should help in keeping the temperature low.

- Other reagents during the digestion/decontamination step should not be refrigerated but kept at room temperature. Lower temperatures reduce the digestion decontamination process of NaOH-NALC.

2. Specimens other than sputum (extra-pulmonary)

a. Pus and other mucopurulent specimens

If the specimen is thick or mucoid and less than 10 ml in volume, digest and decontaminate with NaOH-NALC method similar to the procedure used for sputum specimens. If the specimen is not thick, it may be treated with 2-4% NaOH. The concentration of NaOH depends upon the contaminating bacteria expected to be present in the specimen. If the volume is over 10-12 ml, process only 10 ml or first concentrate by centrifugation at 3000x g for 15-20 minutes. In such a situation, if the specimen is thick, liquefy the specimen by adding a small quantity of NALC only (50-100 mg powder) and mix well. After the concentration step, resuspend the sediment in 5 ml sterile water, decontaminate with NaOH and concentrate again by configuration. Always resuspend the sediment (pellet) in buffer to reduce the pH.

b. Gastric aspirates

Concentrate by centrifugation before decontaminating. Resuspend the sediment in about 5 ml of sterile water and decontaminate with NaOH-NALC or 2-4% NaOH as recommended for sputum. After decontamination, concentrate again prior to inoculation of the sediment into culture media. Due to the low pH, gastric aspirates should be processed as soon as possible (within 4 hours of collection). If the specimen cannot be processed quickly, it should be neutralized with NaOH before transportation or storage.

c. Bronchial washings

All other pulmonary specimens, such as bronchial washings (BAL) may be treated as sputum. If the specimen is up to 10 ml in volume, process the whole specimen. For larger volumes, concentrate the specimen by centrifugation (3000x g, 15-20 minutes). If the specimen is thick or mucoid, liquefy by adding a small quantity of NALC powder (50-100 mg). After centrifugation, resuspend the sediment in 5 ml sterile water and decontaminate like sputum.

d. Laryngeal swabs

Transfer the swab into a sterile centrifuge tube and add 2 ml sterile water. If necessary, break off the swab stick so the cap of the centrifuge tube can be placed on it and tightened. Add 2 ml of NaOH-NALC solution replace the cap and mix well in a vortex mixer. Let stand for 15 minutes. Remove the swab by with forceps, squeezing the liquid out of the swab and discarding it. Fill the tube with phosphate buffer. Mix and centrifuge at about 3000x to 3500 g for 15-20 minutes. Discard the supernatant fluid and resuspend the sediment in 1-2 ml sterile buffer. Use this suspension for smear and culture.

e. Tissue

Tissue biopsies are generally collected aseptically and therefore decontamination procedures are not required. Homogenize the tissue in a tissue grinder with a small quantity of sterile saline or water (2-4 ml). All steps must be done in a biological safety cabinet (BSC) and all equipment must be sterile. Decontaminate the homogenized specimen following the same NaOH-NALC procedure as in sputum. After resuspension of the sediment with phosphate buffer, inoculate 0.5 ml MGIT tube. If the tissue grinder is not available, use a mortar and pestle. Tissue may also be placed in a Petri dish with sterile water (2-4 ml) and be torn apart with the help of two sterile needles. Work under the hood and use sterilized materials.

f. Urine

Isolation of mycobacteria from urine specimens has not been validated due to a very small number of urine specimens in BD clinical trials. Some investigators have successfully used BACTEC 460 TB and MGIT medium for isolation of mycobacteria from urine.[63, 64] As a routine isolation method, a totally voided, early morning urine specimen is used for mycobacterial culture. Pooled or mid-stream urine specimens are not recommended. The specimen is concentrated by centrifugation using several 50 ml centrifuge tubes (with screw caps) for at least 20-25 minutes. Resuspend the sediment in each tube with 1-2 ml sterile water and then pool together (total volume 5-10 ml). Decontaminate the concentrated specimens with 4% NaOH for 15-20 minutes. After decontamination, proceed in a manner similar to sputum.

g. Other body fluids

Body fluids, such as CSF, synovial fluid and pleural fluid are collected aseptically and thus can be inoculated into MGIT medium without decontamination (with the addition of PANTA). However, since sterility is not guaranteed, it is recommended these specimens should be lightly decontaminated. If the specimen volume is more than 10 ml, concentrate by centrifugation at about 3000-3500x g for 15-20 minutes. Liquefy thick or mucoid specimens prior to centrifugation by adding NALC powder (50-100 mg). After centrifugation, resuspend the sediment in about 5 ml of saline and then decontaminate following the procedure similar to that for sputum. Isolation of mycobacteria from blood specimens has not been evaluated thoroughly. A few studies have been published or presented where blood was used with MGIT System after lysis centrifugation.[65] BACTEC Myco/F Lytic medium is recommended for isolation of mycobacteria and fungi from blood samples.

E. Smears for Acid-Fast Bacteria (AFB)

1. Smear preparation

Prepare smears from all processed specimens before inoculation into medium. Details of the procedure are given in the CDC Procedure Handbook[2] or any other reference mycobacteriology book.[1] The procedure is outlined as follows:

a. After digestion/decontamination, concentration and resuspension of the pellet mix the specimen well with a pipette and place about one drop or 2-3 loopfulls on a clean microscope slide.

b. Spread the smear about 1½ cm x 1 cm.

c. Allow the smear to air dry completely.

d. Heat-fix the smear either by passing over the flame three to four times or by heating on a slide warmer at 65-75ºC for 2-3 hours or overnight. Do not overheat or expose smear to UV light.

e. Perform all the above procedures in a biological safety cabinet. Handle the smear carefully since mycobacteria may still be viable.

2. Staining methods

Commercially prepared staining kits are available; for example, BD, Ziehl-Neelsen Staining Kit, BD Kinyoun's Staining Kit, BBL Two-Step Quick Stain, BBL Fluorescent Staining Kit (see Appendix A for details). These stains would give optimal results because they have been quality controlled. However, if the stains are to be made in the laboratory, follow procedures given in Appendix B. If commercially prepared stains are used, follow manufacturer's recommendations. Information about different stains is given in Appendix B.

a. Ziehl-Neelsen staining

This method is used for staining smears made from specimen if fluorochrome staining is not available. It is also used to stain fluorochrome positive smears for confirmation, and for staining smears made from positive cultures.

- Flood the slide with carbol fuchsin stain. You may stain by covering the smear with filter paper and flooding the smear with the stain.
- Heat gently until steam rises (electric stainer may be used for heating.) Do not allow the stain to boil or dry. Keep adding stain as it dries during heating.
- Stain for 5-10 minutes; allow cooling.
- Wash gently with water.
- Decolorize with acid alcohol for 2 minutes or until no more color appears with acid alcohol.
- Wash gently with water. Drain excess water.
- Pour Methylene Blue (counter stain) on the smear and leave for 2 minutes.
- Wash gently with water. Drain excess water.
- Air-dry and observe under microscope. Do not blot dry as it may remove smear accidentally.

b. Kinyoun's staining

The procedure for staining is the same as with the Ziehl-Neelsen method, except heating carbol fuchsin is not necessary since it is a cold staining method (see Appendix B). Kinyoun's stain is used in place of the Ziehl-Neelsen (ZN) method.

c. Two-step staining

The BD Quick Staining Kit is available and does not require heating or decolorizing (see Appendix A). This is a superior method because the decolorizing step has been eliminated by adding the decolorizing agent into the counter stain. This is a replacement for Ziehl-Neelsen or Kinyoun's staining method. The procedure for staining is the same as the Kinyoun's method except there is no decolonization step. After washing the carbol fuchsin stain, apply the counter stain. Follow the procedure recommended by the manufacturer.

d. Fluorochrome acid-fast staining

This method is recommended for quick screening of large numbers of specimens for the presence of acid-fast bacteria (AFB). Commercially prepared stains are available for fluorescent staining (BD Brand Fluorescent Stain – see Appendix A). Among their advantages, fluorochrome stained smears are much quicker to screen, which offers time and labor savings over Ziehl-Neelsen or other carbol fuchsin methods. Screening can be done faster using lower magnification (250x to 450x magnification) compared to the ZN staining (800x-1000x magnification) and thus, a larger area can be covered within the same timeframe. Fluorochrome staining is considered more sensitive than the ZN staining method for detecting AFB on a smear. However, it requires an expensive UV microscope, and since the stained smears are not stable they should be read preferably the same day (overnight refrigerated storage is acceptable). It is recommended that fluorochrome smears that are positive for AFB should be re-stained with any of the carbol fuchsin staining methods to confirm the results, looking for AFB morphology which cannot be easily detected in fluorochrome stains.[2] This practice should be followed at least during the initial phase of starting fluorochrome staining and also for periodic quality control checking of this method. Fluorochrome staining is not recommended for smears made from positive cultures.

Two fluorescent stains commonly used are Auramine O and Auramine-Rhodamine. With Auramine O staining, mycobacteria appear bright yellow fluorescent color while with Auramine-Rhodamine staining mycobacteria develop yellow-orange fluorescent color. The staining procedure is the same for both stains.

Procedures

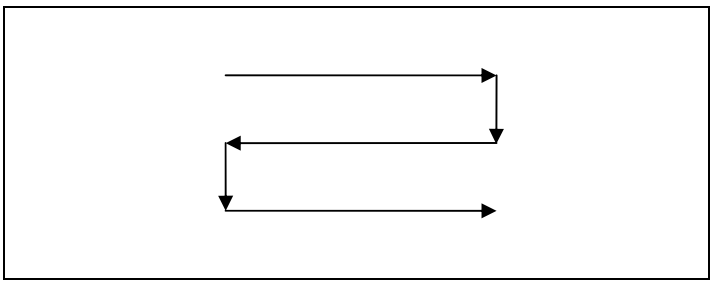
- Shake the bottle of stain and flood the slide with the stain covering the whole smear all the time (no filter paper, no heating).
- After 15 minutes, rinse the slide gently with water. Drain excess water.
- Flood the slide with 0.5% acid alcohol (decolorizer) and leave for 2 minutes.
- Gently rinse the slide with water again. Drain.
- Cover the smear with potassium permanganate solution (counter stain) and leave for 2 minutes. Do not over expose the smear to counter stain.
- Rinse with water again.
- Air-dry and examine the smear under 10x, then 40x objective using a UV light microscope for acid-fast bacilli.

Caution: Use chlorine-free water as chlorine may interfere in the fluorescence. Preferably, use distilled or deionized water. The time for counter stain is critical. Do not exceed 2 minutes with potassium permanganate. Do not blot dry.

3. Microscopy

It is important to examine smears very carefully. Use a good binocular light microscope with oil immersion (100x) objective and a 10x eye piece (total magnification 1000x for ZN smears). Examine a minimum of 100 fields for each smear before reporting as negative. For smears found 3+ to 4+, only a few fields may need to be examined. Report results as soon as possible.

Below is the recommended procedure of smear examination. Lines with arrows indicate movement of the field observation by moving the slide under the lens of the microscope.



4. Reporting

Report smear results as soon as they are available. Negative as well as positive results should be reported. The CDC recommends the smear result should be reported within 24 hours of receiving the specimen.[13]

There are different criteria for degree of positivity which may be followed to quantitatively report the number of AFB seen on a smear. One of the quantitative reporting procedures recommended by the CDC is as follows.[2]

Number of AFB Seen	Report
0	Negative
1-2 AFB / Whole Smear	Doubtful positive. Confirm by observing another smear from the same specimen or from another specimen from the same patient.
1-9 AFB/100 Field	1+
1-9 AFB/10 Field	2+
1-9 AFB/Field	3+
>9/Field	4+

If smear positivity is doubtful with only 1-2 AFB seen on the whole smear, stain and examine another smear made from the same patient. Doubtful fluorochrome stained positive smears should be confirmed by Ziehl-Neelsen or any other carbol fuschin method.

It is important to run a positive and a negative quality control slide with each batch of stains. (See Section II-K for Quality Control.)

F. Preparation and Inoculation for Culture

1. Reagents

a. MGIT medium

The MGIT 960 tube contains 7.0 ml of modified 7H9 broth base. (***Note***: The manual MGIT tube is different in that it contains 4.0 ml of the medium.) The approximate formula, per 1000 ml of purified water, contains:

- Modified Middlebrook 7H9 broth base 5.9 gm
- Casein peptone 1.25g

Adjusted and/or supplemented as required to meet the performance criteria. There is a fluorescent sensor embedded in silicone on the bottom of the tube. The tube is flushed with 10% CO_2 at the time of filling and then capped with polypropylene screw caps. Keep the caps closed until you are ready to make any addition to the medium. Open the cap for as little time as possible.

b. MGIT growth supplement (enrichment)

MGIT growth supplement is provided for the BACTEC MGIT 960, 7 ml tube. For manual MGIT, a different enrichment (BBL MGIT OADC, 15 ml) is used. The enrichment must be added to the MGIT medium prior to inoculation of a specimen. MGIT growth supplement contains 15 ml of the following approximate formula:

- Bovine Albumin ----------50.0 gm
- Dextrose----------20.0 gm
- Catalase----------0.03 gm
- Oleic Acid---------- 0.1 gm
- Polyoxyethyline state (POES) ---- 1.1 gm

MGIT growth supplement, or OADC enrichment, is a sterile product. Handle aseptically. Do not use if turbid or if it appears to be contaminated.

Do not leave MGIT tube caps open after adding OADC. It is important to add the growth supplement in the biological safety cabinet to avoid contaminating the medium.

c. MGIT PANTA™

Contamination can be reduced by supplementing the medium with a mixture of antimicrobial PANTA prior to the inoculation of specimen.

Each vial of MGIT PANTA (for MGIT 960) contains a lyophilized mixture of the antimicrobials with the concentrations, at the time of production, as follows:

- Polymyxin B----------6,000 units
- Amphotericin B---------- 600 µg
- Nalidixic Acid----------2,400 µg
- Trimethoprim---------- 600 µg
- Azlocillin---------- 600 µg

For manual MGIT, the procedure for adding PANTA differs but the final concentrations of PANTA antimicrobials in the medium are the same in both the systems.

2. Procedures

a. Reconstituting PANTA

Reconstitute MGIT PANTA with 15.0 ml MGIT growth supplement. Mix until completely dissolved. Add 0.8 ml of this enrichment to each MGIT tube. The enrichment with reconstituted PANTA should be added to the MGIT medium prior to inoculation of specimen in MGIT tube. Do not add PANTA/enrichment after the inoculation of specimen. Do not store MGIT tube after the addition of enrichment/ PANTA.

b. Inoculation of MGIT medium

- Label MGIT tubes with specimen number.
- Unscrew the cap and aseptically add 0.8 ml of MGIT growth supplement/PANTA to each MGIT tube. Use of an adjustable pipettor is recommended.
- Using a sterile pipette or a transfer pipette, add up to 0.5 ml of a well mixed processed/concentrated specimen to the appropriately labeled MGIT tube. Use separate pipette or pipette tip for each specimen.
- Immediately recap the tube tightly and mix by inverting the tube several times.
- Wipe tubes and caps with a mycobactericidal disinfectant and leave inoculated tubes at room temperature for 30 minutes.
- Work under the biologic safety cabinet for the specimen inoculation.

c. Inoculation of additional media

It is customary to use two different types of media for maximum recovery of mycobacteria. With the MGIT system, maximum recovery of mycobacteria may be achieved by using an additional solid medium, most commonly an egg-based medium such as LJ is used.[13] The decision to use MGIT medium alone or in combination with an additional conventional solid medium should be made after reviewing each institution's own experience and requirements. Usually 0.1 to 0.25 ml of processed/concentrated specimen is inoculated onto solid medium.

d. Precautions

- One of the major sources of contamination in MGIT medium is environmental contaminants introduced during addition of growth supplement.

- Make all additions inside a biosafety hood.
- Do not open several tubes at a time.
- Open MGIT tube for as short a period of time as possible.
- A repeat pipettor is very helpful when adding the growth supplement.
- Always recap the tube tightly. If the cap is left loose, it may affect the detection of fluorescence.
- Volumes greater than 0.5 ml of decontaminated specimen may disturb the pH of the medium and may cause false fluorescence. This may also increase contamination or otherwise adversely affect the performance of the MGIT medium.

e. Incubation

Incubation Temperature: All inoculated MGIT (7mL) tubes should be entered in the BACTEC MGIT 960 instrument after scanning each tube (please refer to the BACTEC MGIT 960 Instrument Manual for details). It is important to keep the cap tightly closed and not to shake the tube during the incubation. This helps in maintaining the oxygen gradient in the medium. The instrument maintains 37ºC $\pm$ 1ºC temperature. Since the optimum temperature for growth of *M. tuberculosis* is 37ºC, make sure the temperature is close to 37ºC.

***Note*:** If a specimen is suspected of containing mycobacteria which require an optimum temperature other than 37°C (for example, *M. haemophilum, M. marinum, M. chelonae* and *M. ulcerans* require 30°C), then two sets of media should be inoculated, one in the instrument at 37ºC and the other in an outside incubator at 30ºC. These tubes can be monitored by using a UV light source (Wood's lamp) and can also be checked visually (refer to the BACTEC MGIT 960 Manual). Specimens from skin and open wounds should always be inoculated into duplicate MGIT tubes, one for 37ºC and the other for 30ºC.

Length of incubation: MGIT tubes should be incubated until the instrument flags them positive. After a maximum of six (6) weeks, the instrument flags the tubes negative if there is no growth. Some species such as *M. ulcerans* and *M. genavense* may require extended incubation time. If such species are expected to be present, incubate further for 2-3 weeks.

f. Detection of positive growth

The instrument signals a tube positive for growth, and an indicator green light shows the exact location of the positive tube in the drawer of the instrument. At this point, the tube should be removed and scanned outside the instrument. The tube should be observed visually. Mycobacterial growth appears granular and not very turbid while contaminating bacterial growth appears very turbid. Growth, especially of the *M. tuberculosis* complex, settles at the bottom of the tube.

Information about the time-to-detection of positive growth can be retrieved from the unloaded positives report. Time-to-detection of positive growth depends on several factors, such as:

- the number of viable AFB inoculated into MGIT tube;
- the type of species of mycobacteria, such as *M. tuberculosis and M. bovis* that grow more slowly than NTM, such as *M. avium* complex;
- certain types of specimens, such as specimens from extra-pulmonary sites, usually take a longer time to turn positive due to lower numbers of AFB present in the specimens;
- the treatment status of patients also plays an important role. Specimens from chronically treated patients with drug–resistant TB take a longer time to grow;
- processing of specimen influences the positivity as well. A high pH or very low pH may cause injury or death to mycobacteria during processing of the specimen. Thus, it takes longer for revival and growth of viable mycobacteria. In some instances, as many as 60-70% of the mycobacteria are killed during processing.[2]
- loss of mycobacteria during centrifugation is also significant.[62] If the centrifuge generates heat it will accelerate killing of mycobacteria during centrifugation. Insufficient centrifugation speed may not bring down all mycobacteria into the sediment since mycobacteria, being hydrophobic, are difficult to concentrate.

It has been reported that in a broth system, most of the mycobacterial species grow better and faster compared to the solid media.[25, 58, 66] This is especially true for slow growing mycobacteria such as the *M. tuberculosis* complex, particularly from treated patients. *M. avium*, which is a slow growing NTM on solid medium, grows much faster in liquid medium.

***Note*:** In some instances, especially if mycobacterial growth is extremely slow or there is less oxygen consumption during mycobacterial growth, there may be growth in the MGIT broth without the presence of fluorescence. It is recommended that at the termination of incubation protocol, all negative tubes should be observed visually for turbidity and growth before discarding. If there is any suspicion of growth, an AFB smear and subculture should be done. This eliminates chances of reporting false negatives. If MGIT results are not satisfactory due to poor recovery, delay in detection or high contamination rate, follow the instructions for troubleshooting (Appendix C). Quality Control for the reagents and products used in the isolation, as well as for the test procedure, is critically important for mycobacteriology laboratories (see Section II-K, Quality Control).

G. Work-up of Positive Cultures

1. AFB smear from a positive MGIT tube

Once a MGIT tube is positive by fluorescence or by visual observation, prepare a smear and stain with carbol fuchsin stain.

Procedure

- Use a clean slide.
- Mix the broth by vortexing and then by using a sterile pipette, remove and aliquot. Place 1-2 drops on the slide and spread over a small area (approx. 1½ x 1 cm).
- Let the smear air dry.
- Heat-fix the smear by passing it over a flame a few times or by using an electric warmer at 65ºC -70ºC for 2 hours to overnight. Do not leave the smear openly exposed to the UV light of the safety cabinet.
- Stain the smear with Ziehl-Neelsen, Kinyoun's or BBL® Quick Stain. Fluorochrome stain is NOT recommended. Air dry but do not blot dry.
- Place a drop of oil on the stained and completely dried smear and screen under a low power objective to locate stained bacteria. Switch to an oil immersion objective lens for detailed observation.
- If the broth appears turbid or contaminated, irrespective of AFB smear results, subculture on a blood or chocolate agar, or TSI, to rule out the presence of contaminating bacteria.
- If the smear is negative for AFB and the tube does not appear to be contaminated, i.e. broth is clear, re-enter the tube into the instrument for further monitoring. Repeat AFB smears after 1-3 days.

2. Dealing with contamination

Liquid media are more prone to contamination than solid media. It is extremely important to process specimens with extreme care, adhering very closely to procedures and recommendations. Following are guidelines for controlling excessive media contamination (for further details, please refer to Appendix C-3 Troubleshooting).

a. Bacterial contamination

The incidence of contamination with bacteria (other than mycobacteria) varies from laboratory to laboratory depending upon several factors. According to the CDC guidelines, up to 5% contamination rate is acceptable in cultures of clinical specimens on solid media.[2] A general recommendation is that 5% $\pm$ 2% is acceptable for all media types. However, for liquid media, slightly higher contamination may be accepted (up to 7-8%). Very low contamination rate (less than 3%) may indicate too harsh a decontamination process, which would also affect growth of mycobacteria and may reduce the positivity rate and increase time-to-detection of positive mycobacterial culture. On the other hand, a higher contamination rate (above 8%) may be due to the following reasons:

- Improper or under decontamination of specimen.
- Very mucoid specimens that are hard to liquefy may result in high contamination.
- Long storage and transportation time of the specimen after collection. In such situations, especially in hot weather, bacteria tend to overgrow and then are hard to kill by routine decontamination procedure.
- Use of non-sterile materials such as pipettes, tubes, etc.

Sometimes if reagents are prepared, stored in bulk and used for long periods of time, they may become contaminated. (For details of troubleshooting, refer to Appendix C-3).

Detection of contamination: Growth of contaminated bacteria will result in positive fluorescence. It is important to observe all fluorescent positive MGIT tubes visually for turbidity and to make an AFB smear.

If a MGIT tube broth is heavily turbid, contamination is suspected even if the AFB smear is positive. Usually contaminating bacteria cause heavy turbidity, although *M. tuberculosis* growth appears as particles without any significant turbidity, while some of the NTM may produce light turbidity. Contamination may be confirmed by the following method:

- Make a smear and stain with Ziehl-Neelsen stain. Presence of non-acid-fast contaminated bacteria on smear confirms contamination.

- Sub-culture a loopful of blood agar. If blood agar is not available use chocolate agar or brain heart infusion (BHI) agar plate. Several specimens (4) may be carefully inoculated on a plate (small streak for each specimen, properly labeled). Divide the plate and identify specimen number by a marker. Incubate these subcultures at 35ºC±1ºC and observe after 24-48 hours. If contaminating growth appears, confirm again by gram and ZN stain.

- If contamination is confirmed with negative AFB smear from the broth, discard the specimen and report as contaminated. If contamination is confirmed with positive AFB smear from the broth, repeat the isolation procedure (see below).

How to control high contamination rate: The following are steps to help reduce a high contamination rate during isolation of mycobacteria from clinical specimens.

Review all the procedures and make sure all recommended steps are followed closely. If high contamination persists, take the following measures:

- Increase the NaOH concentration (not more than 1.5% final concentration in the specimen). The increase in NaOH is known to decrease contamination rate. Do not increase the exposure time more than 25 minutes to NaOH-NALC solution.

- Increase the concentration of PANTA.

- PANTA concentration may be increased by reconstituting with a smaller volume of Growth Supplement. However, the increase of PANTA concentration should be carefully evaluated since higher concentration of some antimicrobials present in PANTA may adversely affect growth of some species of mycobacteria other than *M. tuberculosis*. Instead of 15.0 ml use 10.0 ml to reconstitute PANTA. Add the regular 0.8 ml volume in the MGIT tube.

- Do not change the NaOH concentration and PANTA at the same time. Try one procedure at a time and document improvement of results.

- If there seems to be a common source of contaminating bacteria (same kind of bacteria contaminating repeatedly), check sterility of all reagents. It is a good practice to dispense small quantities of reagents and use only one at a time. Leftovers should be discarded or re-sterilized.

- Try to reduce time between collection of specimens and processing. If a specimen needs to be stored, use refrigeration.

- Transport specimen with ice and in an insulated chest, especially in hot weather.

- Inverting the tube during the decontamination process helps decontaminate the inside surface of the top of the tube.

- If there is a persistent *Pseudomonas* contamination problem, oxalic acid procedure is known to be more efficient for killing these bacteria. However, it has not been validated for MGIT. Azlocillin in PANTA is very effective in the inhibition of *pseudomonas* growth; increasing the PANTA concentration may help.

b. Isolation of mycobacteria from contaminated or mixed cultures

Decontamination of contaminated culture: Usually more than one specimen is collected from a patient and it is not necessary to salvage a contaminated specimen if other specimens from the same patient are positive and not contaminated. However, if it is critical to have results on a particular specimen that was contaminated, the contaminated broth may be reprocessed to recover mycobacteria.

- Transfer the entire MGIT broth into a 50 ml centrifuge tube.
- Add an equal quantity of 4% sterile NaOH solution.
- Mix well and let stand for 15-20 minutes, mixing and inverting the tube periodically.
- Add phosphate buffer pH 6.8 after 15-20 minutes up to 40 ml mark. Mix well.
- Centrifuge at least at 3000x g for 15-20 minutes.
- Pour off the supernatant fluid.
- Re-suspend the sediment in 0.5 ml of buffer and mix well.
- Inoculate 0.5 ml into a fresh MGIT tube supplemented with MGIT growth supplement/PANTA.
- Leave inoculated tubes at room temperature for 30 minutes, and the place in the instrument and follow for observation of growth.

c. Isolation of mixed mycobacterial culture on Middlebrook Agar Plate

AFB-positive cultures with more than one mycobacterial species may be separated by streaking a small loopful of positive broth on a Middlebrook 7H10 or 7H11 agar plate. Spread the loopful on the full surface of the plate to achieve isolated colonies. Incubate the inoculated plate at 37ºC $\pm$ 1C in a plastic bag. Periodically observe for growth (up to 6 weeks).

d. Cross-contamination

Cross-contamination of mycobacteria from specimen to specimen is also known in mycobacteriology laboratories. Usually it happens during the processing of specimens, especially at the time when a NaOH-NALC solution is added to the specimen or when a buffer is added to the tubes. Aerosol generation or splashing during the addition causes cross-contamination by contaminating the next tube or by contaminating the reagent stock solution. Touching the lip of the specimen tube with the reagent container during pouring or adding of the reagent may also lead to high contamination. Sometimes stock solution of a reagent gets contaminated with mycobacteria commonly found in water (*M. gordonae, M. xenopi*). Aliquoting small quantities reduce the chances of cross-contamination. In the event of a cross-contamination episode, all reagents, equipment and biosafety cabinets must be thoroughly checked.

3. Sub-culturing a positive MGIT tube

It is always helpful to subculture a MGIT tube positive for mycobacteria on an LJ slant. At the time positive mycobacterial growth is detected, there is sufficient biomass to use this growth for making a smear, performing drug susceptibility testing, or performing other tests such as species identification. However, growth on solid medium is important for observation of colony morphology and chromogenicity, for biochemical testing, and speciation, or for a future reference. For subculture, inoculate approximately 0.1-0.2 ml of a well mixed positive MGIT broth on an LJ medium. This is especially important if MGIT is used as a stand-alone system. All subcultures should be incubated at 37ºC $\pm$ 1ºC and be examined periodically until good growth is detected.

4. Identification of isolated mycobacteria

Tentative differentiation may be made by the following observations:

a. Rate of growth. Generally, *M. tuberculosis*, *M. bovis* and, to some extent, *M. kansasii* are slow growers and take a longer time to turn positive in a MGIT tube as compared to other mycobacteria (NTM).

b. In liquid medium growth, *M. tuberculosis* appears granular, while growth of most NTM does not show flakes or granules; rather it forms uniform slight turbidity (except *M. kansasii*).

c. Smear made from a positive MGIT broth also helps in tentative differentiation of *M. tuberculosis* complex from other mycobacteria. Cultures belonging to the TB complex form typical clumps and serpentine cords while other mycobacteria appear as loose, smaller clumps and cording or single cells. *M. kansasii* may be difficult to differentiate because its morphology is closer to *M. tuberculosis*.[67] The identification of isolates grown in MGIT tube by molecular methods such as AccuProbe® (GenProbe Corp.) has been reported with good results.[57, 68, 69, 70] According to published reports, AccuProbe testing of positive specimens can be performed within 24 hours of confirmation of

positivity. The lateral flow immunochromatography test (CapiliaTB test) has also been reported for differentiation of the *M. tuberculosis* complex from NTM.[71] For complete speciation, other methods such as biochemical testing can be used.[2] Although HPLC analysis has been successfully carried out on positive 12B vials, HPLC analysis of growth in the MGIT tube has not been published yet.

H. Results / Reporting

If viable mycobacteria are present in an inoculated specimen, they will grow in the MGIT medium and will be detected visually as well as by fluorescence. Report results only when a MGIT tube is positive by the instrument and smear made from the positive broth is also positive for AFB. Do not report a positive culture unless smear made from the positive tube is definitely positive for AFB. In rare cases, a MGIT tube may be negative in the instrument but will be positive by AFB smear and/or subculture. In such a case, report positive results. Reports should be sent as soon as results are ready. In case the identification requires additional time, results may be reported as culture positive for AFB, identification pending. If possible, it is better to identify the *M. tuberculosis* complex by molecular probe or other rapid tests and report results once identification is known.

Negative cultures should be reported after completing the incubation protocol of the instrument and visual observation of the negative tubes. Contaminated cultures must be reported as contaminated after confirmation by smear/or subculture on bacterial medium.

Conventionally, reports are sent at the following points[2]:

a. Smear from specimen (fluorochrome or ZN) – Report positive or negative and the staining method used. (CDC recommendation within 24 hours of receipt of specimen.)

b. Culture – Positive (with confirmation by AFB smear). Preferably after completion of identification – *M. tuberculosis* complex or MOTT bacilli (CDC recommendation within an average of 14 days.) Speciate mycobacteria later and report.

c. Upon completion of the drug susceptibility test – susceptible or resistant to each test drug. (CDC recommendation within an average of 28 days.)

d. Culture – Negative upon completion of the incubation protocol (42 days).

I. Performance Characteristics

Generally, liquid media are reported to yield more positive cultures than the solid media with significant savings in time-to-detection of positive growth. Numerous studies have been presented in scientific meetings or have been published in journals comparing performance of BACTEC MGIT 960 with BACTEC 460TB System and with conventional solid media.[16, 17, 18, 19, 20, 21, 22, 24, 25, 26, 32, 66, 72]

J. Limitations of the Procedure

a. Colony morphology and pigmentation cannot be observed in a liquid medium.

b. Even if a single viable contaminating bacterium survives the decontamination and PANTA inhibition, it may contaminate the entire medium. Contamination may mask mycobacterial growth.

c. A positive culture from a clinical specimen cannot be correlated with colony forming units (CFU) present in the specimen which sometimes is used to establish important NTM infection.

d. MGIT tube that appears positive may contain a mixed growth of more than one type of mycobacteria. Faster growing mycobacteria may develop positive fluorescence prior to slower growing mycobacteria. Therefore, it is important to subculture positive MGIT tube on a Middlebrook agar plate if there is any indication of the presence of more than one species of mycobacteria on the AFB smear made from the culture.

e. Sometimes, excessive carryover of reducing agent or alkali may cause false fluorescence of the sensor for a short time.

f. The use of PANTA antibiotic mixture, although necessary for suppression of contaminating bacteria, may have some inhibitory effect on some mycobacteria other than *M. tuberculosis* complex. This inhibition varies from species to species and isolates within a species. However, overall isolation of NTM is higher in liquid as compared to solid media.

K. Quality Control

1. Quality control of AFB smear staining

Some laboratories only perform quality control with a fresh batch of stain. However, it is recommended to include a positive control and a negative control with each batch of slides for staining.

Prepare smears from positive cultures of *M. tuberculosis* (H37Rv, ATCC #27294 or H37Ra ATCC 21577); mycobacteria other than *M. tuberculosis* complex (NTM) may also be used for positive control. A suspension equivalent to McFarland number 0.5-1.0 turbidity standards must be used for making a positive smear. Smears can also be made from growth on solid medium. Bacterial suspension such as *E. coli* can be used for the negative control. All the controls should be examined before the slides from clinical specimens. The positive control should show good staining of AFB while the negative control should not show any AFB. Certain mycobacteria other than *M. tuberculosis*, especially the rapid growers, are easily decolorized and may give faint staining reactions. To monitor the quality of

microscopy techniques, have a second person look at a selected number of positive and negative smears, as well as those that have very few AFB on the smear. Compare results of the two technicians.

For external quality control (EQC) follow the standard procedures established in the laboratory.

2. Quality control (QC) testing of MGIT medium

Every new lot of MGIT medium and every new lot of the enrichment should be quality control tested by the user upon receipt and before it is used routinely.

a. QC strains

Cultures: The following three mycobacterial cultures are recommended for quality control testing.

- *M. tuberculosis*, H37Rv ATCC 27294
- *M. kansasii* ATCC 12478
- *M. fortuitum* ATCC 6841

If the ATCC reference strains of *M. kansasii* or *M. fortuitum* cannot be obtained, then a laboratory isolate which is well-characterized may be used as a quality control strain. Well-characterized strains will be available from the mycobacterial strain bank of TDR/WHO in late 2006.[73]

b. Preparation of culture suspension

- Subculture the above mycobacteria on several LJ slants.
- Incubate at 37ºC $\pm$ 1ºC.
- Observe growth visually.
- As soon as there is good, confluent and pure growth, use this growth for making suspension.
- Growth should appear within 10-15 days of subculturing and should be used within this period. Aged cultures would not give reliable results.
- Remove growth from the slant by carefully scraping the colonies off the slant with a sterile loop or sterile spatula made from wooden applicator sticks. Take extreme precaution not to scrape off any culture medium (which gives false turbidity measurement).

- Transfer growth into a screw cap tube containing 4 ml of sterile 7H9 broth and glass beads (6-10 beads, 1-2 mm diameter), which helps to break up clumps **(Tube A)**.

- Vortex the tube for at least 1-2 minutes. Make sure the suspension is well dispensed and very turbid (greater than McFarland #1 turbidity).

- Let the suspension stand undisturbed for 20 minutes.

- Using a transfer pipette, carefully transfer the supernatant to another sterile screw cap glass tube **(Tube B)**. Avoid picking up any sediment.

- Let this stand undisturbed for 15 minutes.

- Carefully transfer the supernatant into another screw cap glass tube **(Tube C)** without taking any sediment.

- Adjust the turbidity of suspension in Tube C to McFarland #0.5 turbidity standard by adding more 7H9 broth or sterile saline/deionized water and mix well. If the suspension is too turbid, transfer some of the suspension to another sterile tube and adjust the turbidity to McFarland #0.5 standard. This is the working suspension for QC testing. This suspension may be frozen in small aliquots (1-2 ml) in appropriate tubes/vials at -70ºC + 10ºC. The frozen suspension may be used up to 6 months. Once thawed, do not refreeze.

c. Preparation of dilutions

- Dilute the working suspension McFarland #0.5, freshly prepared or frozen) 1:5 by taking 1.0 ml of suspension and adding into 4.0 ml of sterile water or saline. Mix well **(Tube 1)**.

- Dilute two more times 1:10 by adding 0.5 ml of suspension Tube 1 into 4.5 ml of sterile water or saline **(Tube 2)**. Mix well and then again add 0.5 ml from Tube 2 to 4.5 ml of sterile saline or distilled/deionized water **(Tube 3)**. Mix well. Final dilution 1:500 (**Tube 3**). Stop here for *M. tuberculosis* and use Tube 3 for QC testing.

- For *M. fortuitum*, further dilute Tube 3 1:10. Take 0.5 ml of suspension from Tube 3 and add to 4.5 ml of sterile water or saline and mix well. Final dilution 1:5000 **(Tube 4)**. Use Tube 4 for QC testing.

- For *M.* kansasii, dilute Tube 4 once again 1:10 by adding 0.5 ml from Tube 4 to 4.5 ml of sterile saline/water, mix well. Final dilution 1:50,000 **(Tube 5)**. Use Tube 5 for QC testing.

d. Inoculation/incubation

- Supplement MGIT medium with Growth Supplement and PANTA as recommended.
- Inoculate 0.5 ml from **Tube 3** to each of two MGIT tubes for *M. tuberculosis*. Similarly, inoculate 0.5 ml from **Tube 4** for *M. fortuitum* and **Tube** 5 for *M. kansasii* into each of the two labeled MGIT tubes. Mix.
- Enter the inoculated tubes in the MGIT 960 instrument. Take the tubes out when indicated positive by the instrument. Retrieve data for time to positive.

e. Expected results

- *M. tuberculosis* Tube fluorescence positive in 6 to 10 days
- *M. kansasii* Tube fluorescence positive in 7 to 11 days
- *M. fortuitum* Tube fluorescence positive in 1 to 3 days

If the above criteria are not met, repeat the test. If QC test still does not give satisfactory results, check the viability of the inoculum, age of the culture if stored frozen and other procedures. If everything meets the established specifications, contact Technical Services at BD Diagnostic Systems.

f. Precautions

- Use freshly prepared suspension adjusted to McFarland #0.5 standard. If frozen (-70ºC $\pm$ 5ºC), thaw prior to use for each quality control testing. Do not store or refreeze once thawed.
- All work should be carried out in a proper biological safety cabinet.
- All materials should be sterilized by autoclaving prior to disposal.
- Follow all the recommended safety precautions.

3. Quality control of laboratory procedures

There should be periodic quality control checks of all the reagents used, such as NaOH-NALC and buffer, as well as for procedures followed in the laboratory. For better contamination monitoring, it is important to include a negative control with a batch of specimens to be processed. This could be done on a daily or weekly basis. Periodically, a positive control may also be included to monitor growth performance of a QC organism.

a. Positive and negative controls

For the negative control, use 5 ml of phosphate buffer and for the positive control use 5 ml of *M. tuberculosis* suspension (McFarland #0.5 turbidity) diluted 1:500 (see quality control procedure). Process negative and positive controls along with clinical specimens, using the same digestion, decontamination and concentration methods. Inoculate into fresh MGIT tubes and incubate similarly to other specimens. The positive control should show positive growth and time-to-detection should be within a specified time with each testing (this is established by data collected from the positive control after several tests). The negative control should show no growth within the incubation protocol period. If negative control shows positive fluorescence, check for the presence of bacteria/mycobacteria. If positive for growth, investigate procedures and all the reagents for possible source of contamination.

b. Quality control with laboratory data

For overall performance of laboratory procedure, periodic analysis of results helps in monitoring the lab efficiency and establishing good laboratory practices. Every 3 to 6 months calculate the following statistics:

- Smear Positive*****, Culture Positive******-Total #, Avg. Time-to-Detection
- Smear Negative, Culture Positive: Total #, Avg. Time-to-Detection
- Smear Positive, Culture Negative: Total #
- Smear Negative, Culture Negative: Total #
- Positive Control, Avg. Time-To-Detection
- Contamination: Total #, Avg. Time-To-Detection

***** Smear Positive – smear of the clinical specimen

****** Culture Positive – calculate for both MGIT and any other medium used, culture positive confirmed for AFB

If there is an abrupt shift in any category of specimen data, it would indicate some change in the laboratory practices or reagents. Increase in contamination rate indicates that all the procedures should be reviewed and corrective measures should be taken to achieve satisfactory results. If culture positivity or time-to-detection in MGIT is similar or lower as compared to the solid medium, procedures need to be re-evaluated since overall MGIT performance is expected to be better with earlier time-to-detection.

4. Record keeping

- Record the lot numbers for MGIT tubes, MGIT OADC, or MGIT Growth Supplement, MGIT PANTA and other reagents.

- Keep a record of the batch of specimens processed at one time, date of inoculation, person who did the work, time-to-positivity by fluorescence, smear results from positive tube, contamination, etc.

- Compare results of MGIT with those of solid medium.

Section III: Drug Susceptibility Testing

A. Primary Drug Susceptibility Testing (SIRE)

1. Introduction

Antimicrobial susceptibility testing is critical in prescribing an effective drug regime for a tuberculosis patient, especially in areas where drug resistance incidence is high. It is also important in the follow-up of patients who are on antimicrobial therapy but are not responding to therapy. Drug susceptibility testing is an integral part of the WHO DOTS-Plus program.

The BACTEC MGIT 960 susceptibility testing for Streptomycin (S), Isoniazid (I), Rifampin (R) and Ethambutol (E), called SIRE, is a rapid and qualitative procedure for establishing susceptibility of *M. tuberculosis* for the four drugs using critical test concentrations. In addition, higher test concentrations for S, I and E (high E not available in US) are also available in case of testing against higher concentrations is indicated.

2. Principles of the test

Isolated cultures from TB patients are subjected to growth in the presence of a known concentration of a test drug. A control is also included with no addition of the drug. If the patient's isolate grows in the control but does not grow in presence of the drug, it is considered susceptible. On the other hand, if it grows in both the tubes, then it is considered to be resistant to that drug.

There are several methods for susceptibility testing but the most common one is the proportion method. In the proportion method, resistance is established at the 1% level for most of the drugs. This means that if 1% or more of the total test bacterial population is resistant to a drug it is considered as resistant for clinical purposes. Historically, proportion method has used Middlebrook agar solid medium. After 3 to 4 weeks of incubation, the percentage of colonies on the drug medium, as compared with the drug-free medium, is calculated to establish resistance.

In 1980, a broth-based proportion method known as BACTEC 460TB radiometric susceptibility testing was introduced. In this method, a radiometric medium, BACTEC 12B, with a C^{14}-labeled substrate is used. The bacterial inoculum in the control is a hundredfold less than the inoculum in the drug-containing medium. The $^{14}CO_2$ produced during growth and metabolism of mycobacteria in this medium is measured and designated as the Growth Index (GI). Once the GI in the control reaches 30, usually after 4-6 days incubation (maximum 12 days), comparison of GI values in the drug-containing and drug-free medium establishes the proportion of resistance.[8, 9]

The BACTEC MGIT 960 susceptibility test was also established with similar principles, with the increase in the fluorescence in the sensor measured automatically and designated as growth value (GV). If a drug is added to the medium which is bacteriostatic or bactericidal to the test mycobacteria, it inhibits growth and thus, there is little or no oxygen consumption, therefore little or no fluorescence of the sensor.

The BACTEC MGIT 960 susceptibility test provides results similar to those obtained by BACTEC 460 Radiometric TB System within approximately the same timeframe. The BACTEC MGIT 960 SIRE susceptibility test concentrations are slightly less than those used in the method of proportion on solid media in order to avoid false susceptibility results. The BACTEC MGIT 960 susceptibility testing was evaluated thoroughly by comparing with Middlebrook 7H10 medium as well as with BACTEC 460TB System.[33, 41, 42, 43, 44, 45, 46, 47, 49, 50, 51]

3. Reagents

BACTEC MGIT 960 SIRE Kit for **critical concentrations** contains the following drugs in lyophilized form. Each kit contains one each of S, I, R, and E drug vial and 8 vials of MGIT 960 SIRE Supplement.

a. Drugs

- Streptomycin - approximate lyophilized drug per vial ---------- 332µg
- INH - approximate drug per vial ---------- 33.2 µg
- Rifampin – approximate drug per vial ---------- 332 µg
- Ethambutol – approximate drug per vial ----------1660 µg

b. SIRE supplement

The SIRE supplement vial differs from the MGIT Growth Supplement and contains, per liter of purified water, the following:

- Bovine albumin ---------- 50.0 g
- Dextrose---------- 20.0 g
- Catalase ---------- 0.03 g
- Oleic acid ---------- 0.6 g

c. Storage

Upon receipt, refrigerate the lyophilized drugs at 2-8ºC. Reconstitute prior to use. Once opened and reconstituted, the leftover drug solutions may be frozen in aliquots at -20ºC or lower and stored for up to 6 months or up to the date of original expiry, whichever comes sooner. Once thawed, discard the leftover and do not store or refreeze

4. Procedures

a. Reconstitution of lyophilized drugs

Reconstitute each critical concentration drug vial with 4 ml of sterile distilled/deionized water. Mix thoroughly and make sure the drug is completely dissolved.

b. Addition of a drug to the medium

Add 0.1 ml (100 µL) of reconstituted drug solution into each of the labeled BACTEC MGIT 960 tubes. This will result in the following critical concentration of drugs in the medium.

- Streptomycin ---------- 1.0 µg/ml of medium
- Isoniazid ---------- 0.1 µg/ml of medium
- Rifampin ---------- 1.0 µg/ml of medium
- Ethambutol ---------- 5.0 µg/ml of medium

c. Preparation of the inoculum

Inoculum from the MGIT tube: It is important that the growth is within the following recommended timeframe.

- The day a MGIT tube is positive by the instrument is considered **Day 0.**
- The tube should be kept incubated for at least one more day (**Day 1**) before being used for the susceptibility testing (may be incubated in a separate incubator at 37ºC ± 1ºC).
- A positive tube may be used for drug susceptibility testing up to and including the fifth day (**Day 5**) after it becomes instrument positive. A tube that has been positive for more than 5 days should be subcultured in a fresh MGIT tube supplemented with MGIT 960 Growth Supplement and should be tested in a MGIT 960 instrument until it is positive. Use this tube from one to five days of instrument positivity as described above.
- If growth in a tube is on **Day 1** or **Day 2**, mix well (vortex) to break up clumps. Leave the tube undisturbed for about 5-10 minutes to let big clumps settle on the bottom. Use the supernatant undiluted for inoculation of the drug set.
- If growth is on **Day 3, 4, or 5**, mix well to break up the clumps. Let the large clumps settle for 5-10 minutes and then dilute 1.0 ml of the positive broth with 4.0 ml of sterile saline. This will be a 1:5 dilution. Use this well mixed diluted culture for inoculation.

Inoculum from growth on solid medium: It is important to have fresh growth on a solid medium, such as an LJ slant (within 15 days of appearance of growth on the medium). Older cultures may result in unreliable susceptibility test results.

- Add 4 ml of BBL Middlebrook 7H9 broth to a clean sterile tube (approximately 16 x 128 mm in size) with 8-10 glass beads.

- Use growth on solid medium which is no more than 15 days old within 15 days of appearance of positive growth. Scrape as many colonies as possible with the help of a sterile loop or a sterile spatula made from wooden applicator sticks. Try not to take any medium when removing growth. Transfer the growth into the tube with broth and glass beads. Tighten the cap and Vortex the tube for 1-2 minutes to break the clumps. The turbidity of the suspension should be greater than the McFarland #1.0 standard

- Let the suspension stand undisturbed for 20 minutes.

- Carefully transfer the supernatant suspension with a pipette into another sterile tube. Avoid taking any growth which has settled on the bottom.

- Let this tube stand for another 15 minutes undisturbed.

- Carefully take the supernatant out with a pipette, without disturbing the sediment, and transfer into another sterile tube. The turbidity of this suspension should be greater than McFarland #0.5 standard.

- Adjust the turbidity of this suspension to McFarland #0.5 standard by adding sterile saline and adjusting by visual comparison. Do not make it below this standard.

- Dilute the above suspension 1:5 by adding 1.0 ml of the suspension to 4.0 ml of sterile saline. Mix well and use it as the inoculum for drug susceptibility testing.

d. Inoculation and incubation

- Label 5 MGIT tubes for each test culture. Label one for GC (growth control, without drug), one for STR, one for INH, one for RIF, and one for EMB.

- Aseptically add 0.8 ml of BACTEC 960 SIRE Supplement to each of the MGIT tubes. Use only MGIT SIRE Supplement and not MGIT Growth Supplement.

- Aseptically add 0.1 ml (100 microliter) or properly reconstituted STR drug in the STR labeled tube. Similarly, add other drugs in the other labeled tubes. It is important to add the correct amount of drug to each tube. If possible, use a well calibrated micropipette for each addition. Use a separate pipette or micropipette tip for each drug. Do not add any drug to the GC tube.

Drug	Concentration of drug after reconstitution *	Volume added to MGIT tube	Final concentration in MGIT tube
STR	83 µl/ml	100 ml	1.0 µl/ml
INH	8.3 µl/ml	100 ml	0.1 µl/ml
RIF	83 µl/ml	100 ml	1.0 µl/ml
EMB	415 µl/ml	100 ml	5.0 µl/ml

* The drugs should be reconstituted using 4 ml sterile deionized or distilled water to achieve the indicated concentrations.

Calculations of the dilution factor for MGIT medium: 7.0 ml of medium + 0.8 ml of SIRE Supplement + 0.5 ml of inoculum = 8.3 ml. Addition of 0.1 ml of the drug solution in 8.3 ml of the medium = 1:83 dilution.

- Aseptically add 0.5 ml of the well-mixed culture suspension (inoculum) into each of the drug containing tubes using a pipette. Do not add to the control.

- For the control, first dilute the test culture suspension 1:100 by adding 0.1 ml of the test culture suspension to 10.0 ml of sterile saline. Mix well by inverting the tube 5-6 times. Use this diluted suspension to add 0.5 ml into the growth control tube.

- Tighten the caps and mix the inoculated broth well by gently inverting the tube several times.

- Susceptibility test "Set Carriers" are provided in different numbers of drug combinations. For a routine SIRE test with critical concentration, a Set Carrier of five tubes is used (refer to BACTEC MGIT 960 User's Manual for details). Place labeled tubes in the correct sequence in the set carrier (GC, STR, INH, RIF, EMB).

- Enter the susceptibility set carrier into the BACTEC MGIT 960 instrument using the susceptibility test set entry feature. (Refer to the BACTEC MGIT 960 User's Manual, AST Instructions.) Ensure that the order of the tubes in the AST Set Carrier conforms to Set Carrier definitions. For example, GC, STR, INH, RIF, EMB for the SIRE standard testing.

- If you need to check purity of the inoculum, streak the test culture suspension onto a blood agar plate. If blood agar is not available, use chocolate agar or BHI agar. Incubate at 35 ºC $\pm$ 1ºC for 48 hours and check if there is any growth. If growth appears, do not set up the susceptibility test. It may be important to establish the purity of culture before setting up susceptibility test, particularly if contamination is suspected.

5. Testing at higher drug concentrations

It is recommended that SIRE drugs should always be tested at the critical concentration. However, in certain situations testing at a higher concentration is indicated. This is important in those isolates which have a low level of resistance; that is, an isolate is resistant at the critical concentration but susceptible at the higher concentration of the drug. Thus, many laboratories first test at the critical concentration of a drug and if an isolate is found resistant, then the higher concentration is tested.

Only Streptomycin, INH and Ethambutol are tested at the higher concentration. Among these three drugs, INH is the most important drug as clinicians may choose to continue INH in the therapeutic regime if the isolated culture of a patient is resistant at the critical concentration and susceptible at the higher concentration (called "low-level resistance"). The following higher concentrations of S, I, and E are available for BACTEC MGIT 960 System. BACTEC MGIT STR 4.0 Kit contains one vial of lyophilized streptomycin (664 µg) and two vials of SIRE Supplement.

BACTEC MGIT INH 0.4 Kit contains one vial of lyophilized isoniazid (66.4 µg) and two vials of SIRE Supplement. BACTEC MGIT EMB 7.5 Kit contains one vial of lyophilized ethambutol (1245 µg) and two vials of SIRE Supplement.

For the higher concentration of drugs, reconstitute the lyophilized drug with 2.0 ml of sterile deionized water and then add 0.1 ml to the MGIT medium. The final concentration of the drug in the medium should be:

- STR ---------- 4.0 µg/ml of medium
- INH ---------- 0.4 µg/ml of medium
- EMB ---------- 7.5 µg/ml of medium

6. Results

The instrument monitors the entered susceptibility test set. Once the test is complete (within 4 to 21 days), the instrument will indicate that the results are ready. Scan the susceptibility Set Carrier and print the report. The instrument printout indicates susceptibility results for each drug. Results are qualitative: Susceptible (**S**), Resistant (**R**) or indeterminate (**X**).

- The instrument interprets results at the time when the growth unit (GU) in growth control reaches 400 (within 4-13 days). At this point, the GU values of the drug vial are evaluated.

- **S** = Susceptible – the GU of the drug tube is less than 100.

- **R** = Resistant – the GU of the drug tube is 100 or more.

- **X** = Error – indeterminate results when certain conditions occur which may affect the test, such as GU of the control reaches ≥400 in less than 4 days. In such situations, the test should be repeated with pure, actively growing culture which is confirmed to be *M. tuberculosis* complex. Certain drug resistant strains grow very slowly in the medium and the results may not be achieved within 13 days with the standard inoculum. In such a case, the inoculum should be increased by decreasing the dilution of the culture suspension in order to get reportable results.

7. Reporting

Results must be reported as soon as they are available. When reporting results, it is important to include the name of the method used, the type of drug and its level of concentration.

In case of resistance, check the medium visually and make sure that the test culture is not contaminated (look for turbidity and put 1 drop of the medium on an agar plate) or that the test culture belongs to NTM. In case of unexpected results or mono-resistance against Rifampin, PZA or Ethambutol, repeat the test to verify resistance.

8. Quality control (QC)

It is extremely important to perform a quality control of drug susceptibility testing periodically. The minimum requirement is to test each new batch of reagents, such as SIRE drugs or MGIT medium. If the batch QC fails, all the results obtained within that batch, as well as the new batch of a reagent should be thoroughly reviewed and the testing should be repeated. Use *M. tuberculosis* H37Rv (ATCC [American Type Culture Collection] number 27294) as a QC strain which is susceptible to all anti-tuberculosis drugs. It is not necessary to include a resistant strain, as most of the resistant strains against a drug which are available from ATCC and other culture collections are highly resistant and do not give any added benefit in the quality control. The test procedure for QC organisms is the same as described above for clinical isolates. The inoculum should be from a freshly grown culture in the MGIT medium or on LJ slant. In case the suspension of QC bacteria is made from growth on solid medium, follow the procedure for suspension preparation as described above. The suspension may be stored in aliquots frozen for up to 6 months at -70ºC±10ºC (for details of QC strain preparation see Section II-K-2).

B. Pyrazinamide (PZA) Susceptibility Testing

1. Introduction

Susceptibility testing against PZA is always carried out at a lower pH of the medium, since pyrazinamide (PZA) is active only at the low pH *in vitro*. There are two methods widely used for PZA susceptibility testing. In the proportion method, Middlebrook 7H10 agar medium at pH 5.5 is used, with 25-50 µg/ml PZA. Colony counts, on the drug-free and drug-

containing medium, determine susceptibility. However, the pH of 5.5 is detrimental to mycobacterial growth and a significant number of test results cannot be determined because of poor growth or lack of growth. The other method is the radiometric BACTEC 460TB method. Here, the BACTEC 12B medium is modified by reducing the pH to 6.0. At this pH, mycobacteria grow better than at pH 5.5. To compensate for the increase in the pH, the concentration of PZA is increased to 100 µg/ml. PZA susceptibility testing with BACTEC 460TB system has proven to be satisfactory and has been recommended by the Clinical and Laboratory Standards Institute (CLSI, previously known as NCLS).[74]

The BACTEC MGIT 960 PZA test method is developed on the same principle as the BACTEC 460 method except that it is a non-radiometric method and results are automatically interpreted by the instrument. Results obtained by the MGIT method correlate well with those obtained by the BACTEC 460 method.[53, 54]

2. Principles of the test

The BACTEC MGIT 960 PZA susceptibility test is a qualitative procedure to test susceptibility of *M. tuberculosis* complex against PZA. Results are obtained within 4 to 21 days. The MGIT 960 medium is a modified 7H9 broth with a reduced pH of 5.9. The detection of growth is achieved by the oxygen sensor at the bottom of the tube in the same way as the one in the regular MGIT tube, and the principle of the detection of resistance is the same as for SIRE drug susceptibility testing (please refer to Section III-A-2).

3. Reagents

The BACTEC MGIT 960 PZA medium tube contains 7 ml of broth. It consists of 5.9 g of modified Middlebrook 7H9 broth and 1.25 Casein peptone per liter of purified water adjusted to pH 5.9.

The BACTEC MGIT PZA Drug Kit contains two vials of 20,000 µg of lyophilized PZA and 6 vials of PZA supplement. Each vial of the supplement contains 15 ml of enrichment with the following formula per liter of water.

- Bovine albumin----------50.0 g
- Dextrose ----------20.0 g
- Polyoxyethyline state (POES) ----------1.1 g
- Catalase ----------0.03 g
- Olecic acid ---------- 0.1 g

Storage instructions: PZA medium may be stored at 2-25ºC. Do not freeze, avoid exposure to light and do not use if found turbid.

Upon receipt**,** PZA drug vials should be stored at 2-8ºC. Once reconstituted, the antibiotic solution may be kept frozen at -20ºC or colder for up to six months, but should not to exceed the original expiration date. Once thawed, do not store or refreeze.

The PZA supplement should be stored at 2-8ºC upon receipt. Avoid freezing or overheating and use prior to the expiration date. Minimize exposure to light.

4. Procedures

a. Reconstitution of lyophilized PZA drug

Reconstitute each of the PZA drug vials with 2.5 ml of sterile distilled/deionized water. Mix well. The reconstituted drug solution will contain 8000 µg/ml of PZA.

b. Preparation of the inoculum

The PZA susceptibility test is recommended for a pure culture of *M. tuberculosis* complex. The test culture should be thoroughly checked for its purity and a confirmed identification of *M. tuberculosis*.

Preparation from a positive MGIT tube: Use a freshly positive MGIT tube as described in the section for SIRE testing. Please refer to Section III-A-4).

Day 0 – the day a MGIT tube is positive by the instrument. Re-incubate.

Day 1 or 2 – one or two days after instrument positive. Use undiluted for the susceptibility testing inoculation.

Day 3, 4 or 5 – mix well and dilute 1:5 by adding 1.0 ml of positive broth in 4.0 ml of sterile saline. Mix well. Use this for the susceptibility testing inoculation.

Day 6 and onward – subculture in a fresh MGIT tube and follow the above guidelines.

Caution: Avoid mycobacterial clumps by mixing the growth well (vortex) and let it stand for 5-10 minutes. Take the supernatant broth for inoculation preparation.

Preparation from growth on a solid medium: Follow the same procedure as described for SIRE susceptibility testing. Scrape off as many colonies as possible from the surface of the solid medium using a sterile loop or wooden applicator stick. Transfer into a sterilized tube containing 4-5 ml of sterile 7H9 broth with 8-10 glass beads. Tighten the screw cap and vortex the broth for 1-2 minutes. Leave the culture suspension undisturbed for 20 minutes. Carefully remove the supernatant fluid and transfer to a fresh sterile tube. Vortex again and leave undisturbed for 15 minutes. Transfer the supernatant fluid into a third sterile tube. Adjust the turbidity of the suspension to McFarland #0.5 standard by gradually adding sterile saline.

For susceptibility test inoculation, dilute this suspension 1:5 by adding 1.0 ml of the suspension to 4.0 ml of sterile saline. Use this diluted suspension for setting up the susceptibility.

c. Inoculation and incubation

- Label two MGIT PZA tubes, one as GC (growth control) and one as PZA (drug containing). Using a pipette, aseptically add 0.8 ml of PZA supplement to each of the two tubes.

- Aseptically add 0.1 ml (100 µL) of the reconstituted drug into the PZA tube. If possible, use a micropipette. Try to be as accurate as possible in adding the drug. This will give you 100 µg PZA per ml of the medium. Do not add drug to the GC tube.

- Inoculate 0.5 ml of the culture suspension to the PZA tube using a sterile pipette.

- For growth control inoculation, first dilute the inoculum 1:10 by adding 0.5 ml of the culture suspension (the one used for the drug tube) to 4.5 ml of sterile saline. Mix well by tightening the cap and inverting at least 5-6 times. Add 0.5 ml of this diluted suspension into the tube labeled GC.

Note: For PZA susceptibility test, the inoculum for the control is diluted 1:10 and not 1:100 as in SIRE AST).

- Tighten the caps and gently invert both the MGIT tubes several times to mix.

- Place them in a two AST Set Carriers (refer to the BACTEC MGIT 960 User's Manual for further information) with the sequence of first GC and the PZA.

- Enter the PZA set into the instrument using AST set entry feature (refer to the BACTEC MGIT 960 User's Manual for further information). Make sure the GC is placed first, and PZA second, in the AST Set Carrier. Select PZA as the drug in 2nd tube AST set carrier definition when performing the AST set entry.

- Check the purity of the inoculum by streaking a loopful of the culture suspension onto blood agar plate. If a blood agar plate is not available use Chocolate agar BHI agar. Incubate and check for growth after 48 hours. If growth appears on the streaked plate, discontinue the susceptibility test and do not use results of this susceptibility test. Repeat testing after obtaining a pure culture.

Precautions: All the additions and handling of cultures should be done only inside a biosafety cabinet. To avoid contamination, use properly sterilized tubes, reagents and other items. Proper reconstitution of PZA drug and accurate addition of the drug to the medium is essential for getting correct results. Preparation of inoculum is critical. It should be as homogeneous as possible with the least amount of mycobacterial clumps. Dilution (1:10) of the culture suspension for the growth control and mixing is critical. Use only "PZA Supplement" and PZA medium for the PZA susceptibility test. Make sure the tubes in the AST set carrier are placed in the proper sequence (i.e. GC, PZA).

5. Results

The BACTEC 960 instrument will monitor the inoculated media and will give results within 4-21 days (Growth Control reaches GU 400 or more) once the test is complete. At this point, the susceptibility set can be removed after scanning and a report can be printed. The susceptibility report will be "**S**" (susceptible) or "**R**" (resistant). If the GC tubes become positive in less than 4 days or remain negative up to 21 days, or if some other conditions occur which may affect the test results, the instrument report will show an Error ("**X**"). In such situations, the test needs to be repeated. The instrument interpretation of results is based on GU values as described for SIRE drugs (Section III-A-5).

6. Reporting

Report results as susceptible or resistant, indicating the method and concentration of the drug used. Mono-resistance to PZA is uncommon. In case mono-resistance is observed with a clinical isolate, repeat the test and report results only when it is confirmed. Cultures which are contaminated, or belong to an NTM species, or are a mixed culture of *M. tuberculosis* and other mycobacteria, will give erroneous results. Strains of *M. bovis*, including *M. bovis* BCG, are also naturally resistant to PZA.

7. Quality control

It is extremely important to periodically perform a quality control of drug susceptibility testing for PZA. The minimum requirement is to test each new batch of reagents, such as PZA drug or MGIT PZA medium. If the QC batch fails, all the results obtained within that batch, as well as the new batch of a reagent, should be thoroughly reviewed and the testing should be repeated.

Use *M. tuberculosis* H37Rv (ATCC [American Type Culture Collection] number 27294) as a QC strain which is susceptible to all anti-tuberculosis drugs. It is not necessary to include a resistant strain, as most of the resistant strains against a drug which are available from ATCC and other culture collections are highly resistant and do not give any added benefit in the quality control. The test procedure for QC organisms is the same as described above for clinical isolates. The inoculum could be from a freshly grown culture in the MGIT medium or on a LJ slant. If the suspension of QC bacteria is made from growth on solid medium, follow the procedure for suspension preparation as described above. The suspension may be stored in aliquots frozen, for up to six months, at -70ºC $\pm$ 10ºC (for details of QC strain preparation, see Section II-K-2).

If the QC batch fails, that is, the pan-susceptible H3Rv shows some resistance, then all the results obtained within that batch become invalid and the testing should be repeated.

C. Secondline Drug Susceptibility Testing

Procedures for secondline susceptibility testing in MGT are not provided by BD. However, recently two major studies have been carried out to evaluate susceptibility testing against secondline drugs with MGIT 960. These studies have established that the MGIT 960 system can be efficiently used for testing secondline drugs and have also provided procedures and the test concentrations.[75, 76]

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Section IV: References

[1]Isenberg HD (Editor). Clinical Microbiology Procedure Handbook, Vol.1. American Society for Microbiology (Publisher), Washington, D.C.

[2] Kent PT, Kubica GP. Public Health Microbiology, a Guide for the Level III Laboratory. Centers for Disease Control, Division of Laboratory Training and Consultation. Atlanta, GA, US Department of Health and Human Services, US Government Printing Office, 1985.

[3] Middlebrook O, Cohn ALL. Bacteriology of tuberculosis: Laboratory Methods. Am J Pub Health. 1958;48:844-853.

[4] DeLand FH, Wagner RN Jr. Early detection of bacterial growth with carbon-14 labeled glucose. Radiology 1969;92:154:155.

[5] Kirihara JAL, Hillier SL, Coyle MB. Improved detection times of *Mycobacterium avium* complex and *M. tuberculosis* with the BACTEC radiometric system. J Clin Microbiol. 1985;22:841-845.

[6] Morgan MA, Horstmeier CD, DeYoung DR, Roberts GD. Comparison of a radiometric method (BACTEC) and conventional culture media for recovery of mycobacteria from smear-negative specimens. J Clin Microbiol. 1983;18:384-388

[7] Roberts GD, Goodman NL, Heifets L, et al. Evaluation of the BACTEC radiometric method for recovery of mycobacteria and drug susceptibility testing of *Mycobacterium tuberculosis* from acid-fast smear- positive specimens. J Clin Microbiol. 1983;18:689-696.

[8] Siddiqi SH, Libonati JP, Middlebrook G. Evaluation of a rapid radiometric method for drug susceptibility testing of *Mycobacterium tuberculosis*. J Clin Microbiol. 1981;13:908-912.

[9] Siddiqi SH, Hawkins JE , Laszlo A. Inter-laboratory drug susceptibility testing of *Mycobacterium tuberculosis* by radiometric procedure and two conventional methods. J Clin Microbiol. 1985;22:919-923.

[10] Takahashi H, Foster V. Detection and recovery of mycobacteria by a radiometric procedure. J Clin Microbiol. 1983;17:380-381.

[11] Siddiqi SH, Libonati JP, Carter ME, et al. Enhancement of mycobacterial growth in middlebrook 7H12 medium by polyoxyethylene stearate. Current Microbiol. 1988;17:105-110.

[12] Siddiqi SH, Laszlo A, Butler WR, et al. Bacteriological investigation of unusual mycobacteria isolated from immuno-compromised patients. Diagn Microbiol Infect Dis. 1993;6**:**321-323.

[13] Tenover FC, Crawford JT, Huebner RE, et al. The resurgence of tuberculosis: is your laboratory ready? J Clin Microbiol. 1993;31:767-770.

[14] Bird BR, Denniston MM, Huebner RE, et al. Changing practices in mycobacteriology: a follow-up survey of State & Territorial Public Health Laboratories. J Clin Microbiol. 1996;34:554-559.

[15] Siddiqi SH, Hwangbo CC. A new antimicrobial mixture (polymixin B, amphotericin B, naildixic acid, trimethoprim and azlocillin) for effective suppression of non-mycobacterial contamination during primary isolation of mycobacteria. 1986; ASM Annual Meeting, Washington, DC. Abstract U35.

[16] Abe CK, Hirano K, Wada M, et al. Comparison of the newly developed MB redox system with Mycobacteria Growth Indicator Tube (MGIT) and 2% Ogawa egg media for recovery of mycobacteria in clinical specimens. Kekkaku (Japanese). 1999;74:707-713.

[17] Apers L, Mutsvangwa J, Magwenzi J, et al. A comparison of direct microscopy, the concentration method and the Mycobacteria Growth Indicator Tube for the examination of sputum for acid-fast bacilli. Int J Tuberc Lung Dis. 2003;**7**:376-381.

[18] Chien HP, Yu MC, Wu MH, et al. Comparison of the BACTEC MGIT 960 with Lowenstein-Jensen medium for recovery of mycobacteria from clinical specimens. Int J Tuberc Lung Dis. 2000;4:866-870.

[19] Goloubeva V, Lecocq M, Lassowsky P, et al. Evaluation of mycobacteria growth indicator tube for direct and indirect drug susceptibility testing of Mycobacterium tuberculosis from respiratory specimens in a Siberian prison hospital.
J Clin Microbiol. 2001;39:1501-1505.

[20] Idigoras P, Beristain X, Iturzaeta A, et al. Comparison of the automated nonradiometric BACTEC MGIT 960 system with Lowenstein-Jensen, Coletsos, and Middlebrook 7H11 solid media for recovery of mycobacteria. Eur J Clin Microbiol Infect Dis. 2000;19:350-354.

[21] Jayakumar KV, Forster T, Kyi MS. Improved detection of Mycobacterium spp. using the BACTEC MGIT 960 system. Br J Biomed Sci. 2001;58:54-158.

[22] Lee JJ, Lin CB, Wang JD, et al. Comparative evaluation of the BACTEC MGIT 960 system with solid medium for isolation of mycobacteria. Int J Tuberc Lung Dis. 2003;**7**:569-574.

[23] Macondo EA, Ba F, Toure-Kane NC, et al. Improvement of tuberculosis diagnosis by the Mycobacteria Growth Indicator Tube (MGIT) in a developing country laboratory. Bull Soc Pathol Exot (French). 2000;93:97-100.

[24] Samra Z, Kaufman L, Bechor J, et al. Comparative study of three culture systems for optimal recovery of mycobacteria from different clinical specimens. Eur J Clin Microbiol Infect Dis. 2000;19:750-754.

[25] Somoskovi A, Kodmon C, Lantos A, et al. Comparison of recoveries of *Mycobacterium tuberculosis* using the automated BACTEC 960 system, the BACTEC 460 TB system and Lowenstein-Jensen medium. J Clin Microbiol. 2000;38:2395-2397.

[26] Zaruba R, Kralova M. Evaluation of the effectiveness of the BACTEC MGIT automatic system for culture of mycobacteria in comparison with classical methods of culture. Epidemiol Microbiol Immunol. 2002;51:66-70.

[27] Ardito F, Sanguinetti M, Posteraro B, et al. Comparison of the mycobacteria growth indicator tube with radiometric and solid culture for isolation of mycobacteria from clinical specimens and susceptibility testing of *Mycobacterium tuberculosis*. New Microbiol. 2000;23:151-158.

[28] Badak FZ, Kiska DL, Setterquist S, et al. Comparison of Mycobacteria Growth Indicator Tube with BACTEC®460 for detection and recovery of Mycobacteria from clinical specimens. J Clin Microbiol. 1996;34:2236-2239.

[29] Flanagan PG, Williams R, Paull A. Comparison of two automated systems for the isolation of mycobacteria from clinical specimen. Eur J Clin Microbiol Infect Dis. 1999;18:912-914.

[30] Hanna BA, Ebrahimzadeh A, Elliott LB, et al. Multicenter evaluation of the BACTEC MGIT 960 system for recovery of mycobacteria. J Clin Microbiol. 1999;37:748-752.

[31] Kanchana MV, Cheke D, Natyshak I, et al. Evaluation of the BACTEC MGIT 960 system for the recovery of mycobacteria. Diagn Microbiol Infect Dis. 2000;37:31-36.

[32] Leitritz L, Schubert S, Bucherl B, et al. Evaluation of BACTEC MGIT 960 and BACTEC 460TB systems for recovery of mycobacteria from clinical specimens of a university hospital with low incidence of tuberculosis. J Clin Microbiol. 2001;39:3764-3767.

[33] Palaci M, Ueki SY, Sato DN, et al. Evaluation of Mycobacteria Growth Indicator Tube for recovery and drug susceptibility testing of *Mycobacterium tuberculosis* isolates from respiratory specimens. J Clin Microbiol. 1996;34:762-764.

[34] Pinheiro MD, Ribeiro MM. Comparison of the BACTEC 460TB system and the BACTEC MGIT 960 system in recovery of mycobacteria from clinical specimens. Clin Microbiol Infect. 2000;6:171-173.

[35] Rohner P, Ninet B, Benri AM,et al. Evaluation of the BACTEC 960 automated non-radiometric system for isolation of mycobacteria from clinical specimens. Eur J Clin Microbiol Infect Dis. 2000;19:715-717.

[36] Scarparo C, Piccoli P, Rigon A, et al. Evaluation of the BACTEC MGIT 960 in Comparison with BACTEC 460TB for detection and recovery of Mycobacteria from clinical specimens. Diagn Microbiol Infect Dis. 2002;44:157-161.

[37] Tortoli E, Cichero P, Piersimoni C, et al. Use of BACTEC MGIT 960 for recovery of mycobacteria from clinical specimens: Multi-Center study. J Clin Microbiol. 1999;37:3578-3582.

[38] Tsuyuguchi K, Ikeda T, Nakatani K, et al. Evaluation of the Mycobacteria Growth Indicator Tube system for detection and quantification of mycobacteria from clinical specimens. Kekkaku (Japanese). 2003;78:389-393.

[39] Whyte T, Cormican M, Hanahoe B, et al. Comparison of BACTEC MGIT 960 and BACTEC 460 for culture of Mycobacteria. Diagn Microbiol Infect Dis. 2000;38:123-126.

[40] Witebsky FG, Keiser JF, Conville PS, et al. Comparison of BACTEC 13A medium and Du Pont Isolator for detection of mycobacteria. J Clin Microbiol. 1988;26:1501-1505.

[41] Abe C, Aono A, Hirano K. Evaluation of the BACTEC MGIT 960 system for drug susceptibility testing of Mycobacterium tuberculosis isolates compared with the proportion method on solid media. Kekkaku (Japanese). 2001;76:657-662.

[42] Ardito F, Posteraro B, Sanguinetti M, et al. Evaluation of BACTEC Mycobacteria Growth Indicator Tube (MGIT 960) automated system for drug susceptibility testing of *Mycobacterium tuberculosis*. J Clin Microbiol. 2001;39:4440-4444.

[43] Adjers-Koskela K, Katila ML. Susceptibility testing with the manual Mycobacteria Growth Indicator Tube MGIT) and the MGIT 960 System provides rapid and reliable verification of multidrug resistant tuberculosis. J Clin Microbiol. 2003;41:1235-1239.

[44] Bemer P, Palicova F, Rusch-Gerdes S, et al. Multi-center evaluation of fully automated BACTEC Mycobacteria Growth Indicator Tube 960 system for susceptibility testing of *Mycobacterium tuberculosis*. J Clin Microbiol. 2002;40:150-154.

[45] Bergmann JS, Fish G, Woods GL. Evaluation of BBL MGIT (Mycobacteria Growth Indicator Tube) AST SIRE System for antimycobacterial susceptibility testing of *Mycobacterium tuberculosis* to 4 primary anti-tuberculosis drugs. Arch Pathol Lab Med. 2000;124:82-86.

[46] Bergmann JS, Woods GL. Reliability of mycobacteria growth indicator tube for testing susceptibility of *Mycobacterium tuberculosis* to ethambutol and streptomycin. J Clin Microbiol. 1997;35:3325-3327.

[47] Bergmann JS, Woods GL. Mycobacterial growth indicator tube for susceptibility testing of *Mycobacterium tuberculosis* to isoniazid and rifampin. Diagn Microbiol Infect Dis. 1997;28:153-156.

[48] Cambau E, Truffot-Pernot C, Boulahbal F, et al. Mycobacterial Growth Indicator Tube versus the proportion method on Lowenstein-Jensen medium for antibiotic susceptibility testing of *Mycobacterium tuberculosis*. Eur J Clin Microbiol Infect Dis. 2000;19:938-942.

[49] Huang TS, Tu HZ, Lee SS, et al. Antimicrobial susceptibility testing of *Mycobacterium tuberculosis* to first-line drugs: comparisons of the MGIT 960 and BACTEC 460 Systems. Ann Clin Lab Sci. 2002;32:142-147.

[50] Reisner BS, Gatson AM, Woods GL. Evaluation of Mycobacteria Growth Indicator Tubes for susceptibility testing of *Mycobacterium tuberculosis* to isoniazid and rifampin. Diagn Microbiol Infect Dis. 1995;22:325-329.

[51] Tortoli E, Benedetti M, Fontanelli A, et al. Evaluation of automated BACTEC MGIT 960 system for testing susceptibility of *Mycobacterium tuberculosis* to four major anti-tuberculous drugs: comparison with the radiometric BACTEC 460TB method and the agar plate method of proportion. J Clin Microbiol. 2002;40:607-610.

[52] Walters SB, Hanna BA. Testing of susceptibility of *Mycobacterium tuberculosis* to isoniazid and rifampin by Mycobacterium Growth Indicator Tube method. J Clin Microbiol. 1996;34:1565-1567.

[53] Aono A, Hirano K, Hamasaki S, et al. Evaluation of BACTEC MGIT 960 medium for susceptibility testing of Mycobacterium tuberculosis to pyrazinamide (PZA): compared with the results of pyrazinamidase assay and Kyokuto PZA test. Diagn Microbiol Infect Dis. 2002;44:347-352.

[54] Pfyffer GE, Palicova F, Rusch-Gerdes S. Testing of susceptibility of *Mycobacterium tuberculosis* to pyrazinamide with the non-radiometric BACTEC MGIT 960 system. J Clin Microbiol. 2002;40:1670-1674.

[55] Bastian I, Rigouts L, Palomino JC, et al. Kanamycin susceptibility testing of *Mycobacterium tuberculosis* using Mycobacterium Growth Indicator Tube and a colorimetric method. Antimicrob Agents Chemother. 2001;45:1934-1936.

[56] Piersimoni C, Nista D, Bornigia S, et al. Evaluation of new method for rapid drug susceptibility testing of *Mycobacterium avium* complex isolates by using the Mycobacteria Growth Indicator Tube. J Clin Microbiol. 1998;36:64-67.

[57] Alcaide F, Benitez MA, Escriba JM, et al. Evaluation of the BACTEC MGIT 960 and the MB/BacT systems for recovery of mycobacteria from clinical specimens and for species identification by DNA AccuProbe. J Clin Microbiol. 2000;38**:**398-401.

[58] Katila ML, Katila P, Erkinjuntti-Pekkanen R. Accelerated detection and identification of mycobacteria with MGIT 960 and COBAS AMPLICOR systems. J Clin Microbiol. 2000;38:960-964.

[59] Williams-Bouyer N, Yorke R, Lee HI, et al. Comparison of the BACTEC MGIT 960 and ESP Culture System II for growth and detection of Mycobacteria. J Clin Microbiol. 2000;38:4167-4170.

[60] Yan JJ, Huang AH, Tsai SH, et al. Comparison of the MB/BacT and BACTEC MGIT 960 system for recovery of mycobacteria from clinical specimens. Diagn Microbiol Infect Dis. 2000;37:25-30.

[61] Centers for Disease Control and Prevention. National Institutes of Health. Biosafety in microbiological and biomedical laboratories. HHS Publication No. (CDC) 99-8395. US Government Printing Office. Washington, 1999. Also available at www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm.

[62] Kent PT, Kubica GP. The sputum digestion process in mycobacteriology: centrifugation efficacy and digestant toxicity. Annual Meeting of American Society for Microbiology, St. Louis, MO 1984. Abstract U2.

[63] Fadda G, Roe SL. Recovery and susceptibility testing of *Mycobacterium tuberculosis* from extra pulmonary specimens by the BACTEC radiometric method. J Clin Microbiol. 1984;19:720-721.

[64] Harris G, Rayner A, Blair J, et al. Comparison of three isolation systems for the culture of mycobacteria from respiratory and non-respiratory samples. J Clin Pathol. 2000;53:615-618.

[65] Hanna BA, Walters SB, Bonk SJ, et al. Recovery of mycobacteria from blood in Mycobacteria Indicator Tube and Lowenstein-Jensen slant after lysis-centrifugation. J Clin Microbiol. 1995;33:3315-3316.

[66] Lu D, Heeren B, Dunne WM. Comparison of the Automated Mycobacteria Growth Indicator Tube System (BACTEC 960/MGIT) with Lowenstein-Jensen medium for recovery of mycobacteria from clinical specimens. Am J Clin Pathol. 2002;118:542-545.

[67] Tu HZ, Chang SH, Huaug TS, et al. Microscopic morphology in smears prepared from MGIT broth medium for rapid presumptive identification *Mycobacterium tuberculosis* complex, *Mycobacterium avium* complex and *Mycobacterium kansasii*. Ann Clin Lab Sci. 2003;33:179-183.

[68] Banaiee N, Bobadilla-del-Valle M, Riska PF, et al. Rapid identification and susceptibility testing of Mycobacterium tuberculosis from MGIT cultures with luciferase reporter mycobacteriophages. J Med Microbiol. 2003;52:557-561.

[69] Kontos F, Petinaki E, Nicolaou S, et al. Multicenter evaluation of the fully automated BACTEC MGIT 960 system and three molecular methods for the isolation and the identification of mycobacteria from clinical specimens. Diagn Microbiol Infect Dis. 2003;46:299-301.

[70] Kontos F, Petinaki E, Gitti Z, et al. Combined use of the fully automated BACTEC MGIT 960 System and a PCR-restriction fragment length polymorphism analysis for routine detection and identification of mycobacteria from clinical samples. J Microbiol Methods. 2003;52:137-140.

[71] Hasegawa N, Miura T, Yamaguchi K, et al. New simple and rapid test for culture confirmation of *Mycobacterium tuberculosis* complex: a multi-center study. J Clin Microbiol. 2002;40:908-912.

[72] Levidiotou S, Papamichael D, Gessouli E, et al. Detection of mycobacteria in clinical specimens using the mycobacteria growth indicator tube (MGIT) and the Lowenstein-Jensen medium. Microbiol Res. 1999;154:151-155.

[73] TDR TB Website : http://www.who.int/tdr/diseases/tb/default.htm

[74] CLSI Website : http://www.nccls.org/

[75] Kruuner A, Yates MD, Drobniewski FA. Evaluation of MGIT 960-based antimicrobial testing and determination of critical concentrations of first- and second-line antimicrobial drugs with drug-resistant clinical strains of *Mycobacterium tuberculosis*. J Clin Microbiol. 2006;44**:**811-818.

[76] Rusch-Gerdes S, Pfyffer GE, Casal M, et al. Multi-center laboratory validation of the BACTEC MGIT 960 technique for testing susceptibilities of Mycobacterium tuberculosis to classical second-line drugs and newer antimicrobials. J Clin Microbiol. 2006;44:688-692.

Section IV: Appendices

APPENDIX A

Supplies from Becton, Dickinson & Company (BD)

The following is the list of supplies, from BD, related to mycobacteriology work.

1. Supplies for Culture Work

Catalog number	***Description***
290020	Falcon Sputum Collection System
245111	BBL® MGIT™ Mycobacteria Growth Indicator Tube (manual), 4 ml, carton of 25 tubes
245113	BBL® MGIT™ Mycobacteria Growth Indicator Tube (manual), 4 ml, carton of 100 tubes
245116	BBL® MGIT™ OADC, 15 ml carton of 6 vials. Each vial sufficient for 25 MGIT Tubes (manual)
245122	BBL® MGIT™ Mycobacteria Growth Indicator Tube 7 ml (for BACTEC MGIT 960), carton of 100 tubes
245114	BBL® MGIT™ PANTA™ Antibiotic Mixture, lyophilized, carton of 6 vials. Each vial sufficient for 25 MGIT tubes
245124	BACTEC MGIT Growth Supplement Kit, 6 vials, 15 ml each. BACTEC MGIT lyophilized PANTA Antibiotic Mixture, 6 vials. Each Growth Supplement/ PANTA vial sufficient for 15-18 MGIT tubes (for BACTEC MGIT 960)
220908	BBL® Lowenstein-Jensen Medium Slants, package of 10 (20 x 148 mm tubes with cap)
220909	BBL® Lowenstein-Jensen Medium Slants, carton of 100 (20 x 148 mm tubes with cap)
221174	BBL® Middlebrook and Cohn 7H10 Agar, package of 20
295939	BBL® Middlebrook 7H9 Broth, 8 ml, package of 10 tubes
221818	BBL® Normal Saline, 5 ml, package of 10
231106*	BBL® Taxo™ X Factor Strips, 1 vial, 30 discs
240862	BBL® MycoPrep™ Specimen Digestion / Decontamination Kit, ten 75 ml bottles of NALC-NaOH solution and 5 packages of phosphate buffer
240863	BBL® MycoPrep Specimen Digestion Kit, ten 150 ml bottles of NALC-NaOH solution and 10 packages of phosphate buffer.
297297	McFarland Standard – 0.5297298, McFarland Standard – 1.0.

* needed for growth of *M. haemophilum*

2. Supplies for AFB smears

212522	TB Stain Kit K ▪TB Carbolfuchsin KF ▪TB Decolorizer ▪TB Brilliant Green	For staining mycobacteria by the Kinyoun (cold) acid-fast procedure.	1 1 x 250 ml 1 x 250 ml 1 x 250 ml
212520	TB Stain Kit ZN ▪TB Carbolfuchsin ZN ▪TB Decolorizer ▪TB Methylene Blue	For staining mycobacteria by the Ziehl-Neelsen (hot) acid-fast procedure.	1 1 x 250 ml 1 x 250 ml 1 x 250 ml
212315	TB Quick Stain Kit ▪TB Quick Stain Carbolfuchsin ▪TB Quick Stain Methylene Blue	For a more rapid version of staining mycobacteria by the cold acid-fast procedure.	1 1 x 250 ml 1 x 250 ml
212519	TB Fluorescent Stain Kit M ▪TB Auramine M ▪TB Decolorizer TM ▪TB Potassium Permanganate	For staining mycobacteria by the Morse, Blair, Weiser and Sproat fluorescent procedure.	1 1 x 250 ml 1 x 250 ml 1 x 250 ml
212521	TB Fluorescent Stain Kit T ▪TB Auramine-Rhodamine T ▪TB Decolorizer TM ▪TB Potassium Permanganate	For staining mycobacteria by the Truant, Brett and Thomas fluorescent procedure.	1 1 x 250 ml 1 x 250 ml 1 x 250 ml
212514	TB Auramine M	For staining mycobacteria by the Morse, Blair, Weiser and Sproat fluorescent procedure.	4 x 250 ml
212515	TB Auramine-Rhodamine T	For staining mycobacteria by the Truant, Brett and Thomas fluorescent procedure.	4 x 250 ml
212523	TB Brilliant Green K	For staining mycobacteria by the Kinyoun (cold) and Ziehl-Neelsen (hot) acid-fast procedures.	4 x 250 ml

2. Supplies for AFB smears (continued)

212518	TB Carbolfuchsin KF	For staining mycobacteria by the Kinyoun (cold) acid-fast procedure.	4 x 250 ml
212511	TB Carbolfuchsin ZN	For staining mycobacteria by the Ziehl-Neelsen (hot) acid-fast procedure.	4 x 250 ml
212517	TB Decolorizer	For staining mycobacteria by the Kinyoun (cold) and Ziehl-Neelsen (hot) acid-fast procedures.	4 x 250 ml
212512	TB Decolorizer TM	For staining mycobacteria by the Truant, Brett and Thomas and the Morse, Blair, Weiser and Sproat fluorescent procedures.	4 x 250 ml
212516	TB Methylene Blue	For staining mycobacteria by the Kinyoun (cold) and Ziehl-Neelsen (hot) acid-fast procedures.	4 x 250 ml
212513	TB Potassium Permanganate	For staining mycobacteria by the Truant, Brett and Thomas and the Morse, Blair, Weiser and Sproat fluorescent procedures.	4 x 250 ml
212316	TB Quick Stain Carbolfuchsin	For a more rapid version of staining mycobacteria by the cold acid-fast procedure.	3 x 250 ml
212317	TB Quick Stain Methylene Blue	For a more rapid version of staining mycobacteria by the cold acid-fast procedure.	3 x 250 ml

3. Supplies for drug susceptibility testing

Catalog number	*Description*
245123	BD BACTEC™ MGIT™ 960 SIRE Kit (sufficient for 40 tests)
245119	BD BACTEC™ MGIT™ 960 Manual SIRE Kit (sufficient for 80 tests)
245157	BD BACTEC™ MGIT™ 960 IR Kit (sufficient for 40 tests)
245128	BD BACTEC™ MGIT™ 960 PZA Drug Kit (sufficient for 50 tests)
245115	DB BACTEC™ MGIT™ 960 PZA Tubes (carton of 25 tubes)
245125	BD B2ACTEC™ MGIT™ 960 Streptomycin 4.0 Kit (sufficient for 20 tests)
245126	BD BACTEC™ MGIT™ 960 Isoniazid 0.4 Kit (sufficient for 20 tests)
245127	BACTEC™ MGIT™ 960 Ethambutol 7.5 Kit (sufficient for 20 tests)

APPENDIX B

Miscellaneous Procedures & Information

1. Strains of mycobacteria commonly used

ATCC No.*	Strain
27294	*M tuberculosis,* H37Rv Susceptible to all TB Drugs
35820	*M tuberculosis,* H37Rv Streptomycin resistant
35821	*M tuberculosis,* H37Rv PAS resistant
35822	*M tuberculosis,* H37Rv INH resistant
35826	A-f *tuberculosis,* H37Rv Cycloserine resistant
35827	*M. tuberculosis,* H37Rv Kanamycin resistant
35828	*M. tuberculosis,* H37Rv Pyrazinamide resistant
35829	*M. tuberculosis,* 113 7Rv Thiacetazone (Amithiozone) resistant
35830	*M. tuberculosis,* H37Rv Ethionamide resistant
35837	*M. tuberculosis,* H37Rv Ethambutol resistant
35838	*M. tuberculosis,* 113 7Rv Rifampin resistant
35721	*M. bovis*
35775	*M. kansasii*
35785	*M. scrofulaceum*
13950	*M. intracellulare*
35717	*M . avium*

(*) American Type Culture Collection. Refer to these ATCC strain numbers when ordering.

Procedure for Ordering Cultures

You may order these cultures from the American Type Culture Collection (ATCC) at the following address. Specify the name of the organism and its ATCC number. Refer to the ATCC catalog for details.

American Type Culture Collection
10801 University Boulevard
Manassas, VA 20110-2209, USA

Phone: +1-703-365-2700
Fax: +1-703-365-2750
E-mail: news@atcc.org

Users outside the United States should investigate the import regulations of their respective governments, and obtain custom and/or other government clearances as necessary prior to ordering materials.

2. McFarland turbidity standard

Already prepared McFarland Standards are available from BD. The following procedure is given in case the commercial standard is not available.

a. Prepare 1% solution of chemically pure sulfuric acid in water.

b. Prepare 1% solution of chemically pure barium chloride in water.

c. Mix 0.1 ml of barium chloride solution with 9.9 ml of sulfuric acid solution in a clean, properly washed test tube.

d. Seal the tube with tape or wax. This is equal to McFarland No. 1 Standard.

e. Add 0.05 ml of barium chloride to 0.95 ml of sulfuric acid or add 5 ml water to 5 ml of well-mixed suspension of McFarland No. 1. Standard will yield McFarland No. 0.5.

f. Accurate measurements are critical to achieve a workable McFarland Standard. When comparing any turbidity with a McFarland Standard, shake the tubes well. Use same type and size of tube for both the test and the McFarland Standard.

3. AFB stains

Note: Already prepared stains are available from BD. If it is not possible to obtain commercially prepared stains, follow these procedures to prepare the stains.

a. Ziehl-Neelsen stain

Solution A: Basic fuchsin 1.0 gm in 10 ml of 95% ethyl alcohol. Dissolve completely.

Solution B: 5.0 gm phenol in 95 ml of 95% ethyl alcohol. Mix gently.

***Note*:** The concentration of Basic fuchsin may differ in different procedures recommended in the literature. Higher Basic fuchsin concentration helps in staining AFB better. Filter the final staining solution if particles or precipitate is observed at any time. Use distilled/deionized water or filter the tap water through a bacterial filter. In some instances, high numbers of mycobacteria are present in tap water and may result in false positivity of smear.

Counter Stain: Solution A, 0.3 g Methylene blue chloride in 30 ml of 95% ethyl alcohol. Dissolve completely.

Solution B: 0.01g potassium hydroxide in 100 ml distilled/deionized water.
Mix solutions A and B. Filter, if necessary, to remove particles.

b. Kinyoun's stain

Solution A: Dissolve 4g of Basic fuchsin in 20 µL of 95% alcohol completely.

Solution B: Dissolve 8g phenol crystals in 100 µL of distilled/deionized water (gentle heating may be required). Mix solutions A&B. Filter with filter paper to remove particles.

Acid Alcohol: Add 3 ml of concentrated hydrochloric acid to 95 ml of 95% ethyl alcohol. Mix gently.

Counter Stains: Methylene Blue – Dissolve 0.3 gm Methylene blue chloride in 100 ml of distilled/deionized water.

c. Auramine O fluorescent (fluorochrome) stain

Solution A: Auramine 0 – Dissolve 0.1g of Auramine in 10 ml 95% ethanol.

Solution B: Phenol – Dissolve 3.0 g of phenol crystals in 87 ml of distilled/deionized water. Mix solution A and B.

Acid Alcohol: Add 0.5 ml of concentrated hydrochloric acid in 100 ml of 70% ethanol.

Potassium permanganate: Dissolve 0.5g of potassium permanganate (KMn04) in 100 ml distilled/deionized water.

4. Reagents for digestion decontamination

a. NaOH-NALC reagents

***Note*:** This is the recommended procedure for MGIT System. Commercially prepared NaOH-NALC (MycoPrep) is available from BD. These reagents are thoroughly quality control tested and yield the best results. The following procedures are described in case these reagents are to be prepared in the laboratory.

Preparation

- Prepare 4% NaOH solution by dissolving 4g NaOH pellets into 100 ml distilled/ deionized water. Sterilize by autoclaving. Concentration of NaOH may be varied (3-6% NaOH solution at the beginning).

- Prepare 2.9% sodium citrate solution by dissolving 2.9 g sodium citrate (21120) in 100 ml distilled/deionized water. Sterilize by autoclaving.

Mixing

Prior to use, mix equal quantities of NaOH and sodium citrate solution. Prepare only as much volume as can be used in a day. Add NALC powder to achieve a final concentration of 0.5% (for 100 ml NaOH-Na citrate solution, add 0.5 g NALC powder). Mix well and use the same day. NALC activity is lost if left standing for more than 24 hours.

b. Sodium hydroxide solution

Prepare 4% NaOH solution by dissolving 4g of NaOH in 100 ml distilled/deionized water. Sterilize by autoclaving. This solution can be stored and used for decontamination of (nonmucoid) contaminated cultures and specimens.

c. Phosphate buffer (pH 6.8, 0.067 M)

- Dissolve 9.47g of anhydrous disodium phosphate (Na_2HPO_4) in 1000 ml (1 liter) distilled/deionized water, using a volumetric flask.

- Dissolve 9.07 g monopotassium phosphate (KH_2PO_4) in 1000 ml (1 liter) distilled/ deionized water, using a volumetric flask.

- Mix equal quantities of the two solutions. Check the pH. Adding more solution A will raise the pH; more solution B will lower the pH. The final pH should be 6.8.

- Sterilize by autoclaving.

APPENDIX C

Procedures for Troubleshooting

1. Decrease or no recovery of mycobacteria

First, determine the number and types *of Mycobacterium .sp.* that were missed in the MGIT medium and recovered in conventional media. Generally, there should be a higher number of mycobacterial species isolated in MGIT medium as compared to solid media. If the amount of growth on solid media is very low (e.g., 1-2 colonies), there may be only a 50% chance of recovering the isolate in the MGlT or any other medium Corrective measures should be taken if the number of colonies is higher on solid medium and the MGIT culture is negative.
If there is an overall decrease in the culture positivity rate, investigate the following parameters:

a. Incubation

If an incubator is used, confirm that the temperature of the incubator is 37°C ± 1°C by placing a calibrated thermometer in various locations throughout the incubator or instrument drawers. Monitor the readings several times each day until heating stability is determined. Check temperature of the MGIT 960 by retrieving the information from the instrument. Too frequent opening of the incubator door or the instrument drawers may have some adverse effect on the incubation temperature. In extremely warm climates, the incubation temperature may be difficult to maintain if the laboratory temperature is not well-controlled.

Mycobacteria, which require optimum temperature other than 37°C, may fail to grow at 37°C. Majority of mycobacterial species grow well at 37°C $\pm$ 1ºC. They may grow slowly or may not grow if the temperature drops below 35°C. Mycobacteria that require temperature other than 37°C may not grow in the MGIT 960 instrument. Specimens suspected of having these mycobacteria must be inoculated into two tubes, one incubated at 37°C and the other incubated in another incubator at the optimum temperature required by the expected mycobacteria species.

Decontamination procedure

- Confirm that purity and concentration of all the reagents used in the digestion/ decontamination procedure are satisfactory. Do not use tap water. Use distilled/ deionized water only for preparation of reagents.

- It is better to start with freshly prepared reagents.

- Do not use reagents that are not compatible with the MGIT System, such as benzalkonium chloride (Zephiran) or sodium lauryl sulfate.

- High pH of the specimen inoculated into MGlT medium may influence the performance of MGlT adversely.

- Do not expose the specimen to the decontamination reagent for longer than the recommended time.

- Check if MGlT tubes are positive by visual growth but negative by fluorescence.

b. Centrifugation

- Relative Centrifugal Force (RCF) should be 3,000-5,000 x g. A lower force decreases the recovery of mycobacteria. Make sure the centrifuge is giving required RCF.

- Generation of heat during centrifugation also lowers the recovery as at higher temperature NaOH becomes more lethal for bacteria. Avoid generation of excessive heat. A refrigerated centrifuge is recommended. If refrigerated centrifuge is not available, use chilled phosphate buffer which is added before centrifugation.

c. Use of PANTA

- Check that PANTA is reconstituted with the proper volume. The high concentration of PANTA may inhibit growth of some NTM bacteria, especially if the number is low in the specimen.

2. Delay in the detection time

Detection time can range from 24 hours to six weeks for MGIT and 8 weeks for LJ medium. If the average detection time established in published studies or in a particular setting of a laboratory is significantly longer, follow these instructions:

Digestion/decontamination procedure

- Decrease the NaOH concentration and/or time of exposure to NaOH. Higher concentrations of NaOH or longer exposure time will prolong the detection time of mycobacteria.

- High pH of the final inoculum will prolong the detection time.

- Check if the incubation temperature is within specifications. Lower temperature would delay detection.

- In a few instances, too high concentration of PANTA may delay the detection of certain strains of NTM, especially if the starting number is low.

Procedures Check

- Water used for preparation of reagents should be pure (distilled/deionized).

- All the reagents used should be sterile.

- All pipettes and tubes should be sterile.

- All inoculations should be made in the biological safety hood.

- Growth Supplement/PANTA mixture should be added to MGIT tubes just prior to inoculation.

- It is important that the addition of MGlT OADC or MGIT Growth Supplement/PANTA should be done inside a biological cabinet. Leave the tube open for as little time as possible. Leaving the tube open, especially on an open bench top, would increase the contamination rate.

3. Guidelines to control high contamination

The normal acceptable contamination rate is 5% $\pm$ 2 for solid media. For liquid media, a slightly higher contamination rate is expected. Bacterial contamination is also an indicator of level of effective decontamination procedure. High contamination rate indicates improper decontamination procedure while too low contamination indicates over treatment of the specimen that could also lower the culture positivity rate or increase the detection time. If in the MGIT it is more than 7-8%, then the decontamination procedure is not satisfactory and corrective measures should be taken. If there is a problem of increased contamination, flowing guidelines would be helpful. Some of the procedures are already included in the main section of this manual and are repeated here briefly for convenience.

Specimen collection and transport

- Are specimens collected in sterile containers?

- What are the conditions for specimen transport and storage prior to processing?

- Is transport time too long? The longer the transport time the more likely the probability of contamination.
- Are weather conditions too hot? Higher contamination is more likely in hot weather if specimen is transported.
  - Collect specimens in clean and sterile containers to avoid outside contamination.
  - Keep the specimen in cool conditions during transport, preferably in an insulated ice box.
  - Transport to lab as quickly as possible.
  - Upon receipt, keep in a cool place, preferably in a refrigerator.
  - Process the specimen as soon as possible.

Specimen quality and quantity

- What is the quality of the specimen being digested and decontaminated?
  - Too watery is not satisfactory. It is mostly saliva and not the coughed up sputum. This may yield poor AFB recovery, but would not contribute to contamination.
  - If it is too mucoid, it may need additional mucolytic reagent. If not completely liquefied during the processing, it may contribute to higher contamination. During the digestion procedure, if specimen is found not completely liquefied, add a small quantity of NAC powder.

- What is the volume of the specimen being digested and decontaminated?
  - Should be 2.0–10.0 ml. Lower volumes may yield fewer positive results. Higher volumes may contribute to higher contamination.
  - Should not be pooled. Pooled specimens may result in high contamination.

Specimen processing

- What is the method of specimen digestion/decontamination?
  - NALC-NaOH is the method of choice.
  - Recommended NaOH concentration of 4% is ideal (final concentration in the specimen 1%).
  - Increase in NaOH usually results in lowering the contamination rate. Higher NaOH concentration (up to 1.5% in the specimen) is acceptable in situations where contamination is a serious problem. Once the contamination problem is under control, try to lower the NaOH concentration gradually and bring it to the recommended concentration.

  - Certain digestion/decontamination procedures are not compatible for liquid media, such as Cetyl pyridinium chloride transport and processing method.

- Are reagents made in-house or bought?
  - Commercially prepared reagents such as MycoPrep, are quality controlled. However, the NaOH concentration in these is the standard 4%. If an increase in NaOH is needed, additional NaOH becomes necessary.
  - If prepared in the laboratory, check the concentration of the reagents carefully.
  - If sterilized in the lab, check autoclaving procedures and sterility of reagents.

- If method is NaOH-NALC, is the solution made daily?
  - NaOH and sodium citrate solutions may be prepared, sterilized and kept for a long time.
  - Once NALC is added, the solution is only effective for 24 hours and should be made fresh daily.

- How much NALC is added to the NaOH and Na-citrate?
  - Check NALC concentration. It should be at least 0.5%.
  - An insufficient amount of NALC will not digest the specimens enough to allow NaOH to come in contact with contaminating bacteria. NALC helps in digestion, but does not decontaminate. NaOH does liquefy as well as decontaminate a specimen.
  - Add additional NALC in case of thick and highly mucoid specimens.
  - Presence of blood in a specimen would inactivate NALC.
  - Vigorous shaking may also inactivate NALC.
  - Make sure that NALC powder is not expired.

- How are the reagents used?
  - Bulk reagents, if used repeatedly, become contaminated. Cross-contamination may also occur.
  - Reagents should be kept in aliquots.
  - Once used, the leftover should be discarded or re-sterilized if permitted.
  - Be careful in adding the reagent to the specimen tube. Avoid touching the tube or creating any aerosol.

- Is the time for digestion/decontamination 15-20 minutes?
  - 15-20 minutes of exposure to the reagent is ideal but up to 25 minutes may not hurt mycobacteria.
  - Less than 15 minutes is not long enough and will result in high contamination.

  - More than 25 minutes would kill more contaminating bacteria but may injure mycobacteria, which will result in the increase in time-to-detection.
  - In case of persistent high contamination, time of exposure may be extended to a total of 25 minutes, but it is preferred to increase the NaOH concentration (4-6%) than increasing the time. Do not increase NaOH concentration and time to exposure at the same time.
  - In summary, longer exposure time of NaOH concentration means lower contamination and slower detection time. Shorter exposure time or lower concentration of NaOH means a higher contamination rate and a shorter detection time. You need to consider these factors.

- How often is the specimen mixed after addition of the digestion/decontamination reagent?
  - Mixing of specimen should be done two to four times after the addition of digestion/decontamination reagent.
  - Too much vortexing oxidizes the reagent and makes it less efficient.
  - Hand mixing is better.
  - Invert the tube a couple of times so the lip of the tube is well decontaminated.
  - Insufficient mixing does not allow the reagent to mix with the specimen and may cause higher contamination.
  - Is the phosphate buffer used to QC the sample to 50 ml obtained commercially (MycoPrep, sterile) or autoclaved by the facility?
  - Addition of buffer is better than water. Water is not recommended for MGIT.
  - If autoclaved in-house, check sterility and validate autoclaving procedure.

Addition of PANTA

- Check storage conditions and expiry date of lyophilized PANTA (refrigerated at 2-8ºC). Improper preparation or storage of PANTA can affect the performance or optimal concentrations.

- Is reconstituted PANTA stored properly and used in a timely manner?
  - MGIT 960: 2-8ºC within 5 days. ***May not be frozen***.
  - Manual MGIT: 2-8ºC within 72 hours. May be stored at -20ºC for up to 6 months (must not exceed expiration date). Must be used once thawed and may not be refrozen.

- Was PANTA reconstituted properly?
  - MGIT 960: 15 ml of Growth Supplement per vial.
  - Measure out 15 ml of Growth Supplement, as the vial has little more than a 15 ml capacity.

- Was the Growth Supplement added to the lyophilized PANTA with a sterile transfer device?

- Did PANTA completely dissolve in the solution?
  - If PANTA is not completely dissolved, this will cause contamination problem.

- Was the Growth Supplement added to PANTA inside a safety hood?
  - Addition on the bench top outside a safety hood may introduce environmental contamination.

- Was Growth Supplement/PANTA added to the tubes by following the recommended procedures?
  - MGIT 960: 0.8 ml/tube
  - Do not open all the MGIT tubes at one time.
  - Add into the tubes with a sterile transfer device. Use of a repeat pipettor with sterile cotton plugged tip helps in reducing contamination.
  - To ensure maximum potency of the antimicrobials, PANTA should be freshly reconstituted and added to the MGIT tube on the day the medium will be inoculated
  - Be sure to mix MGIT tubes after all additions. Mix by inverting the tube.

- What if a high contamination rate persists?
  - If a high contamination rate persists, the PANTA concentration may be increased up to a maximum of 2 times the recommended concentration, by reconstituting the PANTA with smaller quantities of reconstituting fluid (Growth Supplement). First, try increasing the PANTA concentrations 25-35% by reconstituting the PANTA with a smaller quantity of water (manual MGIT) or Growth Supplement (MGIT 960). Do not change the quantity of reconstituted PANTA/supplement that is added to the MGIT tube.
  - Do not increase PANTA and NaOH concentration at the same time. Try one at a time. Once the contamination is under control, bring the concentration back to the normal recommended concentration.

Specimen inoculation

- Was specimen inoculation carried out inside the safety hood?
  - Never inoculate outside a safety cabinet.
  - Do not keep specimen tubes close to each other as this may result in cross-contamination.

- Were the tubes inoculated with the correct amount of specimen?
  - 0.5 ml for all systems.
  - Adding more of the specimen can affect PANTA concentrations or overwhelm the antibiotics, resulting in higher contamination.
  - Do not open all the tubes. Open one tube at a time for inoculation

- Were the inoculated MGIT tubes mixed after the PANTA and specimen have been added?
  - Failure to mix may result in contaminating bacteria not coming in contact with the antibiotics.

Other factors

- Was the elevated contamination rate occurring in general, or can isolated incidences of increased contamination be identified?
  - Particular days. A special situation may be responsible, such as construction pollution, a dust storm or dusty environment.
  - Particular season. High contamination rate in summer is common. Improve transport time and conditions.
  - High contamination associated with one person. Some technicians are more experienced or careful than others. Need retraining.

- Was contamination caused by a common contaminant or various contaminants?
  - Establish if the contamination is coming from the environment, from a constant source or from specimens. Usually, contamination with a specific type of bacteria (or mycobacteria) comes from a common source such as the water supply or contaminated reagent(s).
  - If one particular contaminant persists, check the sterility of reagents. Also check the laboratory environment for a possible source.
  - Run negative control to check contamination during the processing

Quality control

- Are you carrying out regular Quality Control testing with negative and positive controls?
  - Recommendation: Process 5 ml sterile buffer (negative control) along with regular batch of specimens processed in a day. Process the negative control in the same way as clinical specimens and inoculate into MGIT tubes. This would indicate if there is a source of contamination during the processing.

- Periodic sterility testing of the reagents, especially a freshly made batch, is required to keep a check on contamination source from the reagents. Use blood agar plate or any other suitable bacteria medium for checking contamination and Middlebrook agar or LJ medium for mycobacterial contamination check.
- Environmental contamination may be reduced by thoroughly disinfecting the lab, working inside a biosafety hood for all the additions and other processes, and fixing the source of contamination, if established.

4. Cross-contamination

Cross-contamination of mycobacteria from one tube to another is common in mycobacteriology laboratories. Take the following precautions to prevent such cross-contamination.

- Use daily aliquots of NaOH-NALC-Citrate solution and PBS. Any leftover should be discarded. The buffer may be sterilized again by autoclaving.
- Keep the specimen tubes tightly closed and clean them from outside prior to vortexing or shaking.
- Pour decontamination reagents or buffer slowly on the side of the tube without causing any splashing. Do not touch the container of reagents to the lip of the tube at the time of addition.
- After vortexing, wait awhile before opening the cap so that aerosol generated during the mixing settles down. Open cap of specimen tube very gently to avoid aerosol generation.
- When adding reagents to the tube, open one tube at a time. Do not keep all the tubes open at the same time.
- Do not place tubes too close to each other in the rack.

APPENDIX D

Guidelines for Susceptibility Testing

1. Initial start and evaluation of BACTEC MGIT 960 susceptibility testing

a. Introduction

The following are the suggested guidelines for those BACTEC MGIT 960 users who would like to evaluate this system for susceptibility testing and compare it with a reference method such as the BACTEC 460TB method. As the BACTEC MGIT 960 susceptibility test (AST) is a rapid broth-based procedure, it compares best with BACTEC 460 radiometric method from a workflow standpoint. However, there are some technical differences in the two broth-based methods and results of the two methods would compare the best if these differences are understood and when the recommended procedures for BACTEC MGIT 960 and the reference method are strictly followed. Some of the procedures are already included in the main drug susceptibility section of this manual and are repeated briefly for convenience. These guidelines are meant to re-emphasize the key points of the BACTEC MGIT 960 procedures.

b. Planning the evaluation

- First review the published data and look for accuracy and reproducibility of the new system. Look for the comparative antimicrobial susceptibility testing (AST) data with BACTEC MGIT 960 and the reference method for both resistant and susceptible strains.

- Evaluate the system in your laboratory by comparing the BACTEC MGIT 960 method with a reference method. Since BACTEC MGIT 960 is a broth-based system, it is often compared with the BACTEC 460TB system.

- BACTEC MGIT 960 susceptibility test can be set up from growth on solid medium or in liquid medium. However, it is recommended that initially it should be done from growth in the BACTEC MGIT 960 medium. A standardized inoculum is critical for susceptibility testing and inoculum from a fresh positive MGIT tube is more easily standardized as compared to inoculum prepared from growth on solid media.

- Develop a protocol for this evaluation. The protocol should include the following details:

    - Start and finish dates of study.

- The personnel who would carry out actual testing. Preferably one person who is well experienced in susceptibility testing should be responsible for the whole evaluation study.

- Selection of a reference method which has been used successfully and routinely in your laboratory. The reference method could be BACTEC 460TB system, Middlebrook agar (7H10 or 7H11) or Lowenstein-Jensen (LJ) medium.

- Which drug concentrations will be used in the test and what will be the reference method? The concentrations should have established equivalency for both critical and higher concentrations.

- How many total cultures are to be tested during the planned evaluation and how many cultures are to be tested in each set-up? Select a reasonable number of cultures (preferably 20-50) with some known resistant strains (8-10). Preferably select some freshly isolated cultures and some from frozen stock cultures.

- How will the discordant results be handled? Cultures with discordant results may be retested with both methods or with a different method. Another option is that an arbiter could be arranged who would test these in a different laboratory with the same or a different method.

- How will the borderline resistant cultures be handled?

- How will the final results be analyzed?

- How will the reproducibility of the test system be evaluated? It helps to test reproducibility of the test method. If reproducibility is to be evaluated, select a small number of test strains (5-10) with a few (2-5) resistant strains included in this testing. Set up each isolate 2-5 times, each on a separate day. Set up the test and the reference method with freshly prepared inoculum each time and use the same inoculum for both methods, which should be carried out at the same time.

c. Preparation of inoculum

- Work under a biological safety cabinet.

- From a positive 7 ml MGIT tube use the following guidelines: (for details refer to Section III-A-4-C)

Day 0 – the day a MGIT tube is positive by the instrument. Re-incubate at least one more day.
Day 1 or 2 – one or two days after the instrument is considered positive. Use undiluted for the susceptibility testing inoculation.
Day 3, 4 or 5 – dilute 1:5 and use for the susceptibility testing inoculation.
More than 5 days – subculture in MGIT and follow the above criteria.

Procedures:

- Once a MGIT tube is instrument positive, make an AFB smear and confirm the presence of typical cords of AFB without any contamination.

- Observe growth in the tube. There should be detectable particulate growth in the tube.

- Make sure it is not contaminated (turbid).

- Make sure it is a pure culture of *M. tuberculosis*. If mycobacteria other than *M. tuberculosis* (NTM) are present, alone or with *M. tuberculosis*, AST results will not be reliable.

- Mix the positive MGIT broth well by vortexing. Leave for 5-10 minutes to allow large clumps to settle. Take inoculum from the supernatant broth.

- If a tube is positive longer than 5 days, or received with unknown biomass, subculture into a fresh 7ml MGIT tube. Follow procedure described below. Mix tube by inversion.

  - Make a 1:100 dilution of the positive tube using a saline or 7H9 broth
  - Inoculate a MGIT tube (supplemented with Growth Supplement without PANTA) with 0.5 ml of 1:100 diluted specimen
  - Cap tube tightly, mix by inversion
  - Enter tube into instrument

- A too heavy or too light inoculum may give unsatisfactory results.

From growth on solid medium

- Use freshly grown cultures within 14 days after colonies appear on the medium.

- Follow the procedure carefully. Make a very homogeneous suspension with turbidity comparable to McFarland #0.5 standard.

Precautions

- Use a pure culture of *M. tuberculosis* with no contamination and no mixed cultures of different mycobacteria.
- Make sure the suspension is well dispersed. Presence of large clumps may influence susceptibility test results.
- Do not scrape off medium along with the growth. It will give false turbidity.
- When making dilutions, mix well by inverting at least 4-5 times or by vortexing.
- Using a suspension that is too heavy or too light or with low viability may alter the AST results.

d. Addition of supplement into MGIT tubes

- Work under a biological safety cabinet to avoid outside contamination
- Use BACTEC MGIT 960 Growth Supplement for growth and BACTEC MGIT 960 SIRE Supplement for susceptibility testing. Using the wrong supplement will alter the AST results.
- Use of automatic pipettor with sterile tips for adding the supplement helps the workflow and would reduce the chance of contamination.
- Open one tube at a time and do not leave the cap off too long.
- Close the cap and mix well.

e. Addition of drugs to the medium

- Work under a biological safety cabinet.
- When reconstituting a drug vial, make sure the drug is completely mixed in the solution. Partially dissolved drug solution may give false resistance.
- Add exactly 100 µL (0.1 ml) of a drug to the properly labeled tube. Use a properly calibrated sterile pipette or a pipettor with sterile tip with a cotton plug.

- Do not add drug to the growth control.
- Always follow the sequence of G, S, I, R, E and make sure each drug is added to its respective, labeled tube.
- Use a separate pipette or pipette tip for each drug.
- Do not leave the MGIT tube cap open for too long.

f. Addition of inoculum

- Work under a biological safety cabinet.
- Add 0.5 ml of the appropriate inoculum into each drug containing MGIT tube. Do not add to the Growth control.
- Dilute the inoculum 1:100 (for SIRE) or 1:10 (for PZA) and inoculate 0.5 ml to the growth control tube.
- Make sure the inoculum is well homogenized and well mixed.
- Use a separate sterile pipette or sterile tip with cotton plug for each test culture.
- Tighten the caps and mix the medium by inverting three to four times.

g. Loading MGIT tubes in the instrument

- Make sure the tubes are in the proper AST Set Carrier and in the proper order, for example Growth Control, Streptomycin, INH, Rifampin, and Ethambutol (C, S I R E), or in case of PZA, Control, PZA.
- Select the proper Set Carrier definition when entering the AST set in the instrument, as the instrument can read the tubes only according to the defined Set Carrier order.
- There are 2, 3, 4, 5, and 8 AST Set Configurations available.

h. Interpretation of results

- BACTEC MGIT 960 SIRE AST is a 4-13 day and MGIT 960 PZA is a 4-21 day qualitative test. Results are interpreted and reported by the instrument as **S**, susceptible or **R,** resistant or **X,** uninterpretable.

- The BACTEC MGIT 960 instrument continuously monitors the fluorescence of tubes in terms of Growth Units (GU). Predefined algorithms compare the GU of a drug-containing tube with the growth control tube.

- When the growth control reaches a GU of >400 between 4-13 days for SIRE and 4-21 days for PZA, the drug containing tubes are evaluated. If the GU of the drug-containing tube is $\geq$100, then the result is interpreted as "Resistant", and if it is less than 100, it is "Susceptible".

- Susceptibility results and GU values are printed in the Unloaded AST Set Report.

- GU values around 100 in the drug-containing tube at the time when the growth control tube GU is around 400 may be due to borderline resistance (typically caused by an isolate with a MIC near the test concentration) but the instrument will interpret strictly according to the above rule.

- For AST sets entered in the instrument as undefined drugs, the instrument marks the AST set complete when the GU of the growth control tube reaches $\geq$400. The Unloaded AST Set Report will print GU values but no interpretations will be provided. A manual interpretation, using the above rules, should be made.

- GU values can be retrieved from the instrument Vial Inventory Report for all ongoing AST sets.

- Reports are given qualitative results, such as "Susceptible" or "Resistant", according to the CLSI (NCCLS) recommendations.

- According to the CDC and CLSI recommendations, always test at the "critical" concentration.

- Testing of higher concentrations is optional. Clinical relevance of higher concentration testing is non-defined.

- Usually higher concentrations are tested when a patient isolate is resistant to the critical concentrations, particularly in the case of INH>.

- If an isolate is resistant at the low level of INH and susceptible at the high concentration, it is considered low-level resistance (CLSI).

- Isolates with low-level resistance may give inconsistent results when repeatedly tested with the same method or even another method.

- If a new patient isolate is found resistant, the resistance should be reconfirmed by the same or different method.

- Mono-resistance, especially in cases of Rifampin, Ethambutol and PZA, is rare and should be confirmed before reporting.

i. How to evaluate BACTEC MGIT 960 susceptibility results

- Carry out parallel testing with BACTEC 460TB (or another reference method) and BACTEC MGIT 960 using the same culture inoculum. Carefully follow the recommended procedure for both methods. Use the critical concentrations of drugs.

- With discrepant results, first check the purity of the culture.

- If the culture is pure, repeat testing with both methods.

- If possible, retest with the critical as well as with the higher concentrations (S, I, E) with both methods. This would help in determining if the culture is borderline resistant.

- Include a QC strain, such as H37Rv, with each susceptibility test set up. H37Rv strain should yield reportable results with complete susceptibility to all the test drugs at the critical concentrations. If any discrepancy is observed with this strain, discard all the results obtained in that set up.

- Analyze the data for “susceptible”, “resistant” and those which had shown “borderline” results. For BACTEC 460TB, the GI values can indicate the borderline situation while 960 does not use borderline designation. The BACTEC MGIT 960 GU values may be helpful in establishing borderline resistance.

- Borderline resistant cultures are isolates with an MIC near the test concentration. Usually borderline resistant isolates yield inconsistent results and may cause discrepancies when two methods are compared. These cultures are found susceptible when tested at the higher drug concentration of a test drug.

- Calculate sensitivity and specificity of the new system as compared to the reference method.

- Look at the repeat testing and evaluate the reproducibility of both methods.

- False resistance by the test method as compared to the reference method is considered a major error, while false susceptibility by the test method as compared to the reference method is considered to be a much graver error since it would be detrimental for patient therapeutic management.

- Calculation of positive and negative predictive values may not represent the real situation as these values depend upon the prevalence of drug resistance in a population.

2. Susceptibility testing for second line drugs

The BACTEC MGIT 960 susceptibility test is based on growth of the *Mycobacterium tuberculosis* isolate in a drug-containing tube compared to a drug-free tube (Growth Control). The BACTEC MGIT 960 instrument continually monitors tubes for increasing fluorescence. Analysis of fluorescence in the drug-containing tube compared to the fluorescence of the Growth Control tube is made by the instrument to determine susceptibility results for SIRE and PZA at the concentrations provided by BD. Predefined algorithms compare the growth unit (GU) in the drug-containing tube to the growth unit in the Growth Control tube. A result of susceptible or resistant is reported by the instrument. Susceptibility tests Set Carriers with different configurations are provided. The scanning of these Set Carriers carrying the inoculated MGIT tubes tells the 960 instrument what drugs are being tested. Since the instrument software is designed for SIRE and PZA drug susceptibility testing only, other drugs if inoculated would not be recognized by the instrument. However, there is a feature built into the system that can accept undefined drugs. If the Set Carrier is entered as undefined drugs, the instrument will monitor the susceptibility, indicate when the test is ready but will not interpret the results. An undefined drug susceptibility test is to be interpreted manually. With the undefined entry feature you can test any secondary or new anti-tuberculosis drug. Make sure the tubes are properly labeled with the name of the test drug and concentration. The first tube in the set carrier should always be the growth control (with 1:100 dilution of the inoculum).

BD has not validated drugs other than SIRE and PZA. However, two studies, including one multicenter study, have been carried out in Europe for testing several secondline anti-tuberculosis drugs with good reproducible results (see Section III C).

If the Set Carrier is entered as undefined, the growth control (GC) tube is monitored until a GU of 400 is reached. At that point in time, the AST set is marked as complete by the instrument.

Take out the susceptibility test carrier after scanning.

Print the Unloaded AST Set Report, which will have GU values for the drug tubes.

Growth Control (GU) ≥ 400

- If the GU of the drug containing tube is 100 or more, the result is reported as "Resistant".
- If the GU of the drug containing tube is less than 100, the result is reported as "Susceptible".

Remember, the Instrument Inventory Report prints GU values for all ongoing AST sets.

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