Standard Operating Procedures (SOPs)
For
MTB Culture & Drug Susceptibility Testing (DST)

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ACKNOWLEDGEMENTS

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SOPs for MTB Culture & DST Development

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Contents

DEFINITIONS

Accident: An undesired event giving rise to death, ill-health, injury, damage, loss or distress.

Antimicrobial susceptibility test interpretive category – a classification based on an *in vitro* response of an organism to an antimicrobial agent. For mycobacteria, two different categories, "critical concentration" and "minimum inhibitory concentration," have been used to categorise the *in vitro* results. For strains of *Mycobacterium tuberculosis* complex, when tested against the lower concentration of some agents, the "critical concentration" category is applied. Testing of an additional higher concentration (a clinical breakpoint concentration) may also be recommended for some agents. However, there is no "intermediate" interpretive category, even when testing is performed both at the critical concentration and the clinical breakpoint concentration.

Antiseptic: Substance that inhibits the growth and development of microorganisms without necessarily killing them. Antiseptics are usually applied to body surfaces.

Biohazard: The potential source of harm caused by biological agents or toxins.

Biosafety: The set of containment principles, technologies and practices that are implemented to prevent the unintentional exposure to biological agents and toxins, or their accidental release

Biosecurity: The set of measures aimed at the protection, control and accountability for valuable biological materials (VBM) and protection of other valuable items (e.g., equipment) within laboratories, in order to prevent their loss, theft, misuse, diversion of, and/or unauthorized access or intentional unauthorized release

Bio-risk management: The analysis of ways and development of strategies to minimize the likelihood of the occurrence of bio-risks.

Critical concentration of an anti-tuberculous agent has been adopted and modified from international convention. The critical concentration is defined as the lowest concentration of an anti-TB agent *in vitro* that will inhibit the growth of 99% (90% for pyrazinamide) of phenotypically wild type strains of *M. tuberculosis* complex.

Clinical breakpoint – is the concentration or concentrations of an antimicrobial agent which defines an MIC above the critical concentration that separates strains that will likely respond to treatment from those which will likely not respond to treatment. This concentration is determined by correlation with available clinical outcome data, MIC distributions, genetic markers, and PK/PD data including drug dose. A dose increase can be used to overcome resistance observed at lower dosing, up until the maximum tolerated dose, and therefore a higher clinical breakpoint above which the particular drug is not recommended for use. The clinical breakpoint is used to guide individual clinical decisions in patient treatment. The clinical breakpoint is not applicable for drug resistance surveillance purposes.

Critical proportion – is the proportion of resistant organisms within a particular cultured isolate that is used to determine resistance to a particular drug. A 1% (10% for pyrazinamide) critical proportion is used to differentiate susceptible and resistant strains. Any culture that shows less than 1% growth on a medium containing a critical concentration of the agent being tested when compared with the growth on a control without the agent is considered to be susceptible; a culture that has 1% or more growth on the medium containing the critical concentration of the agent is considered to be resistant, and the patient whose sample is being tested may not respond to the agent. The critical concentration and proportion criteria are used for testing first-line and second- line anti-TB agents.

Cross-resistance is resistance to multiple anti-tuberculosis agents caused by a single genetic

change (or multiple changes in case the given resistance mechanisms requires several genetic alterations), although in practice, such mutations may not be known.

Decontamination: Any process for removing and/or killing microorganisms. The same term is also used for removing or neutralizing hazardous chemicals and radioactive materials.

Disinfectant: Chemical or mixture of chemicals used to kill microorganisms, but not necessarily spores. Disinfectants are usually applied to inanimate surfaces or objects.

Disinfection: Physical or chemical means of killing microorganisms, but not necessarily spores.

Fire emergency

- an uncontrolled fire or imminent fire hazard
- OR the presence of smoke or the odour of burning
- OR the uncontrolled release of a flammable or combustible substance
- OR a fire alarm sounding.

Growth control: Culture yielded after inoculation of tubercle bacilli on a culture medium without any test drug to exhibit unrestricted growth.

Good microbiological technique (GMT): Working methods designed to eliminate or minimize exposure to pathogens (for example: aerosols, splashes, ingestion, absorption, accidental inoculation)

Inactivation: Process rendering an organism inert by application of heat, or other means.

Incident: An event that gives rise to an accident or has the potential to lead to an accident.

Indirect susceptibility test – a procedure based on inoculation of drug-containing media using organisms grown in culture.

Manageable fire: A small, incipient or early-stage, localized fire (no larger than a wastepaper basket).

Microbicide: Chemical or mixture of chemicals that kills microorganisms. The term is often used in place of "biocide", "chemical germicide" or "antimicrobial".

Minimum inhibitory concentration (MIC) – the lowest concentration of an antimicrobial agent that prevents growth of more than 99% a microorganism in a solid medium or broth dilution susceptibility test.

Potency – All antimicrobial agents are assayed for standard units of activity or potency. The assay units may differ widely from the actual weight of the powder and often may differ between drug production lots. Thus, a laboratory must standardise its antimicrobial solutions based on assays of the antimicrobial powder lots that are being used.

The value for potency supplied by the manufacturer should include consideration for:

- Purity measures (usually by high-performance liquid chromatography assay)
- Water content (e.g.: by Karl Fischer analysis or by weight loss on drying)
- Salt/counter-ion fraction (if the compound is supplied as a salt instead of free acid or base)
- The potency may be expressed as a percentage, or in units of micrograms per milligrams (w/w).

Proportion method: The proportion method was originally proposed by Canetti and colleagues and modified later; it is the most common method used for testing the susceptibility of *M. tuberculosis* complex isolates. In this method, the inoculum used is monitored by testing two dilutions of a culture suspension, and the growth (that is, the number of colonies) on a control medium without an anti-TB agent is compared with the growth (the number of colonies) present on a medium containing the critical concentration of the anti-TB agent being tested; the ratio of the number of colonies on the medium containing the anti-TB agent to the number of colonies on the medium without the anti-TB agent is calculated, and the proportion is expressed as a percentage. For most anti-TB agents, a 1% critical proportion to used differentiate the proportion of resistant organisms within a particular strain that is used to determine clinically significant resistance to a particular drug.

Quality Assurance: Quality Assurance is planned and systematic activities to provide confidence that an organization fulfils requirements for quality

Respirators:

- FFP2 filtering facepiece particulate respirators meet the requirements of European Standard EN149 and are CE-approved.
- N95 filtering facepiece particulate respirators meet the requirements of US Standard 42CFR84 and are CDC/NIOSH-approved.

Resistance of M. tuberculosis complex strains: Resistance of an M. tuberculosis strain according to the proportion method differs from the concept of resistance normally used in clinical microbiology. The proportion method calculates the proportion of resistant bacilli present in a strain. Below a certain proportion, the strain is classified as susceptible; above that proportion, it is classified as resistant. The strain is presumed to be resistant when growth of more than a certain proportion of the inoculum (critical proportion) occurs on culture media containing a defined concentration – the critical concentration – of the drug.

Spill of specimens: Accidental spillage of specimens before they have been subjected to appropriate waste management.

Sterilization: Process that kills and/or removes all classes of microorganisms and spores.

ABBREVIATIONS:

-18 °C	: Refers to temperatures equal to or below –18 °C
7H10	: Middlebrook 7H10 medium
7H11	: Middlebrook 7H11 medium
AMK	: Amikacin
BAL	: Broncho-alveolar lavage
BDQ	: Bedaquiline
BSC	: Biological safety cabinet, class I or class II
BSL	: Biosafety level
CAP	: Capreomycin
CB	: Clinical breakpoint
CC	: Critical concentration
CDC	: Centers for Disease Control and Prevention
CE	: European Community
CFU	: Colony-forming unit
CFZ	: Clofazimine
CLSI	: Clinical & Laboratory Standards Institute
CPC	: Cetyl-pyridinium chloride
CSF	: Cerebrospinal fluid
DCS	: D-Cycloserine
DLM	: Delamanid
DMSO	: Dimethyl sulfoxide
DR-TB	: Drug-resistant tuberculosis
DSM	: Dihydrostreptomycin
DST	: Drug susceptibility testing
DW	: Distilled water
ECOFF	: Epidemiological cut-off value
EMB	: Ethambutol
EN	: European norm
FFP	: Filtering facepiece particulate
FLD	: First Line Drug
FQ	: Fluoroquinolone (e.g., levofloxacin moxifloxacin)
GC	: Growth control
GFX	: Gatifloxacin
GMT	: Good microbiological technique
gNWT	: Genotypically non-wild type
GTB	: Global TB Programme

gWT : Genotypically wild type
HEPA : High-efficiency particulate air
HPLC : High performance liquid chromatography
ID : Patient's specimen identification, usually laboratory number
INH : Isoniazid
KAN : Kanamycin
LFX : Levofloxacin
LJ : Löwenstein–Jensen medium
LPA : Line probe assay
LZD : Linezolid
MDR : Multidrug-resistant
MFX : Moxifloxacin
MGIT : BACTEC™ Mycobacterial Growth Indicator Tube
MIC : Minimal inhibitory concentration
MOTT : *Mycobacteria* other than tuberculosis spp.
MTBC : *Mycobacterium tuberculosis* complex
MW : Molecular weight
NA : Not applicable
NALC : N-acetyl L-cysteine
NIOSH : National Institute for Occupational Safety and Health
NTP : National Tuberculosis Control programme
OACD : Oleic acid, albumin, dextrose, catalase
OFX : Ofloxacin
PK/PD : Pharmakcokinetic / pharmacodynamic
pNWT : Phenotypically non-wild type
PPE : Personal protective equipment
pWT : Phenotypically wild type
PZA : Pyrazinamide
QC : Quality control
R : Resistance/resistant
RCF : Relative centrifugal force
RMP : Rifampicin
RR : Rifampicin resistant
S : Susceptible/susceptibility
SIRE : Streptomycin, isoniazid, rifampicin, ethambutol
SLD : Second Line Drug
SLI(D) : Second-line injectable (drug) (i.e., amikacin)

SRL : Supranational reference laboratory

STR : Streptomycin

TB : Tuberculosis

WHO : World Health Organization

WT : Wild type

XDR : Extensively drug-resistant

PERSONNEL QUALIFICATIONS

Medical fitness

In accordance with national laws and practices, arrangements should be made for appropriate health surveillance of TB laboratory workers:

- before enrolment in the TB laboratory
- at regular intervals thereafter, annually, or bi-annually
- after any biohazard incident
- at the onset of TB symptoms

Ideally, individual medical records shall be kept for up to 10 years following the end of occupational exposure. Laboratory workers should be educated about the symptoms of TB and provided with ready access to free medical care if symptoms arise. Confidential HIV counselling and testing should be offered to laboratory workers. Options for reassignment of HIV-positive or immuno-suppressed individuals away from the high-risk areas of the TB laboratory should be considered. All cases of disease or death identified in accordance with national laws and/or practice as resulting from occupational exposure to biological agents shall be notified to the competent authority.

Education & Training:

The training shall be:

- Adapted to take account of new or changed conditions
- Given before a staff member takes up his/her post
- Repeated periodically, preferably every year
- Strictly supervised.

Basic education and training must be given on the following topics:

- Advanced TB diagnostics tools and techniques (GeneXpert, Solid and Liquid Culture, DST, LPA)
- Biosafety and Biosecurity of TB Laboratory
- Fire safety is relevant to all laboratory staff as fires can occur in both clean areas and contaminated areas of the laboratory.
- Good laboratory practice and good microbiological technique
- Handling of potentially infectious materials
- Hygiene requirements
- Importance of laboratory results for patient management
- Importance of laboratory results for the national TB programme
- Importance of media and reagent quality for laboratory results and patient management
- Knowledge of aseptic technique
- Laboratory design, including airflow conditions
- Organization of workflow
- Potential risks to health (symptoms of TB disease and transmission)
- Precautions to be taken to minimize aerosol formation and prevent exposure
- Prevention of incidence and step to be taken in case of incidence (Biological, Chemical, Electrical and fire hazard)
- Procedures for culture inoculation
- Use, calibration, identification of malfunctions and maintenance of autoclave, balance, pH meter and inspissators and all other equipment used in a TB culture laboratory
- Viability of bacilli and strain characteristics
- Waste handling and Management
- Wearing and use of protective equipment and clothing

Chapter 1: Basic Laboratory SOP

1.1 Scope

This SOP describes the use of personal protective equipment related to the activities necessary for isolation of TB bacilli, culture manipulation and drug susceptibility testing in the Mycobacteriology laboratory.

1.2 Procedure

1.2.1. Principle

Biohazard in the TB laboratory is mainly related to the production of infectious aerosols generated while performing technical procedures.

Specimen processing for isolation of *Mycobacterium tuberculosis* requires liquefaction of the sputum. Liquefied sputa are more prone to generate infectious aerosols than original viscous sputa. Handling *M. tuberculosis* cultures, i.e., aqueous suspensions with high bacillary loads, for identification or drug susceptibility testing (DST) can generate infectious aerosols containing large numbers of infectious organisms. Work in a biological safety cabinet (Class II-A), at least annually certified, is mandatory.

Laboratory personnel should in any case adhere rigorously to good laboratory practice and GMT.

1.2.2. Equipment and materials

Each country/facility must evaluate the risks and decide on the level of protection that is appropriate.

1.2.3. Masks and respirators

Surgical masks do not offer significant protection to laboratory personnel performing aerosol-producing TB diagnostic techniques.

Adequate respirators are the N95 (USA standard) or FFP2 (European Norm EN149). These respirators filter out 94-95% of particles that are equal or greater than 0.3 - 0.4 µm. The FFP3 respirator or N99 is certified for the retention of 99% of diameter 0.4 µm and above. However, the resistance they offer to breathing may make them uncomfortable for some workers: respirators models with exhalation valves are more comfortable.

The decision to use respirators should be based on risk assessment (e.g., laboratory activity, workload, prevalence of TB, prevalence of MDR-TB, rate of failure/relapse), which should be regularly reviewed and revised when necessary. Instruct and train all users in the proper use, fitting and limitations of their respirators.

The respirator must be inspected before every use to ensure that there are no holes other than the punctures around staples and that no damage has occurred. (Enlarged holes resulting from ripped or torn filter material around staple punctures are considered as damage.) Straps and valves have also to be checked. A damaged respirator must be replaced immediately.

Respirators should be correctly fitted to the face to prevent leakage around the face seal. This is done by placing the mask over the nose and mouth with the top elastic band over the crown of the head and the bottom elastic band over the back of the neck. The metal strip covering the nose should be firmly moulded over the bridge of the nose; smooth right and left index fingers and over the bridge towards the ears,

following the contours of the face. Do not pinch the metal over the nose, as this will cause sharp bends in the metal strip and result in poor sealing of the mask. Facial hair between the wearer's skin and the sealing surfaces of the respirator will prevent a good seal. Respirators should therefore not be used with facial hair.

Store respirators in a convenient, clean, dry and sanitary location.

Even if not worn regularly, respirators must be available in laboratories where manipulations of cultures are performed in case of accidental biohazard outside the BSC.

The reuse of N95 (or FFP2) respirators is permitted unless it has been damaged, soiled, or compromised anyway (cumulative 8 hours could be used)

An N95 mask cannot be cleaned or disinfected.

1.2.4. Gloves

In accordance with universal precautions, appropriate gloves should be worn for all procedures that involve handling of body fluids and other specimens. Gloves must be worn in case of hand injury/skin disease or when the risk of exposure to blood-borne pathogens is high; specimens resulting from invasive clinical investigation must be handled with gloves.

Gloves must be worn while handling cultures.

Proper handwashing with soap and adequate care in the handling of contaminated materials are critical elements of safe laboratory practice. Gloves should not be used as a first protection barrier.

Gloves may give a false sense of protection. Contaminated gloves may in fact be the source of hazards for other staff members if used to handle or operate equipment in the laboratory. Change gloves after every session that requires their use and after every interruption of the activity. Never wear gloves outside the laboratory.

Change gloves after every activity session that requires their use and after every interruption of the activity. Every time hands are removed from the BSC, gloves must be pulled out (as indicated below) and discarded in a waste container in the BSC.

Disposable latex, vinyl or nitrile gloves can be used, and the correct size (small, medium or large) should be available for all individuals. Hypoallergenic gloves should be provided in case of allergy to latex proteins and/or to the corn-starch used for powder. Reusing single-use gloves is not advised, discard used gloves as contaminated material.

The procedure for removing gloves safely is to pull the first glove by the cuff, over and off the first hand; before the tips of the fingers are completely out of the first glove, use the first glove to pull the second glove off the second hand completely. This should prevent the skin from contacting the outer surface of either glove. Following the safe removal of gloves, wash hands immediately with water and liquid soap

1.2.5. Gowns

Always use a gown inside the laboratory (never outside) and change daily or at least weekly given the type of gown.

Long-sleeved with narrow cuffs, back-opening gowns or overalls give better protection than laboratory coats and are preferred in microbiology laboratories. When worn, laboratory coats should be fully buttoned.

An area of the laboratory must be designated for storage of used and new clothing. Laboratory gowns must be stored apart from personnel clothing.

Consideration should be given to having extra clothing suitable for visitors, maintenance, and emergency response personnel.

Laboratory coats should not take to home for washing.

Individual workers should wear appropriate size of lab coat.

1.2.6. Protective glasses

Protective glasses should always be worn when handling acids, alkaline and irritant chemicals.

1.3. Detailed instructions

Lab staff should wear the PPE inside anteroom.

Whenever necessary, hands should be thoroughly lathered with soap, using friction, for at least 10s, rinsed in clean water and dried using a clean paper or cloth towel.

PPE must be removed before leaving the laboratory and hands must be washed.

A hand-washing sink should be provided in each laboratory room, preferably near the exit door. Foot- or elbow-operated faucets are recommended. Where not fitted, a paper/cloth towel should be used to turn off the faucet handles to avoid recontamination washed hands.

PPE should be removed in the following order:

1. Disposable gloves
2. Respirator/mask
3. Gown/suit/coat/overalls

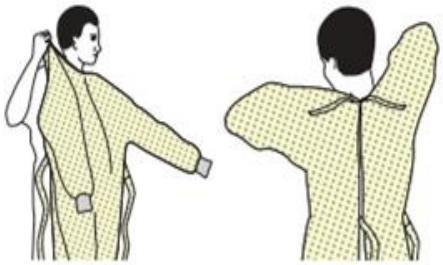
Access to the laboratory by unauthorized persons must be prevented. Entry by maintenance personnel must be allowed only if they are accompanied by laboratory staff, well experienced in biosafety measures; education in risk and appropriate PPE should be provided.

SEQUENCE FOR PUTTING ON PERSONAL PROTECTIVE EQUIPMENT (PPE)

The type of PPE used will vary based on the level of precautions required, such as standard and contact, droplet or airborne infection isolation precautions. The procedure for putting on and removing PPE should be tailored to the specific type of PPE.

1. GOWN

- Fully cover torso from neck to knees, arms to end of wrists, and wrap around the back
- Fasten in back of neck and waist



2. MASK OR RESPIRATOR

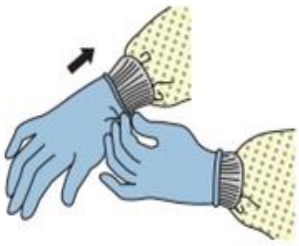
- Secure ties or elastic bands at middle of head and neck
- Fit flexible band to nose bridge
- Fit snug to face and below chin
- Fit-check respirator

3. GOGGLES OR FACE SHIELD

- Place over face and eyes and adjust to fit

4. GLOVES

- Extend to cover wrist of isolation gown



USE SAFE WORK PRACTICES TO PROTECT YOURSELF AND LIMIT THE SPREAD OF CONTAMINATION

- Keep hands away from face
- Limit surfaces touched
- Change gloves when torn or heavily contaminated
- Perform hand hygiene

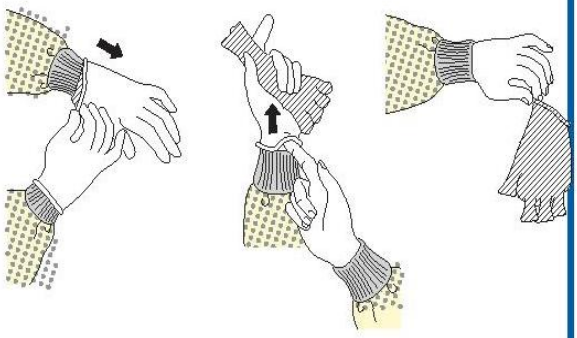
CDC

SEQUENCE FOR REMOVING PERSONAL PROTECTIVE EQUIPMENT (PPE)

Except for respirator, remove PPE at doorway or in anteroom. Remove respirator after leaving patient room and closing door.

1. GLOVES

- Outside of gloves is contaminated!
- Grasp outside of glove with opposite gloved hand; peel off
- Hold removed glove in gloved hand
- Slide fingers of ungloved hand under remaining glove at wrist
- Peel glove off over first glovet
- Discard gloves in waste container

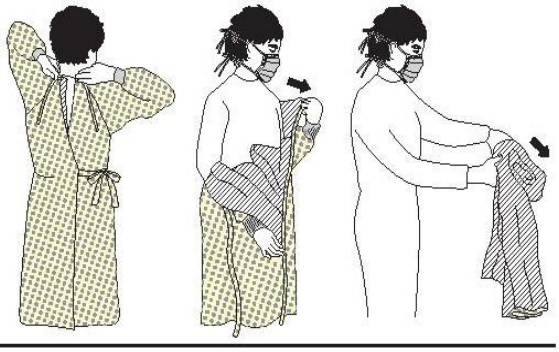


2. GOGGLES OR FACE SHIELD

- Outside of goggles or face shield is contaminated!
- To remove, handle by head band or ear pieces
- Place in designated receptacle for reprocessing or in waste container

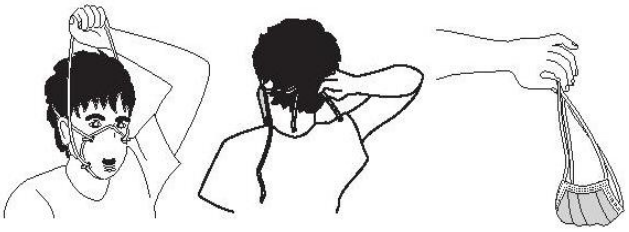
3. GOWN

- Gown front and sleeves are contaminated!
- Unfasten ties
- Pull away from neck and shoulders, touching inside of gown only
- Turn gown inside out
- Fold or roll into a bundle and discard



4. MASK OR RESPIRATOR

- Front of mask/respirator is contaminated — DO NOT TOUCH!
- Grasp bottom, then top ties or elastics and remove
- Discard in waste container



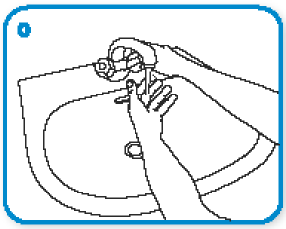
PERFORM HAND HYGIENE BETWEEN STEPS IF HANDS BECOME CONTAMINATED AND IMMEDIATELY AFTER REMOVING ALL PPE

CS250672-A

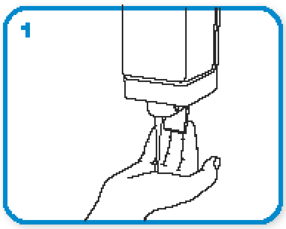
How to handwash?

WASH HANDS ONLY WHEN VISIBLY SOILED! OTHERWISE, USE HANDRUB!

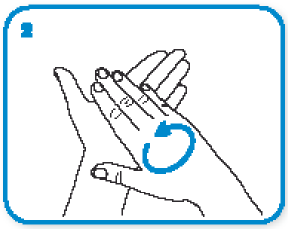
Duration of the entire procedure: **40-60 sec.**



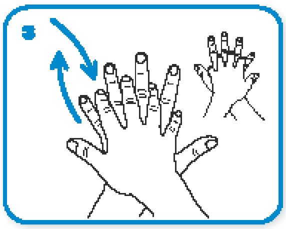
Wet hands with water



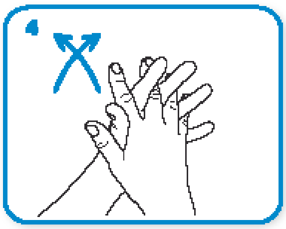
apply enough soap to cover all hand surfaces.



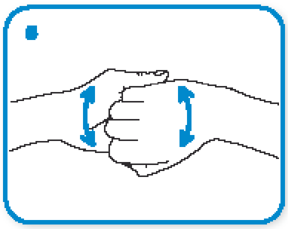
Rub hands palm to palm



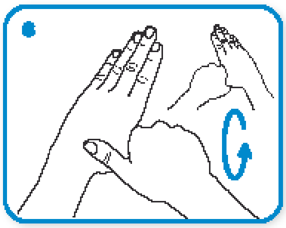
right palm over left dorsum with interlaced fingers and vice versa



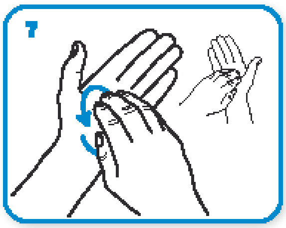
palm to palm with fingers interlaced



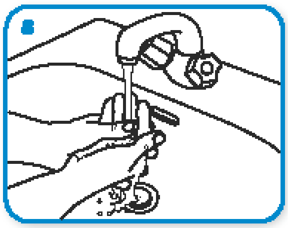
backs of fingers to opposing palms with fingers interlocked



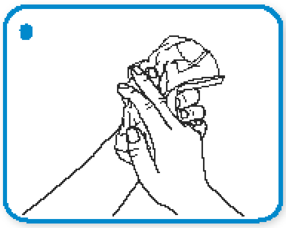
rotational rubbing of left thumb clasped in right palm and vice versa



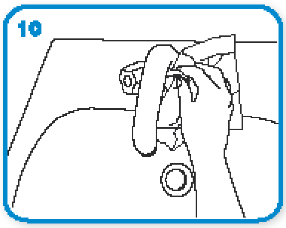
rotational rubbing, backwards and forwards with clasped fingers of right hand in left palm and vice versa.



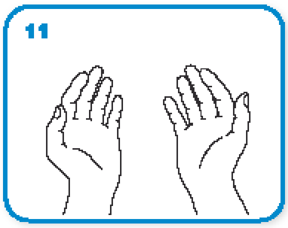
Rinse hands with water



dry thoroughly with a single use towel



use towel to turn off faucet



...and your hands are safe.

WORLD ALLIANCE for PATIENT SAFETY

WHO acknowledges the Hôpitaux Universitaires de Genève (HUG), in particular the members of the Infection Control Programme, for their active participation in developing this material.

World Health Organization

October 2006, version 1.

2.1. **Scope**

This SOP describes the use of disinfectants used in the laboratory to decontaminate surfaces and equipment, also as a pre-decontamination treatment before autoclaving, burning or incinerating waste to be eliminated.

2.2. **Principle**

The temporal killing action of disinfectants depends on the population of organisms to be killed, the concentration used, the duration of contact and the presence of organic debris.

The proprietary disinfectants suitable for use in tuberculosis laboratories are those containing phenols, chlorine, alcohol etc. These are usually selected according to the material to be disinfected.

It is incorrect to assume that a disinfectant which has general usefulness against other microorganisms is effective against tubercle bacilli. Several commercially available disinfectants have no or little mycobactericidal activity, while quaternary ammonium compounds are not effective at the recommended concentrations.

Note: All the above disinfectants are toxic and undue exposure may result in respiratory distress, skin rashes or conjunctivitis. However, used normally and according to the manufacturers' instructions, and national chemical safety regulations, they are safe and effective.

2.3. Equipment and materials

- Glass or plastic bottles of adequate volumes (e.g., 100 ml, 500ml, 10 litres, 50 litres) with leak-proof tops.
- Measuring cylinders
- Plastic or metal funnel
- Balance
- Distilled water

3. Reagents and solutions

2.3.1. Phenol

Phenol should be used at a concentration of 5% in water. However, inhalation and dermal exposure to phenol is highly irritating to the skin, eyes, and mucous membranes. Phenol is also considered to be very toxic to humans through oral exposure. Phenol solutions are used for decontaminating equipment and single use items prior to disposal. They are useful for cleaning up sputum spills in soaked paper towels to cover working surfaces.

Never Autoclave phenol containing substances.

2.3.2. Alcohols

Alcohols, ethanol (denatured ethanol), are used at 70%. Alcohols are volatile and flammable and must not be used near open flames. Working solutions should be stored in proper containers to avoid the evaporation of alcohols. Bottles with alcohol-containing solutions must be clearly labelled to avoid autoclaving.

Alcohols can be used on skin, work surfaces of laboratory benches and biosafety cabinets, and to soak small pieces of surgical instruments. A major advantage of aqueous solutions of alcohols is that they do not leave any residue on treated items. When hands become contaminated, a rinse with 70% ethanol, or isopropyl alcohol followed by thorough washing with soap and water is effective.

To prepare 70% dilutions of alcohol (with different concentrations), follow the ratios below:

- Mix 100 ml of alcohol 95% and 39.1 ml of distilled water
- Mix 100 ml of alcohol 90% and 31.0 ml of distilled water
- Mix 100 ml of alcohol 85% and 23.1 ml of distilled water
- Mix 100 ml of alcohol 80% and 15.3 ml of distilled water
- Mix 100 ml of alcohol 75% and 7.64 ml of distilled water

2.3.3. Chlorine

Chlorine is a widely available disinfectant.

- Sodium hypochlorite solutions, as domestic bleach, contain 50 g/l available chlorine and should therefore be diluted 1:50 or 1:10 to obtain final concentrations of 1 g/l and 5 g/l, respectively. Bleach, either in stock or in working solutions must be stored in well ventilated, fresh and dark areas. In good storage conditions, the 50g/l solution may last as long as 3 months, while diluted solutions should be prepared daily. However, the actual content of available chlorine in domestic bleach may not be reliable in many countries. The two alternatives below should be considered
- Granules or tablets of calcium hypochlorite ($Ca(ClO)_2$) generally contain about 70% available chlorine. Solutions prepared with granules or tablets, containing 1.4 g/l and 7.0 g/l, will then contain 1.0 g/l and 5 g/l available chlorine, respectively.

Storage of stock or working solutions in open containers releases chlorine gas thus weakening their germicidal potential. Bleach can be used as a general-purpose disinfectant and for soaking contaminated metal-free materials (it is highly alkaline and can be corrosive to metal).

2.4. Detailed instructions

Use disinfectant as indicated in the technical procedures.

	Title: Procedure in case of spill of infectious material within the biological safety cabinet		
	SOP Number: 3	Effective Date:	Page **1-3**
	Version: 2.0	April, 2022	
NTP, Bangladesh	July, 2022	Updated in line with the GLI Guideline	

3.1. Scope

This SOP describes hygiene and safety measures for worker protection against laboratory-acquired tuberculosis in the event of an accidental spill of infectious material inside the biological safety cabinet (Class II-A). It refers to spillage of liquids consisting of suspensions of tubercle bacilli or broken tubes of cultures on solid media which generate infectious aerosols and represent a potential hazard for laboratory-acquired tuberculosis.

3.2. Procedure

3.2.1. Principle

When a spill of biohazardous material occurs within a BSC, clean-up should begin immediately, while the cabinet continues to operate. An effective disinfectant should be used and applied in a manner that minimizes the generation of aerosols.

3.2.2. Equipment and materials

- Annually certified BSC
- Absorbent tissue
- Protective clothing
- Gloves
- Forceps
- Autoclavable bags
- Waste containers

3.2.3. Reagents and solutions

Little amount of spill (up to 5ml of liquid sample): Alcohols, ethanol (denatured ethanol, methylated spirits) or iso-propanol, are used at 70%. When hands become contaminated, a rinse with 70% ethanol, or isopropyl alcohol followed by thorough washing with soap and water is effective.

Large amount of Spill (more than 5ml of biological sample) Sodium hypochlorite solutions, as domestic bleach, contain 50 g/l available chlorine and should therefore be diluted 1:50 or 1:10 to obtain final concentrations of 1 g/l and 5 g/l, respectively.

3.2.4. Detailed instructions

- Any laboratory worker in the vicinity of the spill must immediately wash his/her hands and forearms, even if not overtly contaminated by the spill. Use water and soap for cleaning. In case of wounds, cuts or abrasions, medical care should be sought immediately.
- Place absorbent tissue over the spill area and apply disinfectant solution liberally. If BSC walls have been splashed, disinfectant solution should be sprayed or poured onto the contaminated surfaces, which are then covered with a layer of absorbent tissue.
- Leave affected areas covered for a minimum of 2 hours.
- Pick up the contaminated material using forceps, tissue and gloves, and place in an autoclavable bag.
- Any equipment or reusable material (homogenizer, blender, shaker, centrifuge buckets, etc.) that has been splashed should be swabbed with the same disinfectant. Removable parts should be washed with water and dried. Electrical equipment should be checked carefully (integrity of circuit-breakers and earth-fault-interrupters) before use.
- In case of major spillage (large volumes of liquid cultures of tubercle bacilli), the BSC HEPA filters should be fumigated.

3.2.5. Reporting

Notify the incident to head of the laboratory.

Every incident/accident must be documented, and records must be kept in the laboratory archives. All corrective action must be similarly documented.

3.2.6. Quality control

Further work in the BSC must be authorized by the laboratory supervisor.

If a large volume (>20 ml) of infectious material is spilled, an inspection of the BSC may be necessary.

3.2.7. Waste management

All materials used in the clean-up should be treated as infectious waste.

Contaminated-waste containers should be autoclaved. Those containing broken glass should be specifically labelled "CAUTION: GLASS DEBRIS" to prevent further risks of cuts and wounds.

Annex 1. Example of a warning sign

WARNING

DO NOT ENTER

MAJOR BIOLOGICAL HAZARD

Annex 2. Incident report form

Institution:	
Laboratory designation:	
Head of the laboratory:	
Date, time of the incident:	
Nature of the incident (what happened?)	
Extent of the incident: • estimate of the volume of contaminated spill • details of the volume of broken glass (if any) • description of the surfaces contaminated • list of material/equipment contaminated (if any) • list of samples involved in the incident	
List of persons present in the laboratory when the incident occurred	
List of persons with physical injury (if any) Nature of physical injury	
Name of the physician in charge of the first medical aid	
Name of the physician in charge of the medical record and further medical surveillance	
Corrective action: • how to prevent such an incident • how to improve staff adherence to safety procedures	

	Title: Emergency procedure in case of major biohazard incident outside the biological safety cabinet		
	SOP Number: 04	Effective Date:	Page **1-4**
	Version: 2.0	April, 2022	
REVISION			
Revised by	Effective date	Description of change	
NTP, Bangladesh	July, 2022	Updated in line with the GLI Guideline	

4.1. Scope

This SOP describes hygiene and safety measures for worker protection against laboratory-acquired tuberculosis in the event of an accidental spill of infectious material outside the biological safety cabinet. It refers to spillage of liquids consisting of suspensions of tubercle bacilli or broken tubes of cultures on solid media which generate infectious aerosols and represent a potential hazard for laboratory-acquired tuberculosis.

4.2. Procedure

4.2.1. Principle

In case of a major biohazard accident outside the BSC, **evacuation of the area must be the priority.**

4.2.2. Equipment and materials

Warning sign - DO NOT ENTER (see below, under "Related documents")

Emergency spill-kit, stored outside the laboratory but close to the laboratory entrance, containing the following (not Limited to):

- N95 filtering facepiece particulate respirators (meet the requirements of US Standard 42CFR84 and are CDC/NIOSH-approved), at least 10
- head covers, two
- protective glasses (goggles), two
- Gown/apron single use, two
- Shoe cover at least 10 pairs
- autoclavable plastic bags, 1000 x 700 mm, with biohazard sign, at least five
- thick-walled household rubber gloves (to pick up shards and sharps), at least two pairs
- latex gloves, one package each of medium and large
- forceps or tongs
- container for sharps
- absorbent paper tissue, at least 500 x 500 mm, about 1 kg
- concentrated disinfectant for dilution
- bottle with spray head, 1-litre capacity
- alcoholic disinfectant or equivalent, 1 litre (see SOP on use of disinfectants).

4.2.3. Reagents and solutions

Sodium hypochlorite solutions, as domestic bleach, contain 50 g/l available chlorine and should therefore be diluted 1:50 or 1:10 to obtain final concentrations of 1 g/l and 5 g/l, respectively

Bleach, either in stock or in working solutions must be stored in well ventilated, fresh, and dark areas. In good storage conditions, the 50g/l solution may last for 3 months, while diluted solutions should be prepared daily.

Follow the manufacturer's instruction to prepare and use the reagents.

4.2.4. Detailed instructions

- The laboratory worker who caused or noticed the accident **must alert all other** persons present (in the vicinity of the emergency spill situation) and order them to evacuate the laboratory.
- Once outside the laboratory, preferably in the anteroom, ensure that all workers previously working in the area have now **vacated the laboratory**.
- Workers must **remove protective clothing**, specifically gowns that may have been splashed during the spill (and shoes in case of walking across the contaminated floor area).
- Workers **need to wash parts of their bodies** have been splashed, with water and soap. Apply appropriate skin disinfectant on wounds, cuts, and abrasions (see SOP on the use of disinfectants). Seek medical attention, as necessary.
- Place the DO NOT ENTER warning sign on the door of the laboratory.
- **Notify** the laboratory supervisor of the accident, giving as much information as possible on the location, nature, and extent of the accident, as well as any possible contamination of equipment.
- Based on this information, the head of the laboratory must decide how and when the decontamination will be carried out, and by whom.
- In a laboratory equipped with HVAC system (directional air flow), with rooms sealable for decontamination and air-ducting systems constructed to permit gaseous decontamination, fumigation the laboratory may be considered and must be carried out only by a certified professional.
- When fumigation of the room is not necessary, the laboratory supervisor must determine the minimum time necessary for infectious aerosols to be cleared from the laboratory, taking into consideration the number of changes of air per hour. **The minimum time that must elapse before anyone re-enters the laboratory should be 2 hours**.
- **After the appropriate time, prepare to re-enter** the laboratory to disinfect the contaminated area. Assemble a clean-up team of at least two persons. Put on protective clothing including gown, thick rubber gloves for protection against cuts from glass debris, overshoes, goggles and a single-use disposable FFP2/N95 or FFP3/N100 respirator. Re-enter the contaminated area and assess the extent of contamination.
- Spilled infectious substances and broken containers contaminated with **infectious substances should be covered with absorbent tissue.** Disinfectant should then be poured over. If walls have been splashed, disinfectant solution should be sprayed or poured onto the contaminated surfaces which are then covered with a layer of absorbent tissue.
- **Allow adequate time for disinfection – a minimum of 2 hours**.
- Once disinfection is completed, the waste can be discarded into suitable waste containers. Sharp containers should be used for glass debris and other sharps. Autoclavable bags are suitable for the other waste (disposable respirator and overalls, overshoes, absorbent tissue, etc).
- Laboratory gowns are decontaminated using the standard procedure. The thick rubber gloves should be discarded if damaged; otherwise, they can be disinfected. If splashed, goggles must be cleaned with disinfectant.

- If laboratory forms or other printed or written materials are contaminated, the information should be copied onto other forms and the originals discarded in the waste container.
- All contaminated equipment should also be wiped with a suitable disinfectant. Removable parts should be washed with water and dried. Electric equipment should be checked carefully (integrity of circuit-breakers and earth-fault-interrupters) before use.
- The clean-up team leaves the laboratory ensuring that all items of protective clothing, including respirators, have been removed. Once all contaminated material has been appropriately disposed of and the area adequately decontaminated, the DO NOT ENTER warning sign can be removed from the door (Annex 1).

4.2.5. Reporting

Every incident/accident must be documented, and records must be kept in the laboratory supervisor's archives. All corrective action must be similarly documented.

The list of workers present in the laboratory when the incident occurred must be sent to the relevant medical service, together with documentation of the incident, for further medical surveillance.

4.2.6. Waste management

All materials used in the clean-up should be treated as infectious waste.

Contaminated-waste containers should be autoclaved. Disposable things should be incinerated.

Those containing broken glass should be specifically labelled "CAUTION: GLASS DEBRIS" to prevent further risks of cuts and wounds.

Annex 1. Example of a warning sign

WARNING

DO NOT ENTER

MAJOR BIOLOGICAL HAZARD

Annex 2. Incident report form

Institution:	
Laboratory designation:	
Head of the laboratory:	
Date, time of the incident:	
Nature of the incident (what happened?)	
Extent of the incident: • estimate of the volume of contaminated spill • details of the volume of broken glass (if any) • description of the surfaces contaminated • list of material/equipment contaminated (if any) • list of samples involved in the incident	
List of persons present in the laboratory when the incident occurred	
List of persons with physical injury (if any) Nature of physical injury	
Name of the physician in charge of the first medical aid	
Name of the physician in charge of the medical record and further medical surveillance	
Corrective action: • how to prevent such an incident. • how to improves staff adherence to safety procedures	

	Title: Emergency procedure in case of fire		
	SOP Number: 05	Effective Date:	Page **1-3**
	Version: 2.0	April, 2022	
REVISION			
Revised by	Effective date	Description of change	
NTP, Bangladesh	July, 2022	Updated in line with the GLI Guideline	

5.1 Scope

This SOP describes emergency procedures to be followed in case of fire and the responsibilities of workers exposed to a fire hazard in a TB laboratory.

5.2. Procedure

5.2.1. Principle

Laboratory workers are not required to fight fires and should evacuate the premises in case of fire.

Individuals who have been trained in the proper use of fire extinguishers and are confident in their ability to cope with the hazards of a fire may use a portable fire extinguisher to fight small, incipient or early-stage, localized fires (no larger than a wastepaper basket).

5.2.2. Equipment and materials

Portable carbon dioxide extinguisher(s), well-maintained (at least once a year, recorded in a written document) and within its shelf-life.

Bucket(s) full of water

5.2.3. Detailed instructions

5.2.3.1. Small fires

- Small, localized fires can be extinguished without evacuating the premises, but there must be constant evaluation of the evolution of the fire and readiness to evacuate if it cannot be controlled.
- Fire involving cotton, paper, cardboard, wood, fabric: use water.
- Fire involving flammable liquids and gases or alkali metals, and electrical fires: use carbon dioxide extinguisher. Only personnel trained to use fire extinguishers may use them. Always aim the extinguisher at the base of the fire.
- Fire-fighting efforts must be terminated as soon as it becomes apparent that there is risk of harm from smoke, heat, or flames.

5.2.3.2. In all other cases of fire discovery

- Alert people in the area of the need to evacuate the premises.
- Telephone the fire service emergency number, indicating the location and extent of the fire.
- Evacuate promptly.
- Close doors behind you
- **Never enter a smoke-filled room.**

5.2.4. Reporting

Every accident/incident must be documented, and records must be kept in the laboratory supervisor's archives (See Annex). All corrective action must be similarly documented.

5.2.5. Quality control

Use a checklist (non-exhaustive) for further corrective action

- Electrical circuit overloading, frequently due to wires of inappropriate cross-section in relation to fuses used.
- Poor electrical maintenance (poor and perished insulation on cables, extension leads with unprotected plugs lying on the floor, etc).
- Inappropriate circuit-breakers or earth-fault-interrupters.
- Absence or misuse of transformers where required.
- Excessively long gas tubing or long electrical leads.
- Equipment left switched on unnecessarily.
- Equipment not designed for use in a laboratory environment.
- Open flames.
- Deteriorated gas tubing.
- Improper handling and storage of flammable materials.
- Improper segregation of incompatible chemicals.
- Sparking equipment near flammable substances and vapours.
- Improper or inadequate ventilation.
- Appropriate location of water buckets, extinguishers (near room doors and at strategic points in corridors) and fire blankets.

5.2.6. Safety precautions

The effects of fire on the possible dissemination of infectious material must be considered with the laboratory supervisor and any necessary action to maintain biosafety must be taken promptly.

Annex 1. Incident report form

Institution:	
Laboratory designation:	
Head of the laboratory:	
Date, time of the incident:	
Nature of the initial incident (what was the fire source?)	
Extent of the incident:	
Name of the physician in charge of the first medical aid, if requested	
List of persons injured during the incident	
Corrective action: how to prevent the start of such a fire how to limit the spread of fire how to improve staff adherence to safety and emergency procedures	
Measures for biosafety if any	

	Title: Sample conditions and transport for culture procedure		
	SOP Number: 06	Effective Date:	Page **1 -7**
	Version: 2.0	April, 2022	
REVISION			
Revised by	Effective date	Description of change	
NTP, Bangladesh	July, 2022	Updated in line with the GLI Guideline	

6.1. Scope

This SOP specifies the minimum requirements for the quality and quantity of biological specimens sent to a TB laboratory for culture and conditions for transportation of specimens to the laboratory.

6.2. Procedure

6.2.1. Principle

Specimen quality –from the moment of collection to the arrival of specimens at the laboratory where they will be cultured – is the responsibility of the setting in which specimens are collected, that is, either the peripheral laboratory where patients were given sputum containers or the clinics where sampling/biopsy is performed.

Since the laboratory is usually the only place where there is quality control of specimens received, laboratories at all levels must monitor quality indicators, e.g., the proportion of saliva sputum specimens, frequent late arrival of specimens, and report problems so that corrective action may be taken wherever necessary.

Specimens sent to the laboratory should be of adequate volume, as specified below, accurately labelled for identification, and accompanied by a written laboratory request form supplied by NTP

Specimens should be sent to the laboratory as soon as possible after collection, by following NTP sample transportation guideline.

6.2.2. Equipment and materials

Wide-mouthed, unbreakable, leakproof, screw-capped containers. Containers should have a volume capacity of 50 ml and made of translucent material in order to observe specimen volume and quality without opening the container.

6.2.3. Detailed instructions for the procedure

6.2.3.1. Sample collection

A. Sputum

Most specimens received for diagnosis are sputum samples.

- If good specimens are to be obtained, patients must be instructed in how to produce sputum. Specimens should be collected in a separate, ventilated room or preferably outdoors. Keeping both hands-on hips, cough forcibly and collect sputum in the mouth; spit the sputum carefully into a wide-mouthed, unbreakable, leakproof container and close the lid tightly.
- Ideally, a sputum specimen should be 3–5ml in volume, although smaller quantities are acceptable if the quality is satisfactory.
- If specimens are to be cultured using a centrifugation method (see SOP Specimen processing for culture), sputa should preferably collect directly into 50-ml centrifuge tubes to avoid the need for their transfer from one container to another.
- Label each specimen with the unique identification number from the laboratory request form.
- Collect one specimen from each patient according to the NTP policy

B. Laryngeal swab

Laryngeal swabs may be useful in children and patients who cannot produce sputum or may swallow it.

- Collect laryngeal swabs in the early morning, before patients eat or drink anything.
- Use a sterile absorbent cotton swab for collection.
- Transport each specimen in a container with a few drops of sterile 0.9% saline solution to keep the swab wet.

C. Other respiratory specimens

- Bronchial secretion (2–5 ml) and BAL (20–40 ml)
- Pleural effusions (20–50 ml)
- Transbronchial and other biopsies taken under sterile conditions should be kept wet during transportation by adding few drops of sterile 0.9% saline to the tissue.

Surgeons should be reminded not to use formalin or bleach for samples that are sent to the laboratory and if possible, to send the samples the day before surgery.

D. Gastric lavage

Gastric lavages often contain MOTT and are therefore rarely used for adults; they are indicated for children, however, who produce almost no sputum

- Make the collection early in the morning when the patient has an empty stomach.
- Neutralize the specimen by adding 100 mg of sodium bicarbonate to the gastric aspirate and transport it immediately to the laboratory.

E. Extrapulmonary specimens

The laboratory may receive a variety of specimens for diagnosis of extrapulmonary TB- body fluids, tissues, urine etc. These specimens may be broadly divided into two groups which are processed in different ways:

- Aseptically collected specimens (spinal fluid, pericardial, synovial and ascitic fluid, blood, bone marrow, etc.), which are usually free from contaminating flora.
  - All liquid specimens should be collected in sterile glass containers without using any preservative.
  - Specimens can be inoculated directly into liquid vials and transported to the laboratory for culture.
- Specimens must be transported to the laboratory immediately; they should be processed as soon as possible or kept at 2-8 ^{0}C.
- The optimal volumes are at least 3 ml of cerebrospinal fluid Specimens with resident or contamination flora.
  - A urine specimen should consist of a single, early-morning, midstream sample of, collected in a wide-mouthed sterile vessel (of at least 200 ml capacity).
  - Sperm and prostate should send without any addition
  - Menstrual blood should be discouraged
  - Stool samples should be discouraged; however, stool sample from immune compromised patients may be used, mainly to detect MOTT
  - List of Extra pulmonary specimen with required volume should be clearly mentioned.

6.2.3.2. Transport conditions

Sputum should be transported to the laboratory as soon as possible. If a delay of a few days cannot be avoided, keep specimens cool (refrigerated but not frozen). Up to a week in cold conditions will not significantly affect the positivity rate of smear microscopy; however, the additional growth of contaminants will result in an increased contamination rate on culture media. If the delay exceeds 3 days, an equal volume of Cetyl pyridinium chloride (CPC; solution of 1% CPC in 2% sodium chloride) should therefore be added to sputum (see SOP preparation of reagents for culture). Sputum containing CPC can be kept for up to 7 days but must be kept at room temperature (>20 °C since CPC crystallizes at lower temperatures). The addition of CPC must be indicated on the accompanying documents because CPC has to be removed before culturing.

CPC must not be used in sputum for liquid culture and LPA.

6.2.4. Transport packaging

Steps of labelling and packing samples before transportation:

Step 1: Label sample ID number to the falcon tube

Step 2: Transfer Sputum from Sputum cup to falcon tube

Step 3: Tightening the cap and insert the falcon tube into small poly bag

Step 4: Insert the tube to the Zip lock poly bag and close properly

Step 5: Take the transport box and put a biohazard sticker

Step 6: Put an address sticker to the opposite site of the box

Step 7: Insert the tube inside the transport box

Step 8: Close the box carefully

Step 9: Close the box carefully and pack

Step 10: Complete box

Step 11: Insert the complete box inside the large poly bag

Step 12: Ready for shipment

Step 13: transport to nearest GeneXpert lab/culture lab

After collection of sputum sample, it should be labelled properly before shipment.

Three Layer Packaging

Figure 1: Label sample ID number to the falcon tube

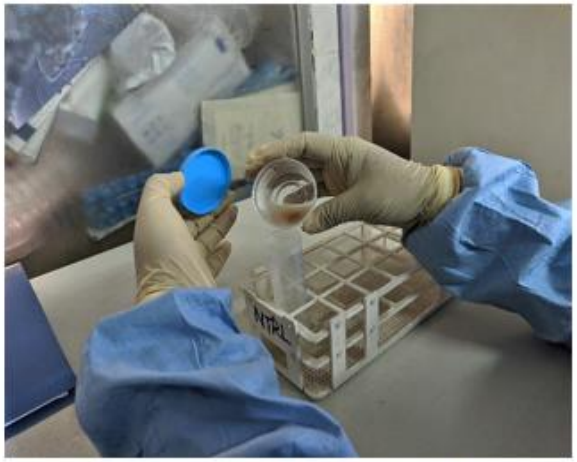


Figure 2: Transfer Sputum from Sputum cup to falcon tube

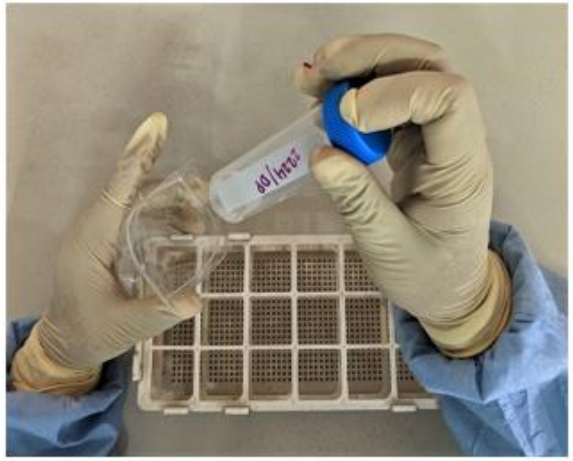


Figure 3: Tightening the cap and insert the falcon tube into small poly bag

Figure 4: Insert the tube to the Zip lock poly bag and close properly

Figure 5: Take the transport box and put a biohazard sticker

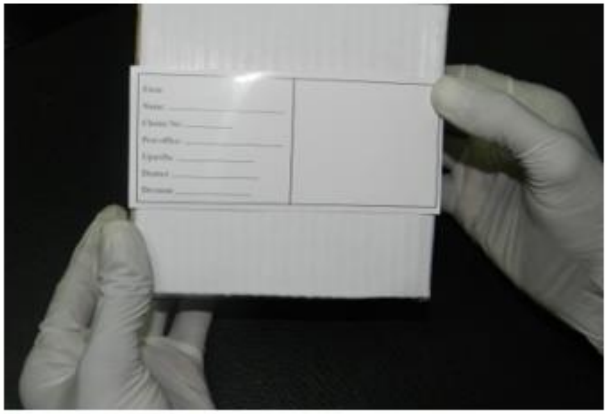


Figure 6: Put an address sticker to the opposite site of the box

Figure 7: Insert the tube inside the transport box

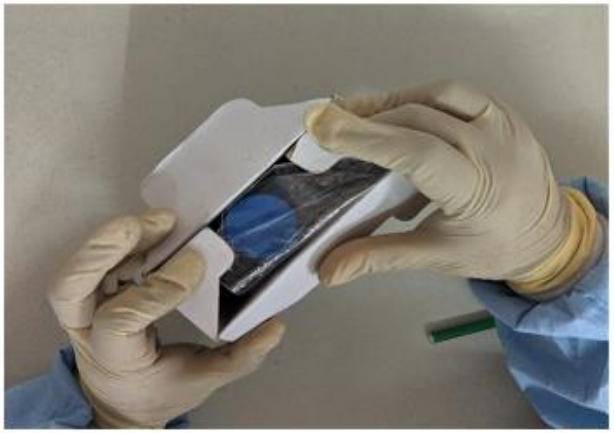


Figure 8: Close the box carefully

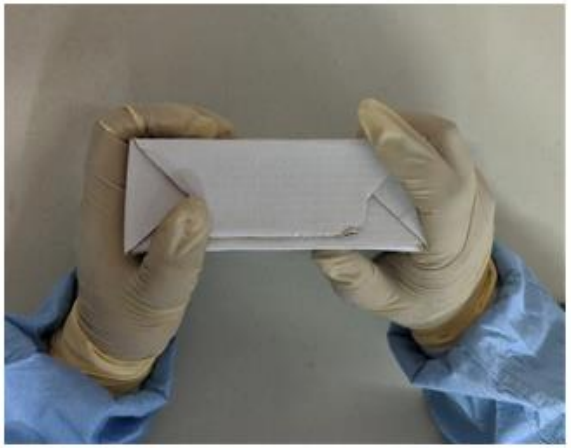


Figure 9: Close the box carefully and cover the pack

Figure 10: Complete box

Figure 11: Insert the complete box inside the large poly bag

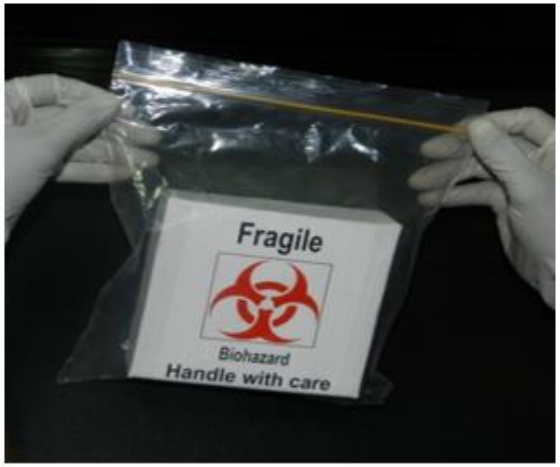


Figure 12: Ready for shipment

6.2.4.1. Responsibilities of Laboratory technologist during transportation of samples

- Ensure good quality and sufficient quantity of specimen collection
- The request form should be properly filled in
- The sample should be matching with the request form
- The cork of the falcon tube should be tightly screwed
- The sample should be kept in the transport box and wrapped with absorbent paper/cotton
- Address sticker containing sender's and receiver's address should be properly filled up
- Bio-hazard sticker should be properly tagged on the transport box

6.3. Reading and reporting

Use form of NTP supplied DR TB 06

6.4. Quality control

Before specimens can be accepted in the laboratory, the accompanying request forms must be checked carefully for identity (sample and request form labelled with the same number). Specimens that cannot be identified exactly will be not processed and should be informed to the sender immediately.

Specimens should be examined on receipt of the sample, to ensure that they correspond in type, quantity, quality and volume to the appropriate criteria. Any deviations must be documented and noted on the lab register since they may affect the results.

The transport conditions and duration must be checked. The samples should reach the laboratories as quickly as possible, preferably within 72 hours of collection. Delays in transportation and/or exposure of specimens to extremes of temperature without protective measures must be documented and noted in the report.

Sample should be processed immediately after receiving of reference laboratory without any delay.

	Title: Maintenance of mycobacterial strains		
	SOP Number: 07	Effective Date:	Page **1-2**
	Version: 2.0	April, 2022	
REVISION			
Revised by	Effective date	Description of change	
NTP, Bangladesh	July, 2022	Updated in line with the GLI Guideline	

7.1. Scope

This SOP describes optimal storage conditions for mycobacterial strains (clinical and reference strains) to ensure their viability and retention of biological characteristics over time.

7.2. Procedure

7.2.1. Principle

Mycobacterial strains must be stored in conditions that preserve their viability and protect specific strain characteristics.

7.2.2. Samples

Mycobacterial strains

Reference strains (H37Rv)

7.2.3. Equipment and materials:

- BSC, class II-A, annually certified
- Solid cultures in tubes/vials
- Liquid cultures in tubes/vials
- Cryovials with screwcaps (external screw on the tube body)
- Freezer at -20°C and /or -80°C
- Platinum loop or one time using plastic loop
- Permanent marker pen
- Strain preservation box

7.2.4. Reagents and solutions

- Sterile Distilled Water
- Skimmed milk
- 7H9 medium

7.2.5. Detailed procedure

7.2.5.1. Short term storage

Cultures on egg-based medium should be preferably stored up to 1 year at -20^{0}C.

Scrape as many colonies as possible from an egg-based/sterile distilled water/skimmed milk and 7H9 medium slant. Suspend colonies in a 2-ml cryovial containing 1.5 ml of distilled water. Store at –20°C for several years or at –80°C for decades. The viability of tubercle bacilli declines much more rapidly at –20°C than at –80°C: only 1% is still viable at –20°C after 2 years compared with 100% at –80°C. It is thus crucial to prepare the heaviest bacterial load possible in order to compensate for the loss of viability.

In deciding whether they actually need a –80°C freezer, laboratories must consider the objectives of long-term storage of mycobacterial cultures. Storage of cultures for up to 5 years after the initial isolation can be achieved at –20°C provided that cultures (or subcultures) showed luxuriant growth and were abundant. A freezer at –80°C may be required for national reference laboratories involved in research

programmes or long-term epidemiological studies. For laboratories performing culture identification, maintenance has to be extended to MOTT species, specifically *M. terrae*, used for quality control of biochemical tests. Survival of MOTT is usually much better but which according to species and strain. Most bacterial species other than tubercle bacilli survive equally well at –20°C and –80°C (100% survival after 3 years at either temperature).

7.2.5.2 Long-term storage of reference cultures

Reference strains for quality control of biochemical tests, drug susceptibility testing and medium preparation (*M. tuberculosis* H37Rv) must be permanently maintained in the laboratory. To avoid serial subculturing, which could lead to genetic drift and alter the phenotypic biological characteristics of strains, it is advisable to adopt the following procedure:

Scrape as many colonies as possible from egg-medium slants of the reference strain. Suspend colonies in a tube containing 15 ml Distilled water.

Dispense the suspension in 10 cryotubes, 1.5 ml per tube. Label each tube with the strain reference and date. Store at –20°C or –80°C.

Thaw one tube and inoculate 10 tubes of egg-based medium/MGIT tubes which will be used as the source of bacilli for quality control whenever necessary. Thaw another tube when the 10th tube source is exhausted.

Monitor strain stocks

7.3. Waste management

Liquid and solid culture media should be autoclaved before disposal.

Chapter 2: Solid Culture and DST

8.1. Scope

This SOP describes the preparation of all reagents used during specimen processing for culture and identification of *Mycobacterium tuberculosis*.

8.2. Procedure

8.2.1. Principle

Reagents should be prepared in small aliquots to avoid reuse of stock solutions.

8.2.2. Equipment and materials

- Balance (sensitivity 0.01 g),
- Spoons or spatulas,
- Weighing paper
- Autoclave
  pH meter
- Dispenser device
- Sterile glassware (as per requirement) and sufficient sterile screw-cap tubes for dispensing the reagents
- Dark bottles
- Refrigerator
- General equipment of a media preparation laboratory; (see SOP on Preparation of plain egg-based media)
- Micro Pipettes
- Pipetting Tips
- Magnetic stirrer
- Beakers and funnels
- Filter paper
- Aluminium foil
- Absorbent paper

8.2.3. Reagents and solutions

8.2.3.1. Reagents for the NALC–NaOH method:

A. Sodium hydroxide (NaOH), 4% and Trisodium citrate, 2.94%

Solution 1: Add 4gm Sodium hydroxide (NaOH) into 100ml distilled water followed by autoclaving at 121°C for 15 minutes.

Solution 2: Add 2.94gm Trisodium citrate·$2H_2O$, analytical grade 100ml distilled water followed by autoclaving at 121°C for 15 minutes.

Mix equal volumes of solutions 1 and 2.

Alternative Method: Sodium hydroxide (NaOH), 4% and Trisodium citrate, 2.94%

Components	**Amount**
1. Sodium hydroxide pellets (analytical grade)	2 g
2. Trisodium citrate·$2H_2O$, analytical grade	1.47 g
3. Distilled water	Up to 100 ml

Dissolve both components in the distilled water. Sterilize by autoclaving at 121°C for 15 minutes.

B. NALC–NaOH solution freshly prepared

Add 0.5g N-acetyl L-cysteine (NALC) in 100 ml Sodium hydroxide and Trisodium citrate solution just before use.

C. Phosphate buffer, 0.067 mol/litre, pH 6.8

Stock solution A: Anhydrous Disodium Phosphate, Na_2HPO_4, 0.067 mol/litre

Dissolve 9.47 g of anhydrous Na_2HPO_4 in 1 litre of distilled water.

Stock solution B: Monopotassium Phosphate, KH_2PO_4, 0.067 mol/litre

Dissolve 9.07 g of KH_2PO_4 in 1 litre of distilled water

Mix 500 ml of solution A and 500 ml of solution B to make a final volume of 1 L.

Alternative Method: Phosphate buffer, 0.067 mol/litre, pH 6.8

Solution A (Disodium Phosphate)	
1. Anhydrous Disodium Phosphate, Na_2HPO_4	4.74 gm
2. Distilled Water	500 ml

Dissolve Na2HPO4 in the distilled water and make up to 500 ml.

Solution B (Monopotassium Phosphate)	
1. Monopotassium Phosphate, KH_2PO_4	4.54 gm
2. Distilled Water	500 ml

Dissolve KH_2PO_4 in the distilled water and make up to 500 ml.

Mix 500 ml of solution A and 500 ml of solution B.

- Check pH for 6.8. Adjust as necessary. Add solution A if pH is low than required and add solution B if pH is high than required to adjust the pH at 6.8.
- Distribute the solution per 400 mL in the 1L thick glass bottles.
- Sterilize by autoclaving at 121^0C for 15 minutes. Prior to use, check the sterility by Incubating overnight at 37^0C: discard if the solution becomes cloudy
- Store up to 3 months at 4^0C.

8.2.3.2. Reagents for the CPC/NaCl method

Ingredients	Amount
Cetylpyridinium Chloride	10 g
Sodium Chloride	20 g
Distilled Water	1000 ml

Mix and sterilize by autoclaving at 121^{0}C for 15 minutes.
CPC solution is self-sterilizing and remains stable if protected against light, extreme heat and evaporation. The stock solution should be stored in dark bottles at room temperature. Stability is 4 months.

8.2.3.3. Other reagents

1. Saline solution

   Sodium chloride (NaCl) 0.9 g

   Distilled water 100 ml

   Sterilize by autoclaving at 121 °C for 15 minutes.

2. McFarland turbidity standard No.1

   The McFarland No.1 standard is the most widely used turbidity standard for the visual comparison of bacterial suspensions. The density of the resulting barium sulphate precipitate is used as a proxy to approximate the colony count of bacterial suspensions. McFarland No.1 is approximately equivalent to 3 x 10^8 CFU/ml.

   Sulfuric acid (H_2SO_4), 1%

   H_2SO_4 1 ml

   Distilled water 99 ml

   Always add acid to water.

   Barium chloride ($BaCl_2$), 1%

   $BaCl_2$ 1 g

   Distilled water 100 ml

   McFarland preparation

   Barium chloride solution, 1% 0.1 ml

   Sulfuric acid, 1% 9.9 ml

   Label the suspension and indicate the last day of use (1 month after preparation).

8.3. Quality control

Use high-grade reagents, sterile distilled water and a balance that is regularly checked for sensitivity.

New reagent batches used for decontamination procedures can be checked by processing a control tube containing all reagents except specimen, centrifuging it and inoculating a culture medium tube/vial with some of the liquid left in the tube. Growth of organisms would lead to the reagent batch being discarded.

9.1. Scope

This SOP describes the preparation of egg-based media suitable for the culture of *Mycobacterium tuberculosis*.

9.2. Procedure

9.2.1. Principle

A variety of solid and liquid culture media have been developed for the isolation of *M. tuberculosis* on culture media. Egg-based media are widely used since they can be prepared locally and offer several other advantages – low cost, stability of the chemicals needed, long shelf-life, and characteristic morphology and luxurious growth of tubercle bacilli. However, it may be as much as 8 weeks before cultures become positive, particularly if specimens contain few bacilli.

Egg mass of whole eggs is mixed with ingredients needed for growth of tubercle bacilli. Coagulation yields a solid medium. Malachite green in the medium helps to minimize the growth of contaminants.

9.2.2. Equipment and materials

- Balance (sensitivity 0.01 g)
- Spoons or spatulas
- Weighing paper
- Autoclave for sterile preparation
- Inspissator and racks to hold slopes in correct position
- pH meter
- Simple dispenser device – either an automatic pipetting aid or a mechanical pipetting aid with thumb wheel or rubber teats
- Appropriate sterile glassware and sufficient sterile screw-capped tubes for media dispensing

  [Note: Because of the risk of infection if glass tubes break, thick-walled, shock-resistant glass tubes or vials should be used.]
- Material for homogenization of egg mass (5-mm glass beads for manual shaking, a plate with magnetic stirrer)
- Thick-walled conical flask, 2500 ml
- Forceps
- Funnel with sterile sieve, for filtration of homogenized egg preparation
- Brown bottles
- General equipment and glassware of a laboratory for media preparation

9.2.3. Reagents and solutions

Best-quality chemicals of certified purity and freshly distilled, sterilized water should be used for the preparation of media.

9.2.3.1. Ethanol, 70%

9.2.3.2. Malachite green solution, 2%, prepared separately

Malachite green dye 2 g

Sterile distilled water 100 ml

Alternative (For 1-liter Löwenstein–Jensen Media)

Malachite green dye 1 g

Sterile distilled water 50 ml

Using aseptic techniques, dissolve the dye in sterile distilled water. To avoid precipitation, dissolve the dye in lukewarm autoclaved distilled water and prepare just before media preparation. Store in dark bottles. This solution is not stable long-term: if precipitation occurs, discard and prepare a fresh solution.

9.2.3.3. Salt solution (For 1-liter Löwenstein–Jensen Media)

Components	**Amount**
Monopotassium Dihydro Phosphate (KH_2PO_4), anhydrous	1.5 g
Magnesium Sulphate ($MgSO_4 \cdot 7H_20$)	0.15 g
Magnesium Citrate	0.375 g
L-Asparagine	2.25 g
Glycerol	7.5 g
Distilled Water	375 ml

- Dissolve the ingredients in order in the distilled water. **(Heat if required, pH 6.8)**
- Autoclave at 121°C for 30 minutes to sterilize.
- Cool to room temperature.

Note: Pre-prepared salt solution mixtures are commercially available and have the advantage that their quality is guaranteed by a certified manufacturer.

9.2.3.4. Hens' eggs

Hens' eggs should be fresh (not more than 7 days old). Prefer middle sized eggs (to keep the relation of egg white and yolks balanced), which means about 20 - 25 eggs per litter of egg mass.

9.3. Detailed *procedure*

- Clean and disinfect the work area
- Clean the eggs carefully with plain soap and water, soak them with 70% ethanol for about 15 minutes and let dry
- Crack each egg into a small sterile beaker and transfer the whole egg into the 3- or 5-liter flask containing a magnet or about 30-40 sterile glass marble. Use magnetic homogenizer at 800-1000 rpm for 30 minutes or shake to homogenize the fluid covering the beaker with aluminium foil.

- Filter through sterile tea filter/ sterile gauze into the measuring cylinder and measure to obtain the final volume 625 ml.

Procedure to prepare complete medium:

- Switch on the inspissator in order to quicken the heating

  [Note: Switch the inspissator at the beginning of media preparation as it takes 3-4 hours which is very long time to raise the temperature at 85°C also, if necessary, switch off the air condition, cool flow will disturb the inspissation]
- Bring the salt solution into room temperature (375 ml salt solution, autoclaved earlier)
- Add 12.5 ml malachite green to the salt solution carefully and mix
- Add salt solution to 625 ml of egg homogenates in the flask containing about 30-40 sterile glass marbles
- Mix all components gently to avoid bubbles to homogeneity
- Dispense in 6-8 ml in sterile McCartney bottles
- Lay down the tubes with half circle loosen the cap on the slopes of inspissator which is already heated to 85°C and keep for 45 minutes for coagulation
- Take the tubes out of the inspissator and allow to cool
- Tighten the screw caps when tubes are at room temperature & label the batch (otherwise condensation will produce excess water)
- Incubate at 37°C for 48 hours to check contamination (keep 5% tubes for 4 weeks and check for further contamination)
- Place the tubes (fresh media) in the refrigerator at upright position preferably into plastic container for up to 4 weeks.
- Record batch data in log sheet

9.4. Quality control

9.4.1. Colour

Tubes from the same batch of media showing different shades of green may be the result of poor homogenization or of the presence of material residues in the tubes. A very dark shade of green can be caused by an excess of malachite green or by a very low (acidic) pH. Yellowish colouring of the media can indicate poor-quality malachite green or very high (alkaline) pH. Discolouration of the coagulated medium may also be due to excessive temperature.

9.4.2. Texture

If the medium is liquid or disintegrates easily when tubes are tapped on the hand (one or two tubes are randomly selected from the batch), the inspissation temperature may have been too low. Tubes containing media of poor texture are not suitable for culture inoculation.

9.4.3. Humidity

The bottom of the tube should show some condensation water. However, an excess of water indicates either that tubes have been tightly capped too soon after inspissation or that the composition of the medium is not standard. The medium should not roll when tubes are moved.

9.4.4. Homogeneity

If bubbles appear in the medium during inspissation, it is possible that the medium has been subjected to excessive temperature and its quality has deteriorated. The presence of clumps in the medium indicates poor homogenization.

9.4.5. Sterility

The batch is tested for sterility. No bacterial growth should be present after 1-day incubation at 37 °C.

9.4.6. Sensitivity

Check sensitivity (ability to yield good growth of tubercle bacilli) of the batch of prepared media with *M. fortuitum* for 72 hours

9.4. Storage and shipping

9.5.1. Storage

The media should be stored in a clean, cool and humid place, preferably in a refrigerator at 2-8 °C, for no more than 1 month. Labels on the tubes should indicate batch number or date of preparation.

9.5.2. Shipping

Tubes can be shipped at ambient temperature (preferably 2-8^{0}C) provided that they are protected from sun and light exposure, overheating and desiccation.

Annex 1. Log-sheet Media Preparation

Preparation: SALT SOLUTION		
Operator's name:		
Date of preparation:		
Quantity:		
Autoclave cycle	**Operator's name:**	
	Time (min)	**Temperature (°C)**
Date:		

Preparation: L-J MEDIA		**Preparation date:**
		Operator's name:
Reagents	**Quantity Prepared (ml or g)**	**Quantity Required (ml or g)**
Salt solution		
Malachite green		
Eggs	**No. of eggs:**	

Inspissator cycle	**Operator's name:**	
	Time (min)	**Temperature (°C)**
Date:		

L-J Batch No:	

Annex 2. Log-sheet – Quality Control

<table>
<tr><td colspan="3">Sterility check: 37°C - 72 hours</td><td></td></tr>
<tr><td colspan="3">Preparation date _____/_____/20___ Batch No.</td><td rowspan="4"></td></tr>
<tr><td>Total slopes produced (A)</td><td>Total slopes contaminated (B)</td><td>Contamination rate (B/A) x 100)</td></tr>
<tr><td></td><td></td><td></td></tr>
<tr><td colspan="3"></td></tr>
<tr><td colspan="4">Sensitivity check</td></tr>
<tr><td colspan="3">Inoculation date _____/_____/ 20___</td><td rowspan="2"></td></tr>
<tr><td></td><td>Time for growth</td><td colspan="2">Growth (minimal)</td></tr>
<tr><td>M.tuberculosis</td><td>(4 weeks max)</td><td colspan="2">(at least 3+)</td></tr>
<tr><td>M.fortuitum (or other rapid grower)</td><td>(72 hours max)</td><td colspan="2">(at least 3+)</td></tr>
<tr><td colspan="4"></td></tr>
<tr><td colspan="2">QC responsible (sign.)</td><td colspan="2">QC</td></tr>
<tr><td colspan="2"></td><td>Passed ☐</td><td>Failed ☐</td></tr>
</table>

	Title: Preparation of Lowenstein–Jensen drug-containing media		
	SOP Number: 10	Effective Date:	Page **1-9**
	Version: 2.0	April, 2022	
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10.1. Scope

This SOP describes the preparation of drug-containing media required for drug susceptibility testing of *Mycobacterium tuberculosis* using the proportion method

10.2. Procedure

10.2.1. Principle

Lowenstein–Jensen (LJ) drug-containing media are used for drug susceptibility testing of *M. tuberculosis* according to the proportion method (Canetti et al., modified).

The critical concentration of drugs to be used in the proportion method has been evaluated from clinical data. While Canetti et al. originally based their method on three concentrations per drug, they later proposed economic versions based on just one or two critical concentrations. Today, a single concentration is most commonly used and is the method described here.

10.2.2. Equipment and materials

The equipment and materials needed are the same as those for the preparation of plain egg-based media (see relevant SOP) plus some specific material for drug storage and handling.

- Balance (sensitivity 0.01 g or 0.001 g)
- Spoons or spatulas
- Weighing paper
- Autoclave
- Inspissator and racks to hold slopes in correct position
- pH meter
- Simple dispenser device – either an automatic pipetting aid or a mechanical pipetting aid with thumb wheel or rubber teats
- Appropriate sterile glassware and sufficient sterile screw-capped tubes for media dispensing

Note: *Because of the risk of infection if glass tubes break, thick-walled, shock-resistant glass tubes or vials should be used.*

- Material for homogenization of egg mass (5-mm glass beads for manual shaking, a plate with magnetic stirrer)
- Thick-walled conical flask, 2500 ml
- Bunsen burner
- Forceps
- Funnel with sterile gauze, for filtration of homogenized egg preparation
- Water-bath

- Brown bottles
- Vortex mixer
- General equipment and glassware of a laboratory for media preparation
- Small desiccator, device for drug storage in dry conditions
- Silica gel
- Refrigerator

10.2.3. Reagents and solutions

10.2.3.1. Drug-containing media

The LJ medium is prepared according to SOP. Drugs are added to the still-liquid LJ medium before inspissation. Final drug concentrations are indicated in Table 1.

Each test-tube must be labelled with the following information:

- name of drug and its concentration
- batch number
- date of production
- expiry date (the last day the medium can be used for DST tests).

Table 1. Final concentrations of drugs for quality control and for test strains

Test drugs		**Solvent**		**Final drug concentration in the culture medium [µg/ml]**
Designation	**Abbrev.**	**Solvent**	**Dilution**	**For test strains**
Isonicotinic acid hydrazide	INH	Sterile dw	Sterile dw	0.2
Rifampicin	RMP[a]	DMSO	Sterile dw	40.0
Ethambutol	EMB	Sterile dw	Sterile dw	2.0
p-nitrobenzoic Acid	PNB	DMSO	Sterile dw	500
Levofloxacin	LFX	DMSO or 0.1 M NAOH	Sterile dw	2.0
Moxifloxacin	MFX	DMSO or 0.1 M NAOH	Sterile dw	1.0
Amikacin	AMK	Sterile dw	Sterile dw	30

[a] If RMP is in sodium salt format, dissolve directly in distilled water.

10.2.3.2. Drugs

The drugs solution (Stock solution) shall be stored at -20°C Freezer.

There are two different ways of preparing the drug containing media:

- Version 1 by adding 1% volume of aqueous drug solution to the plain culture medium – used without any corrections for the volume change.
- Version 2 by adding 10% volume of aqueous drug solution. In this case, a correction for the volume of the drug solution added has to be taken into account when preparing the basic culture medium (Not followed). The advantage of version 2 is the ease of complete homogenisation of drug into the egg mass in a volumetric flask.

10.2.3. Detailed instructions

Prepare a standard batch (1620 ml) of LJ basic culture medium according to the SOP "Preparation of plain egg-based media". If multiple growth controls are included in the DST schema, the volume of this standard batch must be increased enlarged accordingly.

Isoniazid (INH): For the dry and pure INH, the correction factor is 1. Following is the procedure:

I. Solution I **(Stock Solution):** 100mg dissolved in10 ml sterile distilled water (10,000 µg/ml)

   Stock solution can be aliquoted (1ml) into sterile cryovials and stored frozen at –20 °C for 3 months.

II. Solution II: Mix 1ml Solution I into 09 ml sterile distilled water (1,000µg/ml)

III. Solution III: Mix 1ml Solution II into 09 ml sterile distilled water (1,00µg/ml)

IV. Solution IV **(Working Solution)**: Mix 1ml Solution III into 04 ml sterile distilled water (20µg/ml)

V. Solution V **(QC Solution)**: Mix 03ml Solution IV into 03 ml sterile distilled water (10µg/ml)

Add 2ml of Solution IV into 198 ml of LJ media = 0.2µg/ml

Final drug concentration in media (µg/ml):

	0. 2 µg/ml	**0.1 µg/ml**	**0.05 µg/ml**
Medium (ml)	198	19.9	19.8
Solution 4 (ml)	2	-	-
Solution 5 (ml)	-	0.1	-
Solution 5 (ml)	-	-	0.2
Final volume (ml)	200	20	20

Single-channel adjustable microlitre-pipettes with sterile tips must be used for accurate delivery.

Rifampicin (RMP) For RMP usually the correction factor is 1 for a pure RMP or 1.03 for sodium salt.

I. Solution I **(Stock Solution):** 100 mg dissolved in10 ml DMSO (10,000 µg/ml)

   Stock solution can be aliquoted (1ml) into sterile cryovials and stored frozen at –20 °C for 3 months.

II. Solution II **(Work Solution)**: Mix 1.2 ml Solution I into 1.8 ml sterile distilled water (4,000µg/ml)

III. Solution III **(QC Solution)**: Mix 0.5 ml Solution II into 1.5 ml sterile distilled water (1,000µg/ml)

Add 2ml of Solution II into 198ml of LJ media= 40

Final concentration in drug media (µg/ml):

	40 µg/ml	**20 µg/ml**	**10 µg/ml**
Medium (ml)	198	19.9	19.8
Solution 2 (ml)	2	-	-
Solution 2 (ml)	-	0.1	-
Solution 3 (ml)	-	-	0.2
Final volume (ml)	200	20	20

Single-channel adjustable microlitre-pipettes with sterile tips must be used for accurate delivery.

Ethambutol (EMB): For EMB dihydrochloride, the correction factor for EMB is 1.36

I. Solution I **(Stock Solution):** 136 mg dissolved in10 ml sterile distilled water (10,000 µg/ml)

II. Stock solution can be aliquoted (1ml) into sterile cryovials and stored frozen at –20 °C for 3 months.

III. Solution II: Mix 1 ml Solution I into 9 ml sterile distilled water (1,000µg/ml)

IV. Solution III **(Work Solution)**: Mix 1 ml Solution II into 4 ml sterile distilled water (200 µg/ml)

V. Solution IV **(QC Solution)**: Mix 3 ml Solution III into 9 ml sterile distilled water (50 µg/ml)

Add 2ml of Solution III into 198 ml of LJ media=2 µg/ml

Final concentration in drug media (µg/ml):

	2 µg/ml	**1 µg/ml**	**0.5 µg/ml**
Medium (ml)	198	19.6	19.8
Solution III (ml)	2	-	-
Solution IV (ml)	-	0.4	-
Solution IV (ml)	-	-	0.2
Final volume (ml)	200	20	20

Single-channel adjustable microlitre-pipettes with sterile tips must be used for accurate delivery.

p-nitrobenzoic Acid (PNB):

I. Solution I **(Stock Solution):** 1 gm dissolved in10 ml DMSO (100,000 µg/ml)

   Stock solution can be aliquoted (1ml) into sterile cryovials and stored frozen at –20 °C for 3 months.

II. Solution II **(Work Solution)**: Mix 1 ml Solution I into 1 ml sterile distilled water (50,000 µg/ml)

Add 1 ml of Solution II into 100 ml of LJ media= 500 µg/ml

Levofloxacin (LFX):

I. Solution I **(Stock Solution):** 100 mg dissolved in10 ml DMSO (10,000 µg/ml)

   Stock solution can be aliquoted (1ml) into sterile cryovials and stored frozen at –20 °C for 3 months.

II. Solution II: Mix 1 ml Solution I into 9 ml sterile distilled water (1,000µg/ml)

III. Solution III **(Work Solution)**: Mix 1 ml Solution II into 4 ml sterile distilled water (200 µg/ml)

IV. Solution IV **(QC Solution)**: Mix 3 ml Solution III into 9 ml sterile distilled water (50 µg/ml)

Add 2ml of Solution III into 198 ml of LJ media=2 µg/ml

Final concentration in drug media (µg/ml):

	2 µg/ml	**1 µg/ml**	**0.5 µg/ml**
Medium (ml)	198	19.6	19.8
Solution III (ml)	2	-	-
Solution IV (ml)	-	0.4	-
Solution IV (ml)	-	-	0.2
Final volume (ml)	200	20	20

Single-channel adjustable microlitre-pipettes with sterile tips must be used for accurate delivery.

Moxifloxacin (MFX):

I. Solution I **(Stock Solution):** 100 mg dissolved in10 ml DMSO (10,000 µg/ml)

   Stock solution can be aliquoted (1ml) into sterile cryovials and stored frozen at –20 °C for 3 months.

II. Solution II: Mix 1 ml Solution I into 9 ml sterile distilled water (1,000µg/ml)

III. Solution III **(Work Solution)**: Mix 1 ml Solution II into 9 ml sterile distilled water (100 µg/ml)

IV. Solution IV **(QC Solution)**: Mix 3 ml Solution III into 6 ml sterile distilled water (25 µg/ml)

Add 2ml of Solution III into 198 ml of LJ media=1 µg/ml

Final concentration in drug media (µg/ml):

Single-channel adjustable microlitre-pipettes with sterile tips must be used for accurate delivery.

	1 µg/ml	0.5 µg/ml	0.25 µg/ml
Medium (ml)	198	19.6	19.8
Solution III (ml)	2	-	-
Solution IV (ml)	-	0.4	-
Solution IV (ml)	-	-	0.2
Final volume (ml)	200	20	20

Amikacin (AMK)

I. Solution I (Stock Solution): 100 mg dissolved in10 ml Sterile distilled water (10,000 µg/ml)

Stock solution can be aliquoted (1ml) into sterile cryovials and stored frozen at –20 °C for 3 months.

II. Solution II **(Work Solution)**: Mix 1.5 ml Solution I into 3.5 ml sterile distilled water (3,000µg/ml)

III. Solution III **(QC Solution)**: Mix 0.5 ml Solution II into 4.5 ml sterile distilled water (3,00µg/ml)

Add 2ml of Solution II into 198ml of LJ media= 30

Final concentration in drug media (µg/ml):

	30 µg/ml	15 µg/ml	7.5 µg/ml
Medium (ml)	198	19.0	19.5
Solution 2 (ml)	2	-	-
Solution 3 (ml)	-	1	-
Solution 3 (ml)	-	-	0.5
Final volume (ml)	200	20	20

Single-channel adjustable microlitre-pipettes with sterile tips must be used for accurate delivery.

Version 2

Preparation Drug containing media:

The L-J medium is prepared according to SOP for preparation of plain egg-based media. Drugs are added to the still liquid L-J medium before inspissation as per direction given in respective diagram. Final drug concentrations are indicated in Table 1.

Each test tube must be labelled with the following information:

- name of drug and its concentration
- batch number

- date of production
- expiry date (the last day the medium can be used for DST tests).

10.2.3.2. Inspissation

Proceed as indicated in SOP for preparation of plain egg-based media. Allow the culture media to solidify while subjected to a temperature of 85 °C and a humidity of at least 80% for 45 minutes. It is essential to maintain the temperature and time exactly: too high a temperature and/or duration of inspissation will reduce the activity of drugs in the medium.

Once tubes have cooled, label them for the lot and date them. Record the data on log-sheets (see annex)

Each label should indicate

- name of drug and its concentration
- batch number
- date of production
- expiry date (the last day the medium can be used for DST tests).

10.3. Quality control

Quality control of media – colour, texture, humidity, homogeneity and sterility – is carried out as indicated for plain egg-based media.

Sensitivity (capacity to detect resistant strains) and specificity (capacity to detect sensitive strains) of drug-containing media should be tested using reference strains. The procedure is described in DST using the proportion method.

10.4. Storage

The drug-containing media can be stored protected from the light at a temperature of 2–8 °C and should be used within one month.

Annex 1. Log-sheet Media Preparation

Preparation: SALT SOLUTION
Operator's name:
Date of preparation:
Quantity:

Autoclave cycle	**Operator's name:**	
	Time (min)	**Temperature (°C)**
Date:		

Preparation: LJ containing [name of drug]		
Operator's name:		
Preparation date:		
Reagents	**Quantity (ml or g)**	**Batch no.**
Salt Solution		
Malachite green		
Drug solution		
Eggs		No. of eggs:

Inspissator cycle	**Operator's name:**	
	Time (min)	Temperature (°C)
Date:		

Sterility check	

Date of refrigeration:	**Number of tubes prepared:**	**Number of tubes refrigerated:**

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REVISION		
Revised by	Effective date	Description of change
NTP, Bangladesh	July, 2022	Updated in line with the GLI Guideline

11.1. Scope

This SOP describes methods of specimen processing and other laboratory procedures for purposes of culturing *Mycobacterium tuberculosis* on solid or liquid media.

11.2. Procedure

11.2.1. Principle

Culture examination detects fewer bacilli than microscopy and increases the number of TB cases found by 20–50%, depending on local incidence. Culture methods provide definitive diagnosis by establishing the viability and identity of the organisms and allow the detection of drug resistance.

Specimens for isolation of tubercle bacilli contain associated bacterial and/or fungal flora which have to be eliminated before the specimen is inoculated onto culture media.

The decontamination method in use in this laboratory is the Petroff's method in which after adding a solution of sodium hydroxide and allowing a certain time of contact, the pH of the mixture is neutralised by addition of phosphate buffer of pH 6.8.

11.2.2. Samples

Refer to SOP "Sample conditions and transport for culture procedure" for checking the quality of specimens. Standard amount of Sample (2-5 ml) to be processed.

The following specimens should not be processed:

- dried swabs
- saliva
- specimens in broken containers
- specimens collected more than 7 days previously except if collected with a preservative (e.g.: CPC).

11.2.3. Equipment and materials

- BSC, class I or II, annually certified
- Slides
- Slide warmer
- Refrigerated centrifuge with safety shield, a minimum RCF of 3000*g*, operated at 4 degrees
- Centrifuge tubes, preferably 50-ml capacity, clear plastic or thick-walled glass, with screwcaps, resistant to RCF of >3000*g* (RCF)
- Rack for tubes
- Balance
- Pasteur pipettes for 1.0 ml (with graduation), sterile, single-use, plastic (non-sterile pipettes must be sterilized on site before use)
- Pipetting aids
- Mini blender (for biopsy)

- Sterile forceps (for swabs)
- Pipette
- Inoculation loop
- Disinfectants (see relevant SOP)
- Separate waste containers, autoclavable, for pipettes and disposals
- Autoclave
- Buckets, stainless steel or polypropylene
- Vortex mixer
- Timer
- Incubator
- General laboratory glassware
- Refrigerator
- Decontamination reagents and solutions (refer to SOP " Preparation of reagents for culture and drug-susceptibility testing").

11.2.4. Reagents and solutions

LJ slopes tubes/vials for culture media. For culture on solid media use egg-based media, Lowenstein Jensen). For culture on liquid media, use commercially available liquid media.

The reagents and solutions needed are described in the SOP "Preparation of plain egg-media" and SOP "Preparation of reagents for specimen processing for culture".

Avoid using reagent bottles that have already been opened. Use aliquoted stock solutions

11.2.5. Detailed instructions

Important points about specimen processing procedures

- Process clinical specimens as soon as possible.
- Properly label the media to be inoculated to avoid any mix-up of the specimens.
- Minimize aerosol production by opening specimen containers slowly, letting the tubes stand for a few minutes after shaking and before opening, and avoiding expulsion of the last drop from the pipette.
- Process only one specimen at each time. Do not allow open containers or open centrifuge tubes in the BSC. Use aliquots of buffer and decontamination solutions. Use a fresh pipette at every step to avoid transfer of bacilli from one specimen to the other.
- Aseptic technique is important to avoid contamination by bacteria other than tubercle bacilli and especially cross-contamination by tubercle bacilli from other specimens.
- Remember that most techniques require exposure time to disinfectant to be strictly controlled.
- If liquid media are used, it is recommended that solid media are also inoculated to provide back-up cultures in case of contamination of liquid media or in case of malfunction problem if an automated system is used.
- If solid media are used, it is recommended that liquid media are also inoculated to increase the sensitivity of recovery – especially for tissue biopsy, CSF or other small volume of aseptically collected body fluid.
- Prepare smears for staining *after* all media have been inoculated.

A. Sputum processing

Specimens must be processed in centrifuge tubes. If collected in standard containers, sputa must be transferred into centrifuge tubes, which increases the risk of cross-contamination and labelling error. It is thus practical to use 50-ml centrifuge tubes to collect specimens for culture.

The first step of these less time-dependent methods can be performed in microscopy laboratories, outside a BSC. Transport to a biosafety level 2 laboratory must be organized within one week for the CPC method. Ideally perform 6-8 samples at a time is better to maintain the time difference among the first and last samples.

11.2.5.1. Decontamination using NALC–NaOH:

Sodium hydroxide is toxic, both for contaminants and for tubercle bacilli, strict adherence to the indicated timings is therefore essential.

The mucolytic agent *N*-acetyl *L*-cysteine (NALC) enables the decontaminating agent, sodium hydroxide, to be used at a lower final concentration. Sodium citrate is included to bind the heavy metal ions that might be present in the specimen and that could inactivate NALC. The method is also suitable for inoculation in liquid media.

Procedure

1. Mark the volume of sputum on the centrifuge tube (at least 2 ml, not more than 5 ml). Add an equal volume of the NALC–NaOH solution and tighten the screw-cap.
2. Vortex for not more than 20 seconds.
3. Keep at Room temperature for 15 minutes for decontamination.
4. Add phosphate buffer saline upto 45ml. Mix gently.
5. Centrifuge at 3000*g* for 15 minutes.
6. Carefully pour off the supernatant into a discard containing mycobacterial disinfectant (1% hypochlorite or chlorine tablet).
7. Resuspend the deposit in approximately 0.3-0.5 ml phosphate buffer (when using only solid media) or approximately 1-2 ml phosphate buffer (when using liquid media).
8. Inoculate deposit on two slopes of egg-based medium (marked as S1 and S2) and/or into a vial of liquid medium labelled with the ID number. Use a pipette to inoculate each slope/vial with 3–4 drops (approximately 0.1–0.15 ml).

Note: If contamination is found after 24 hours of incubation in both S1 and S2 vial/bottle of the LJ solid media, decontamination should be done following the same procedure only excluding the addition of NALC powder in the NAOH- Trisodium citrate solution.

11.2.5.2. Decontamination using CPC/NaCl method

This soft decontamination method is a means of digesting and decontaminating specimens in transit. Duration of transport should not exceed 7 days otherwise the probability of survival of TB bacilli is low. Specimens should not be refrigerated during transport to the laboratory because CPC precipitates at lower temperatures.

Cetylpyridinium chloride (CPC) is used to decontaminate the specimen; it is bacteriostatic for mycobacteria and neutralization is not needed in the digestion process. A centrifugation step is needed to remove CPC and sediments should be inoculated onto egg-based media only (as egg yolk neutralizes CPC to a certain extent) and not into liquid media

Centrifugation must be done at room temperature because CPC precipitates at lower temperatures.

Note: Staining after exposure to CPC leads to many false-negative results because smears do not adhere well to slides. There is no added value in smearing of the sediment inoculated into culture medium.

Reagents

1% CPC + 2% NaCl solution

Procedure

1. Mark the volume of sputum on the centrifuge tube (at least 2 ml, not more than 5 ml). Add an equal volume of CPC–NaCl to the specimen. Transport to a processing laboratory within 7 days at the most.
2. If not delivered in a centrifuge tube, transfer the liquefied specimen into a centrifuge tube.
3. Add phosphate buffer saline upto 45ml. Mix gently.
4. Centrifuge at 3000*g* for 15 minutes. Set the centrifuge temperature to 20–25 °C (CPC will precipitate at lower temperatures).
5. Carefully pour off the supernatant into a discard can containing mycobacterial disinfectant. Repeat the step 3 and 4 again if needed.
6. Inoculate the deposit on two slopes of egg-based medium in tubes labelled with the ID number. Use a pipette (not a loop) to inoculate each slope with 3–4 drops (approximately 0.1–0.15 ml).

B. Specimens other than sputum (Solid Culture)

11.2.5.3. Laryngeal swabs

Smear examination is not done for laryngeal swabs. Swabs must be cultured on the day that they are received.

Procedure

1. Use sterile forceps to transfer the swab to a sterile centrifuge tube.
2. Add 2 ml of sterile distilled water.
3. Decontaminate following section 2.5.1

Note: Before adding the phosphate buffer solution, remove the swab from the tube with forceps.

11.2.5.4. Gastric lavages

Gastric lavage specimens should be processed as soon as possible after collection.

Note: *The collection tube must contain 100 mg of sodium bicarbonate.*

Proceed as for sputum. If specimen is watery, centrifuge at 3000*g* for 15 minutes, pour off the supernatant, resuspend the sediment in 5 ml of sterile distilled water and then continue as for sputum.

11.2.5.5. Other body fluids

If collected aseptically, centrifuge and inoculate sediment directly onto culture media, preferably liquid media.

If not aseptically collected:

a) when volume is 10 ml or less, handle as for sputum

b) when volume is more than 10 ml, centrifuge first and decontaminate the sediment

11.2.5.6. Tissue

Homogenize using a mini-blender and inoculate into the medium, preferably liquid medium.

11.2.5.7 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 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.

11.2.5.8 Laryngeal swabs

Transfer the swab into a sterile centrifuge tube, add 2 ml sterile water, vortex and wait for 15 minutes. Then remove the swab, treat the sample as sputum, and follow the decontamination process.

11.3. Reading, interpretation, recording and reporting

11.3.1. Incubation of cultures

Incubate tubes/vials at 36 ±1 °C.

For solid media, tubes should be incubated in a slanted position, with screwcaps loose, for 24 hours to ensure even distribution and absorption of inoculum. After 24 hours of incubation, caps are tightened to minimize evaporation and drying of the media. Tubes may then stand upright to save space in incubators.

11.3.2. Reading, interpretation, recording and reporting

Reading and interpretation using solid media

Check colony formation every week and record data on culture register.

Contaminated cultures and rapidly growing mycobacteria (colonies apparent in less than 7 days) are removed. Report results immediately and ask for another specimen.

M. tuberculosis colonies should be well developed within 3–4 weeks. The following characteristics of isolated colonies may yield a tentative identification of *M. tuberculosis*:

- Formation of visible colonies 10–28 days after incubation at 37 °C.
- Greyish white or buff, dry, cauliflower-like colonies with no late production of pigment (up to 28 days).

In case of obvious or doubtful colonies, confirm the presence of MTBC by MPT64 protein detection-based immunochromatographic test. Report results immediately after detection and identification**.**

MPT64 protein detection-based immunochromatographic test

The test cassettes strips were incubated with 100µl (colony dissolved in 200 µl sterile distilled water) solid media or positive liquid media for 15 minutes at room temperature. The pink band in the 'C' region confirmed the test validity. An additional pink band in the 'T' region was interpreted as positive for the MPT64 Ag (often appear within 3-5 minutes). Only the pink band in the 'C' region and no band in the 'T' region were considered negative for the MPT 64 antigen (not more than 15 minutes). No band in 'C' region was interpreted as an invalid test. H37Rv was taken as a positive control for each new kit.

For confirmation of *M. tuberculosis* ZN staining and GeneXpert is recommended. Cultures should be kept in incubator for up to 8 weeks before being reported as negative.

General remarks

Growth on culture media shall be recorded in accordance with the following table

No growth	Negative
Less than 50 colonies	Actual count
50 to 100 colonies	1+
100 - 200 colonies, light bacterial lawn	2+
200 - 500 colonies, almost confluent	3+
More than 500 colonies, confluent growth	4+

Laboratory register

Record in the laboratory register:

- The date of detection of growth and the colony characteristics of positive cultures
- Negative and contaminated tubes – individually entered at the end of the recommended incubation time or when detected
- Reporting date.

Reporting

Results should be reported in accordance with qualitative and quantitative criteria.

Fill out the DR TB 06 culture reporting portion to deliver the result.

11.4. Quality control

11.4.1. Sensitivity of plain egg-based medium

Serious problems affecting the sensitivity of culture medium, i.e., its capacity to sustain consistent growth of tubercle bacilli, can be detected by seeding a 1/10 000 dilution of a suspension of *M. tuberculosis* calibrated to McFarland No. 1 (equivalent to a bacterial suspension containing 1 mg/ml of tubercle bacilli)

- Prepare a McFarland No. 1 suspension with a *M. tuberculosis* reference strain.
- Dilute the suspension with 10-fold dilutions to the 10^{-4} dilution.
- Inoculate five tubes of a previous batch of medium and five tubes of the new batch of medium with 0.2 ml of the 10^{-4} diluted suspension.
- Incubate at 36 ± 1 °C.
- Read and interpret as usual, following instructions given above in section.
- If the number of colonies obtained on the recently prepared or purchased batch of medium is significantly lower than that on the reference batch, the sensitivity of the new medium, whether prepared or purchased, is not adequate.
- It is convenient to keep a register of the following type:

Medium: Lowenstein–Jensen

Batch no.	**Volume (ml)**	**Date**			**Sterility control**	**Sensitivity control**				
		Preparation or purchase delivery	Start of use	End of use	Contamination detected Yes/No	Strain/ specimen inoculated	Growth detected at 20 days (colonies)		Growth detected at 60 days (colonies)	
							Control batch	Present batch	Control batch	Present batch

This register allows the identification and elimination of deficient media batches.

In the case of egg-based media, 20 days of incubation are usually enough to determine whether the sensitivity of the batch is satisfactory. If sensitivity is not satisfactory, negative culture results obtained with tubes inoculated with the deficient medium will be invalidated and these cultures will have to be repeated.

Media batches that are contaminated or not homogeneous or contaminated, those that were exposed to high temperatures of inspissator, and those with inadequate sensitivity should be discarded without delay immediately.

11.4.2. Specimen handling

Reception

Make sure that the samples received are clearly identified and are accompanied by a completed NTP request form. It is critical for the laboratory to be able to differentiate specimens received for diagnostic purposes from those received for control of treatment. This will allow proper interpretation of results and guide the sequence of necessary bacteriological studies for each patient. Lack of this information rules out the use of monitoring of bacteriological results as a method of internal quality control.

Make sure also that the laboratory register shows the date on which the sample was cultured as well as all the above-mentioned information and any relevant details concerning the processing of the specimen (e.g., decontamination of aseptically collected specimens).

Organization of work

The routine of laboratory work will determine the maximum possible number of days per week that can be allocated to specimen processing. Each day of delay in inoculation of the specimen on growth media diminishes culture positivity rate. Do not delay the processing of the specimens beyond a period of 7 days following collection. Use commonly recommended methods of preservation, such as the CPC/NaCl method, when transportation delays are expected. The processing of gastric washings from children should be expedited as much as possible.

Maintain a systematic and traceable sequence of processing the specimens during the workday and process smear-positive specimens (potential sources of cross-contamination) last. Do not process culture isolates alongside clinical specimens.

Decontamination and cross-contamination

Take all precautions to avoid the transfer of bacilli from one specimen to another:

- use aliquoted reagent solutions
- do not reuse the reagent solution aliquots opened during the workday
- do not open a specimen container or a tube before capping the previous one
- dispense solutions without touching the neck of the tubes with the pipettes or dispensers; and
- *carefully* decant supernatants into a flask containing a 5% phenol aqueous solution to avoid splashing (e.g., along a funnel).

The total contact time (15 minutes) of the sample with the decontaminant must be strictly controlled. Too short a time results in high contamination rates, too long a time causes loss of viability of the bacilli.

Note that some manufacturers of commercially available liquid media based on modified Middle brook media recommended the NALC decontamination method only.

Concentration by centrifugation

When using a centrifuge verify that the rotor reaches and maintains the required RCF of 3000*g* for 15 minutes to obtain good recovery of the mycobacteria. Non-refrigerated centrifuges are not suitable because temperatures reached during centrifugation typically exceed 37 °C and will affect the viability of

the bacilli. However, using CPC for decontamination/digestion of sputa, centrifugation must be done at room temperature because CPC precipitates at lower temperatures.

Incubation

Record the temperature of the incubator daily with a thermometer accurate to ±1 °C. The temperature should not fluctuate beyond the 35–37 °C range. When an incubation chamber is used, place thermometers at different and easily visible places to ascertain temperature uniformity. Maximum–minimum thermometers provide more information on temperature variation.

Whenever the temperature exceeds 38 °C or falls below 35 °C, the event should be entered in the laboratory registry. Excessive temperature will invalidate culture-negative reports for the specimens that were incubated during the occurrence. New specimens should be obtained from these patients for retesting.

When using automated systems for liquid cultures, refer to the manufacturer's manual.

Reading

- Solid media

  Make sure that cultures are checked at regular intervals:

  - at 24-48 hours of incubation to detect and record early contamination.
  - weekly to detect growth as early as possible.

  Confirm that new specimens have been requested whenever smear-positive specimens turn out to be culture-negative or when all inoculated tubes/vials are contaminated.

Recording and reporting of results

Make sure that:

- the date of detection of growth and the colony characteristics of positive cultures are entered in the laboratory register
- for liquid media, the date and number of days to detection of growth are recorded
- negative results are entered at the end of the recommended incubation time
- contaminated tubes are individually entered when detected
- the laboratory register also records the reporting date
- results are reported to clinicians as soon as they are available
- results are reported according to qualitative and quantitative criteria

Using liquid media, contaminated or mixed cultures may be interpreted as positive. A preliminary check for the presence of mycobacteria is carried out by microscopic examination of a smear prepared from the growing organisms at the bottom of the liquid culture (do not mix as the growth is mainly at the bottom of the tube).

Fill out an individual form for each patient for whom specimens for diagnosis were submitted. Future results of all bacteriological testing will be added to the form. This facilitates the detection of anomalies, e.g., patients who systematically show smear-positivity and culture-negativity, non-reproducible reports, patients positive after the third month of treatment. It also allows the determination of performance indicators, such as the contribution of the culture to diagnosis.

The supervisor, who oversees all technical aspects of TB laboratory work, will monitor monthly the compliance with guidelines in the preparation of reagents, media, handling of samples, recording and reporting etc. The supervisor will also check records of pH readings of the salt solutions used in the preparation of egg-based media and sterilization records obtained with temperature monitoring devices.

11.4.3. Daily monitoring routines

Daily monitoring allows early correction of errors. The following paragraphs deal with "alarm signals" that require attention.

Smear-positive/culture-negative specimens

Find out whether the specimen was collected for control of treatment. If this is the case, a negative culture could simply reflect the fact that the patient is shedding dead bacilli and that treatment is effective.

If the sample was collected for diagnostic purposes, watch for a recurrence of this type of result.

Ensure that:

- the concentration of the decontaminating solution and the time of contact with the specimens are those recommended in the technical guidelines
- the temperature of the incubator did not exceed acceptable limits
- the sensitivity of the culture batch being used has been thoroughly checked.

Contaminated tubes/specimens

Find out whether the time that elapsed between specimen collection and specimen processing was too long. If so, corrective measures will have to be introduced in the specimen transportation system, in the laboratory work routine or in both.

Recurrent contamination

Recurrent contamination can occur in specimens processed during a particular day or in decontaminated specimens or in specimens collected in one particular place. In such cases, the sterility of the decontamination reagent solutions, of the whole decontamination process or of the specimen collection/transportation system will have to be checked; if errors are detected, immediate remedial action must be implemented. If the problem is traced to the technologist performing the procedure, he or she should be immediately retrained.

If contamination of specimens from the same patient recurs, a harsher decontamination procedure may have to be used for further specimens from the patient. Increase the reagent concentration, *not* the time of exposure. Use two volumes of decontaminant solution to one volume of specimen. Do not apply the modified procedure to all specimens – only to contaminated specimens.

Clustering of culture-positive specimens

Cross-contamination between specimens from epidemiologically unrelated patients can cause a sequence of positive culture isolations in a short interval of time. The occurrence of cross-contamination should be investigated to rule out false-positive culture diagnoses. The following circumstances will be investigated:

- some of the patients involved do not have clinical symptoms compatible with tuberculosis
- other specimens from the same patient are not culture-positive
- one or several specimens involved, which yielded cultures with very few colonies, were processed immediately after a highly smear-positive specimen.

If, in laboratories processing numerous extrapulmonary, supposedly sterile specimens, it is found that the positive cultures were derived from decontaminated specimens only, this would strongly imply that the transfer occurred via the decontamination solutions or the laboratory equipment. If cross-contamination is suspected in such circumstances, the cultures involved in the contamination episode should be submitted to a reference laboratory for genotyping.

If cross-contamination cannot be ruled out, check that the following precautions are being respected:

- solutions are dispensed without touching the necks of the tubes
- aliquoted reagent solutions are being discarded after single use

- the processing sequence of specimens is maintained, i.e., smear positive specimens are processed last
- tubes are not uncapped simultaneously or immediately after being taken from the centrifuge
- supernatants are discarded carefully
- gloves, if worn, are frequently changed, and never reused.

In busy laboratories in high-incidence settings, it may be advisable to have a BSC dedicated to processing of smear-positive specimens since the probability of cross-contamination increases with the number of smear-positive specimens.

11.4.4. Periodic monitoring routine

Depending on the workload and the prevalence of bacteriologically positive cases, analysis of the results obtained during a month, a quarter or a semester allows the detection of systematic errors. These analyses are key to the quality control of diagnostic cultures of pulmonary TB in adults (but do not apply to follow-up).

Classification of specimens from adult pulmonary TB patients investigated for diagnosis:

a smear-positive and culture-positive
b smear-positive and culture not done
c smear-negative and culture-positive
d smear-positive and culture-negative
e smear-positive and culture contaminated
f smear not done and culture-positive

From this classification, calculate the following indicators:

- Contribution of culture to diagnosis for a given setting

$$\frac{c+f}{a+b+c+d+e+f} * 100$$

- Contribution of culture to diagnosis over microscopy

$$\frac{c}{(a+c+d+e)} * 100$$

  Culture is more sensitive than smear microscopy and is expected to contribute at least 20% to the bacteriological confirmation of adult pulmonary TB cases.

- Percentage of smear-positive and culture-negative diagnostic cases

$$\frac{d}{(a+c+d+e)} * 100$$

- This percentage should be very low, typically around 2-3 %. Exceptionally, patients are found with persistent smear-positive and culture-negative diagnostic specimens. These are usually undisclosed treatment control specimens. Higher percentages could be the result of decontamination procedures that are too harsh or of transport delays.

11.4.5. Determination of the contamination rate

The contamination rate is a valuable indicator of the efficiency of procedures used for specimen processing. It is calculated as the percentage of contaminated tubes among all inoculated tubes or vials and not as the percentage of samples. It should be within the range 2–5% and not exceed 5% if the Petroff decontamination method is used.

When available, computer databases should be preferred to hard-copy forms for registering and monitoring results of **positive patients and culture quality indicators.**

11.4.6. Alarm signals

The indicators in the following table are valid for specimens from adult pulmonary TB patients investigated for diagnosis (but do not apply to follow-up):

Indicators of culture performance	Normal value (%)	Much higher: investigate	Much lower: investigate
Contribution of culture to bacteriological diagnosis of tuberculosis	20	A	B and C
Percentage of smear positive/culture negative specimens	2–3	C and D	Not a problem
Percentage of contaminated tubes	2–5 6–8 with liquid media	E	F

A	Smear microscopy reading errors: false negatives" A high percentage of incipient pulmonary TB and paediatric TB cases are being tested (not a problem)
B	Inadequate use of culture: patients who are not TB suspects are being examined, rather than incipient TB cases
C	Excessive delay between specimen collection and specimen processing Over-harsh specimen decontamination procedures (excessive concentration and/or too long a contact time with the decontaminant) Low relative centrifugal force or overheating of centrifuge Low culture media sensitivity (lack of homogeneity, overheating during inspissation, too much malachite green, too acidic a pH) Incubation at too high or too variable a temperature Misclassification of a follow-up specimen
D	Smear microscopy reading errors: false positives
E	Un-refrigerated storage of specimens Excessive delay between collection and processing of specimens Low decontaminant concentration Too short a contact time between decontaminant and specimen Deficiency in the sterilization procedure Careless use of the Bunsen burner, heavy people movement in the work area, generation of air draughts by fans or air-conditioning systems, etc,
F	Too high a concentration of decontaminant Too long a contact time of the specimen with the decontaminant Poor specimen neutralization Too high a concentration of malachite green in the culture medium Incubation at too high or too variable a temperature

11.4.7. Delay in the delivery of reports

Culture procedures for tuberculosis bacteriology are notoriously time-consuming,

often taking weeks or months to complete. For this reason, the following schedule is recommended:

- If the cultures have been contaminated, a report should be sent out immediately

  and a repeat specimen requested

- If cultures are positive and growth has been identified as *M. tuberculosis* a

  report should be sent out immediately

- At eight weeks a final report should be issued for the negative cultures

11.5. Waste disposal

All inoculated tubes and vials, whether negative or contaminated, should be autoclaved as potentially infectious material.

12.1. Scope

This SOP describes the susceptibility testing of *Mycobacterium tuberculosis* and other members of the *M. tuberculosis* complex (*M. africanum* and *M. bovis* are the most frequent) to anti-TB drugs on Löwenstein–Jensen medium using the proportion method. This method is considered to be a reference standard against which other routine methods should be assessed. Other methods are not covered here.

Because suspensions with high concentrations (starting from $>10^9$ bacilli/ml) of viable, infectious bacteria are handled, strict compliance with safety and protection measures is mandatory. The procedure must be carried out in GLI TB laboratory safety guideline.

12.2. Procedure

12.2.1. Principle

The proportion method (Canetti et al., modified) determines the percentage of growth (number of colonies) of a defined inoculum on a drug-free control medium versus growth on culture media containing the critical concentration of an anti-TB drug.

The critical drug concentration, as well as the critical proportion of resistant colonies, has been evaluated from clinical data. While Canetti et al. originally based their method on three concentrations per drug, they later proposed more economical versions based on only one or two concentrations. Today, a single concentration is most used and is the method described here (see Table 1).

Table 1. Critical concentrations of first-line and second line drugs

Drug	INH	RMP	EMB	PNB	LFX	MFX	AMK
Critical concentration (µg/ml)	0.2	40	2	500	2	1	30

12.2.2. Equipment and materials

1. Autoclave
2. BSC, class I or II, annually certified
3. Incubator
4. Refrigerator
5. Safety Bunsen burner, with device to light on demand, or micro-incinerator
6. Rack for glass tubes
7. Closed platinum/iridium loop, 3 mm internal diameter, calibrated to 10 µl, and/or short, sterile graduated pipette or single-channel microlitre pipette with sterile tips, for delivery of 0.1-ml volumes
8. Pipettes, graduated, for 0.1 ml. 0.2 ml and 1.0 ml
9. Pipetting aids
10. Buckets, stainless steel, or polypropylene

11. Vortex mixer
12. Sterile glass beads, diameter 3 mm
13. Thick-walled glass tubes with tightly fitting screwcaps (16 x 125 mm) or Bijou Bottle (20 x 50mm)
14. Sterile NaCl solution, 0.9%
15. McFarland turbidity standard no. 1 (as in SOP "Preparation of reagents for culture and DST"
16. Separate waste containers (autoclavable) for pipettes and disposables
17. Drug-containing media as in SOP " Preparation of Lowenstein–Jensen
18. drug-containing media". (All LJ tubes containing drugs should be from the same batch.).
19. Plain culture media as in SOP " Preparation of plain egg-based media".
20. LJ tubes containing drugs at the critical concentration and LJ tubes with no drug added.

12.2.3. Reagents and solutions

Disinfectants (see relevant SOP)

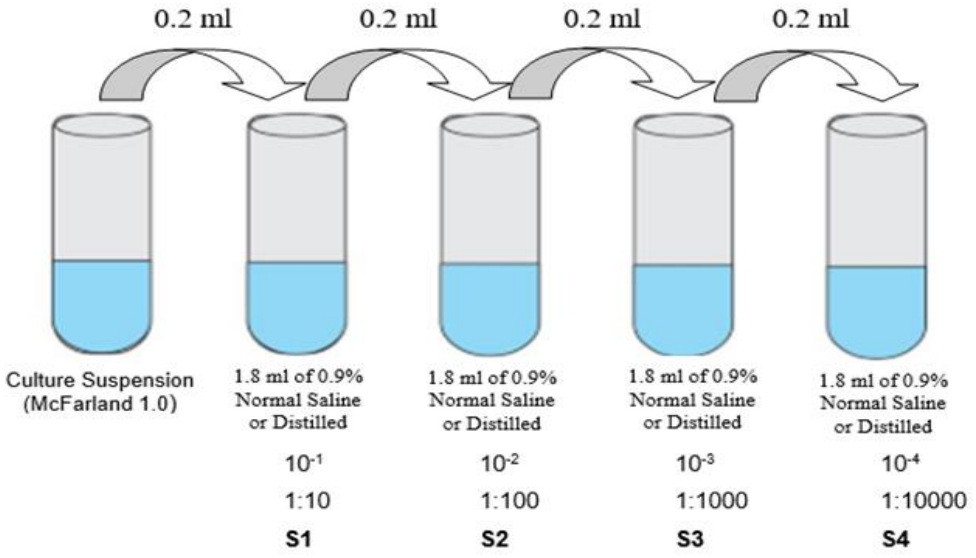
Sterile distilled water

12.2.4. Detailed instructions

Preparation of the calibrated bacterial suspension

1. With a loop, scrape colonies from all over the culture (try to pick up portions from all colonies).
2. Use a sterile, small, thick-walled screw-capped glass tube containing 5–7 sterile glass beads (approximately 3 mm in diameter).
3. Gently shake the loop over the beads.
4. Repeat steps 1–3.
5. Add 2 drops of sterile saline or distilled water, shake, add 2 further drops (total of 0.1ml), shake/vortex.
6. Let stand for 10-15 minutes to allow the larger aggregates of bacteria to settle. Using a pipette, match the suspension with McFarland 1.0 Standard by adding saline or distilled water until turbidity matches McFarland 1.0.

Dilution of the calibrated bacterial suspension:



1. Dispense 1.8 mL of sterile 0.09% normal saline or distilled water in 4 sterile dilution tube (dilution 1/10, 1/100, 1/1000 and 1/10,000).

2. Transfer 0.2ml of the matched McFarland No1 bacterial suspension in the first sterile dilution tubes (S1) containing 1.8 mL of sterile 0.09% normal saline or distilled water.

3. Mix thoroughly to give a 1/10 dilution.

4. Transfer 0.2 mL from the 1/10 dilution prepared above into the second sterile dilution tube (S2) containing 1.8 mL of sterile 0.09% normal saline or distilled water.

5. Mix thoroughly to give a 1/100 dilution.

6. Transfer 0.2ml from the 1/100 dilution to the 3rd sterile dilution tube (S3) containing 1.8 mL of sterile 0.09% normal saline or distilled water).

7. Mix thoroughly to give a 1/1000 dilution.

8. Repeat the same procedures for 4th sterile dilution tube (S4), mix thoroughly to give 1/10,000.

Inoculation

The objective of the technique is to achieve a growth of 30–100 colonies on the growth control (drug-free) medium using the most dilute suspension for inoculation.

1. Mark all sets of culture media properly with patient's identification (at least laboratory number).
2. Remove condensed moisture from the slants before inoculation.
3. The inoculation may be performed with pipettes (delivering 0.1 ml) or with a calibrated loop of diameter 3 mm (delivering 10 µl).

Inoculum 10 µl, calibrated loop

Although use of the calibrated loop needs some skill, a small inoculum volume is recommended in areas with high humidity to avoid accumulation of water in the culture medium tubes.

Inoculate suspension dilutions as indicated in **Table 2.a & 2.b** on drug-free and drug containing tubes. Drug free culture media consist of growth control (GC). The two tubes labelled GC2 are inoculated with suspension S2 (dilution 10^{-2}), the two tubes labelled GC4 are inoculated with suspension S4 (dilution 10^{-4}). Tubes containing 1st line (INH, RMP or EMB) or 2nd line (LFX, MFX, AMK) drugs (one tube per drug) are inoculated with S2 (dilution 10^{-2}) and S4 dilution 10^{-4} and one tube for PNB with suspension S2 (dilution 10^{-2}) for identification.

Table 2.a: Drug dilution inoculation (1st line)

Suspension Dilution	Drug-free tubes (Growth control, GC)	INH 0.2 µg/ml	RMP 40 µg/ml	EMB 2 µg/ml	PNB
Culture Suspension	**X**	**–**	**–**	**–**	**–**
S1; 10^{-1}	**–**	**–**	**–**	**–**	**–**
S2; 10^{-2}	**XX (GC2)**	**X**	**X**	**X**	**X**
S3; 10^{-3}	**–**	**–**	**–**	**–**	**–**
S4; 10^{-4}	**XX (GC4)**	**X**	**X**	**X**	**–**

– = no inoculation

Table 2.b: Drug dilution inoculation (2nd line)

Suspension Dilution	Drug-free tubes (growth control, GC)	LFX 2 µg/ml	MFX 1 µg/ml	AMK 30 µg/ml
Culture Suspension	**X**	**–**	**–**	**–**
S1; 10^{-1}	**–**	**–**	**–**	**–**
S2; 10^{-2}	**XX (GC2)**	**X**	**X**	**X**
S3; 10^{-3}	**–**	**–**	**–**	**–**
S4; 10^{-4}	**XX(GC4)**	**X**	**X**	**X**

– = no inoculation

Incubation

Care must be taken to distribute the inoculum evenly over the lower part of the culture medium; avoid inoculating the upper (thin) part of the slant. The tube cap should allow a little gas exchange but also prevent drying out. Screw caps meet these needs.

The incubation temperature shall be 36 ± 1 °C.

The inoculated media are examined for contamination after 1 week of incubation and for DST interpretation after 4 and 6 weeks of incubation.

12.2.5 Reading, interpretation, recording and reporting

12.2.5.1. General remarks

Slants should be read after 4 weeks of incubation for as a provisional result and after 6 weeks of incubation for definitive interpretation.

Growth on culture media should be recorded as follows:

No growth	0
Fewer than 50 colonies	Actual count
50–100 colonies	+
100–200 colonies, light bacterial lawn	++
200–500 colonies, almost confluent	+++

Growth on the GC4 tube should allow easy counting of 30 to 100 isolated colonies. If there is no growth on drug-free media after 6 weeks, the test cannot be interpreted and should be repeated.

A strain is considered to be **susceptible** if there are no colonies or considerably less than 1% growth on the drug medium compared with GC with 1% inoculum.

A strain is considered to be **resistant** if the number of colonies on the drug-containing medium exceeds that on the GC with the 1% inoculum.

The first reading of drug susceptibility test results is done after 4 weeks of incubation. At that time all strains showing drug resistance can be reported as drug resistant. Because some (especially multidrug-resistant) strains grow very slowly, a further 2 weeks of incubation are needed before reporting susceptibility.

12.2.5.2. Reading, interpretation

$$\text{Calculation} = \frac{\text{Number of colonies on the drug media}}{\text{Number of colonies on the control (drug free) media}} \times 100 = \% \text{ Proportion Resistant}$$

Result is 1 or > 1 is resistant. Result <1 is sensitive

"Borderline cases" (with about 1% growth on drug-containing medium) should be reported as resistant (under reservation) and retested.

If fewer than 20 colonies have grown on GC2, a reliable interpretation is possible only for resistant strains. For susceptible strains, the result should be repeated.

If more than 100 colonies have grown on GC2, interpretation is possible only for strains that did not grow on drug-containing media and can therefore be safely interpreted as sensitive. If there is some growth on drug-containing media, the 1% proportion cannot be estimated with confidence and the test must be repeated.

12.3. Reporting

Use **DR TB 06** for reporting.

12.4. Quality control

Every new batch of drug-containing media prepared for DST must be quality-controlled. A suspension of a freshly subcultured *M. tuberculosis* H37Rv strain is diluted and inoculated into drug free and drug containing media in the same manner described above.

If the batch fails the quality control criteria, DST of the patients' strains must be repeated with another batch of drug-containing media.

External quality assessment should be organized and supervised annually by the national reference laboratory, using a panel of 20 test strains provided by the SRL network.

12.5. Waste management and other safety precautions

Used pipettes, tubes and all material handled are collected inside the BSC in appropriate autoclavable containers and autoclaved.

Gloves and other waste may be collected in an autoclavable plastic bag, which should be sealed before autoclaving.

Chapter 3: Liquid Culture and DST

Section 1: 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. 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 followed by the decontamination procedure.

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 GLI TB Laboratory safety guideline has recommended the facility with directional airflow accompanied by HVAC system for mycobacterial work.

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 discoloured, cloudy or appears to be contaminated.

C. Specimen Handling

1. Collection

2-10 ml early morning specimens should be collected in clean, preferably sterile containers with a tight-fitted lid or cap. For patients with respiratory symptoms, the specimens should be expectorated sputum and not saliva.

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

It is extremely important to follow the standard procedure for decontamination recommended for MGIT 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.

NaOH-NALC procedure

This is the standard recommended procedure to be used with MGIT, which is also recommended by CDC. 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%.

Materials and Methods

Materials Required:

- Disposable 50 ml plastic tubes (Falcon tubes)
- Sterile NaOH-NALC-sodium citrate solution
- Sterile Phosphate buffer pH 6.8 (0.067M)
- Refrigerated Centrifuge (with covered bucket & swing rotor) with a minimum 3000g (rcf) force.
- Vortex mixer, shaker
- Timer
- Pipettes/transfer pipettes or a pipettor with cotton plugged pipette tips
- Analytical balance
- Precision balance
- NALC powder
- Racks for falcon tubes
- pH meter
- Sterile transfer pipette with graduation
- Pipette aid
- Paper towel
- Discard bucket with biohazard bag containing appropriate disinfectants
- Hot Plate

Procedures:

- Labelling 50 ml falcon tube with proper ID no.
- Transfer sputum to a 50 ml centrifugetube 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 wholetube 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/vortex/shaking by hand and shake lightly during the whole time.
- Make sure the specimen is completely liquefied. If still mucoid, add a small quantity of NALC powder 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).

  Note: Addition of sterile water is not a suitable alternative for the phosphate buffer.
- Centrifuge the specimen at a speed of 3000 g for 15-20 minutes. Use of refrigerated centrifugation at a higher speed is known to increase recovery of mycobacteria.
- After centrifugation, allow tubes to sit for 5 minutes to allow aerosols to settle. Then take the tubes carefully inside the BSC and 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

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 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.
- Temperature increase during centrifugation increases the killing effect on mycobacteria which will decrease the positivity rate and increase time-to-detection.
- 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.
- **CPC containing sputum must not be used for liquid culture processing**

E. Smears for Acid-Fast Bacteria (AFB)

Smear preparation

Prepare smears from all processed specimens before inoculation into medium. 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 loopfuls on a clean microscope slide.

b) Spread the smear about 2 cm x 3 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 65-75^{0}C 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.

1. **Staining methods**

a. Ziehl-Neelsen staining

F. Preparation and Inoculation for Culture

1. <u>Reagents</u>

a. MGIT medium

The MGIT 960 tube contains 7.0 ml of modified 7H9 broth base.

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 beadded 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 antimicrobialPANTA 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. <u>Procedures</u>

a. Reconstituting PANTA

Reconstitute MGIT PANTA with 15.0 ml MGIT growth supplement. Mix until completelydissolved. 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

- All procedures should be done inside the biologic safety cabinet.
- 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 labelled MGIT tube. Use separate pipette or pipette tip for each specimen.
- Immediately recap the tube tightly and mix by inverting the tube several times.

c. Precautions

- Make all additions inside a biosafety hood.
- One of the major sources of contamination in MGIT medium is environmental contaminants introduced during addition of growth supplement.
- Do not open several tubes at a time.
- Open single MGIT tube at a time.
- 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 themedium and may cause false fluorescence. This may also increase contamination or otherwise adversely affect the performance of the MGIT medium.

d. 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 andnot to shake the tube during the incubation. This helps in maintaining the oxygen gradient inthe medium. The instrument maintains $37^0C \pm 1^0C$ temperature. Since the optimum temperature for growth of *M. tuberculosis* is 37^0C, make sure the temperature is close to 37^0C.

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.

e. 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, ittakes longer for revival and growth of viable mycobacteria. In some instances, as manyas 60-70% of the mycobacteria are killed during processing
- loss of mycobacteria during centrifugation is also significant. 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 andfaster compared to the solid media. 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 growthbefore 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. Quality Control for the reagents and productsused in the isolation, as well as for the test procedure, is critically important for mycobacteriology laboratories.

G. Work-up of Positive Cultures

Growth of contaminated bacteria will result in positive fluorescence. It is important to observe all fluorescent positive MGIT tubes visually for turbidity. After the machine indicate positive to any MGIT tube, confirm the MGIT tube with

MPT64 protein detection-based immunochromatographic test

The test cassettes strips were incubated with positive liquid media for 15 minutes at room temperature. The pink band in the 'C' region confirmed the test validity. An additional pink band in the 'T' region was interpreted as positive for the MPT64 Ag (often appear within 3-5 minutes). Only the pink band in the 'C' region and no band in the 'T' region were considered negative for the MPT 64 antigen (not more than 15 minutes). No band in 'C' region was interpreted as an invalid test. Test should repeat with another strip of MPT64 kit in case of invalid test result. H37Rv was taken as a positive control for each new kit.

If the sample interpret as negative at MPT 64 antigen test kit, follow the steps below:

- Inoculate one BHI agar (or Blood agar/chocolate agar) media from positive MGIT tube. Incubate for 24-48 hours. Check the growth. If no growth occurs in BHI agar plate document the findings into register for laboratory purpose. If growth occur in BHI agar, ZN microscopy is suggested to identify MOTT complex.
- Also, transfer the whole liquid from MGIT tube into 50ml falcon tube and re-process the sample by using 4% NaOH and re-inoculate the sample into another MGIT tube and incubate again
- The tubes interpreted as negative by MGIT machine will consider as negative result
- The tubes interpreted as positive by the machine will further checked by MPT64 antigen kit for 2nd time
- Inoculate the incubated samples from MGIT tube with negative result by MPT64 antigen again in BHI agar (or Blood agar/Chocolate agar) and incubate for 24-48 hours

- Growth in BHI agar media indicate the contaminated result whereas no growth in BHI agar indicate the negative result.

1. Dealing with contamination

Liquid media are more prone to contamination than solid media. It is extremely important toprocess specimens with extreme care, adhering very closely to procedures and recommendations. Following are guidelines for controlling excessive media contamination

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. A general recommendation is that 5% ± 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.

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 ispositive. Usually contaminating bacteria cause heavy turbidity, although *M. tuberculosis* growth appears as particles without any significant turbidity. Contamination may be confirmed by the following method:

- 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 labelled). Divide theplate and identify specimen number by a marker. Incubate these subcultures at35°C±1°C and observe after 24-48 hours. If contaminating growth appears, interpret the result as contaminated.

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. Ifhigh 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 inPANTA 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 collectedfrom a patient, and it is not necessary to salvage a contaminated specimen if other specimensfrom 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.

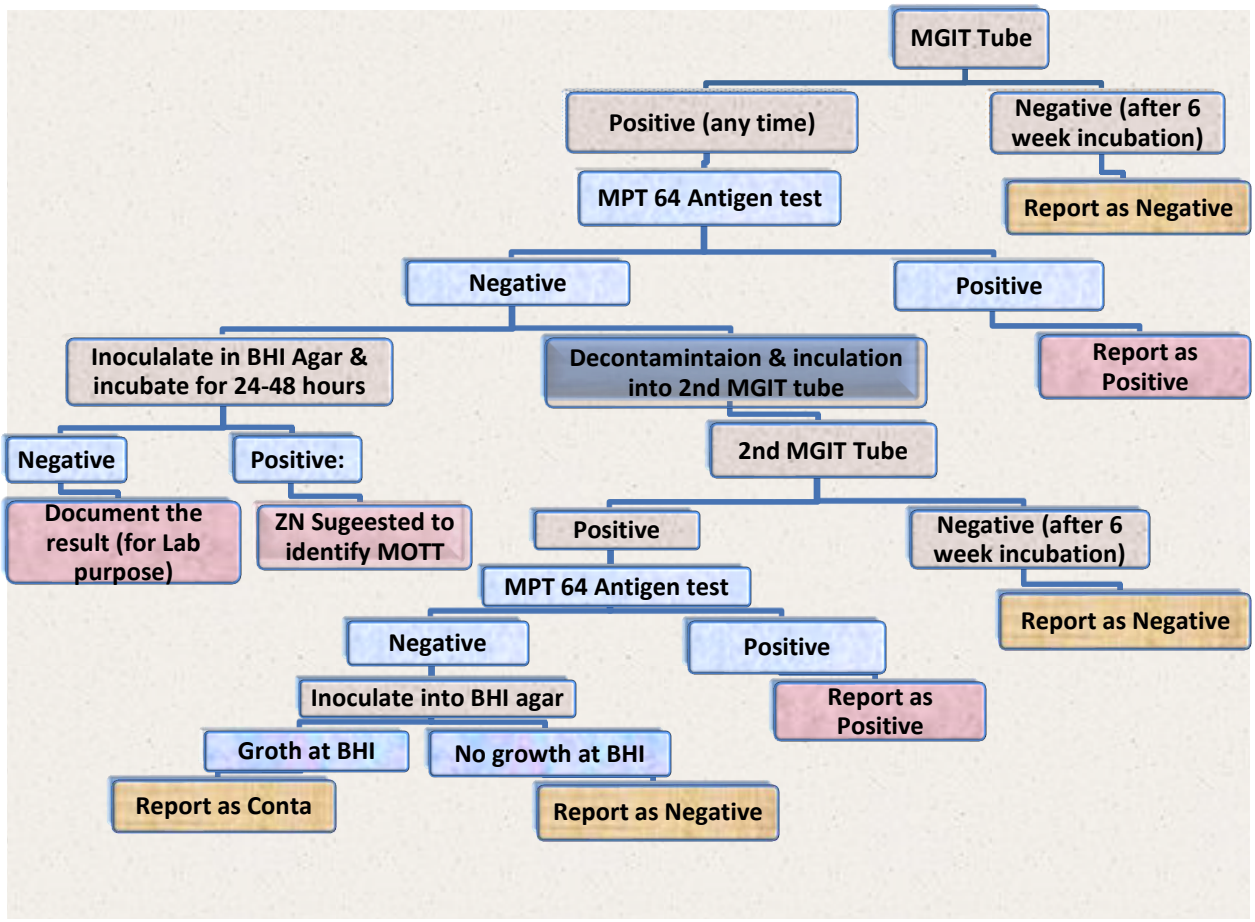
- Transfer the entire MGIT broth into a 50 ml centrifuge tube.
- Add an equal quantity of 4% sterile NaOH solution.
- Mix well and let stand for 15-20 minutes, mixing and inverting the tube periodically.
- Add phosphate buffer pH 6.8 after 15-20 minutes up to 40 ml mark. Mix well.
- Centrifuge at least 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.

c. 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 oradding 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 eventof a cross-contamination episode, all reagents, equipment and biosafety cabinets must be thoroughly checked.

H. Results / Reporting

Result will be recorded following the flow-chart below:



I. Limitations of the Procedure

- Colony morphology and pigmentation cannot be observed in a liquid medium.
- Even if a single viable contaminating bacterium survives the decontamination and PANTA inhibition, it may contaminate the entire medium. Contamination may mask mycobacterial growth.
- A positive culture from a clinical specimen cannot be correlated with colony forming units (CFU) present in the specimen which sometimes is used to establishimportant NTM infection.
- 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.
- Sometimes, excessive carryover of reducing agent or alkali may cause false fluorescence of the sensor for a short time.
- 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 speciesand isolates within a species. However, overall isolation of NTM is higher in liquid as compared to solid media.

J. Quality Control

1. 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.

b. Preparation of culture suspension

- Subculture the above mycobacteria on several LJ slants.
- Incubate at 37°C ± 1°C.
- 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 dispensedand 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 andadjust the turbidity to McFarland #0.5 standard. This is the working suspension forQC testing. This suspension may be frozen in small aliquots (1-2 ml) in appropriatetubes/vials at -70°C + 10°C. 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 bytaking 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 Tube3 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 4.5ml 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 labelled 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 atBD Diagnostic Systems.

f. Precautions

- Use freshly prepared suspension adjusted to McFarland #0.5 standard. If frozen (-70°C $\pm$ 5°C), 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.

2. 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.

3. 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 from positive tube, contamination, etc.

Section 2: Susceptibility testing for anti-TB agents using liquid media

Principles

The MGIT medium consists of modified Middlebrook 7H9 broth. The automated MGIT system requires an instrument called the BACTEC 960 (other sizes are also available). Tubes in the automated system require 7.0 ml of medium. For routine culture of specimens, a growth supplement known as OADC (consisting of oleic acid, albumin, dextrose, and catalase) is available from the manufacturer. This growth supplement is added to the medium prior to inoculation to complete the medium. It is essential for the growth of many types of mycobacteria, especially those belonging to the *M. tuberculosis* complex.

In addition to the Middlebrook 7H9 liquid medium, 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 by CO_2. The depletion of free oxygen results in fluorescence of the sensor within the MGIT tube when visualized under ultraviolet light. The intensity of fluorescence is directly proportional to the extent of oxygen depletion. The BACTEC MGIT 960 instrument automatically detects this fluorescence.

Susceptibility testing for second-line agents can be performed based on the same principle as that of first-line agents. Two MGIT tubes are inoculated with the test culture. A known concentration of a test agent is added to one of the MGIT tubes, and both tubes are incubated. Growth in the tube containing the agent is compared with that in the MGIT tube without the agent (that is, the control tube). If the test agent is active against the mycobacteria, it will inhibit the growth and, thus, there will be suppression of fluorescence while growth in the control tube will be uninhibited, and fluorescence will increase.

Growth is monitored by the MGIT 960 instrument and is recorded in values known as Growth Units (GU). In the case of first-line agents, the instrument automatically interprets the difference in GU values between the control tubes and the tubes containing the agent; results are reported as susceptible or resistant. GU values are available for second-line or newer agents, but the user must interpret the values manually.

The principles of the proportion method have been used to establish procedures for DST for both first-line and second-line agents. To assess the 1% criteria, the inoculum of the control is diluted 100-fold compared with the inoculum for the tube containing the anti-TB agent. Growth in the tubes containing the anti-TB agent is compared when growth in the control tube reaches a predetermined threshold, expressed in GU. The protocol for the BACTEC MGIT 960 susceptibility test usually takes for 4-13 days for both first-line and second-line agents. In some instances, it may be necessary to extend the incubation period for some drug-resistant strains that may take longer than 14 days to grow. PZA use a 21-day protocol and a 1:10 growth control (GC) is used for this drug.

Preparing the medium

The medium used with the BACTEC MGIT 960 system is modified Middlebrook 7H9 broth; 7ml are required. For DST, one tube is needed as the control (that is, this tube does not contain the anti-TB agent); for each agent and concentration to be tested, an additional tube is needed. Specific DST carriers are also needed to place the tubes in the instrument; the number of carriers needed is based on the number of tubes that are being used for DST.

Equipment and Materials for first-line DST

BACTEC MGIT 960 SIRE kit (cat No. 245123 BD Cat No. GDF 106028) contains one each lyophilised vials of streptomycin, isoniazid, rifampicin and ethambutol and 8 vials of SIRE supplement.

Streptomycin	: 332.0 µg
Isoniazid	: 33.2 µg
Rifampicin	: 332.0 µg
Ethambutol	: 1660.0 µg

Reconstitute each BACTEC MGIT SIRE kit drug with 4ml of sterile distilled water to achieve the desired stock concentration

BACTEC MGIT 960 PZA kit (cat No. 245128BD Cat No. GDF 106033) contains two lyophilized vials of pyrazinamide and six vials of PZA supplement.

Reconstitute each BACTEC MGIT PZA kit drug with 2.5 ml of sterile distilled water to achieve the desired stock concentration (Table 4)

Table 4. Reconstitution volumes and final concentrations for first-line anti-TB agents

Drug lyophilised	Volume added	Concentration of reconstituted agent	Volume added to each MGIT tube	Final concentration in MGIT tubes
Isoniazid	4ml	8.3	100	0.1 µg/ml
Rifampicin	4ml	83	100	1.0 µg/ml
Ethambutol	4ml	415	100	5.0 µg/ml
Pyrazinamide	2.5ml	8000	100	100 µg/ml

The MGIT medium is complete only after the growth supplement has been added to the medium. If the Becton Dickson SIRE supplement is not available, then the BBL OADC growth supplement may be used (catalogue number: 245116, Cat No. GDF 106033) in the same volume as the SIRE supplement (i.e., 800 µl). For pyrazinamide DST, use pyrazinamide (PZA) DST supplement.

Table 7. Reconstitution volumes and final concentrations for lyophilize second-line anti-TB agents available form BD

Drug lyophilised	BD catalogue No.	GDF catalogue No.	µg/ vial	Volume added	Concentration of reconstituted agent µg/mL	Volume added to each MGIT tube	Final concentration in MGIT tubes
Moxifloxacin	215404	106585	249	3 ml Dilute	83 20.75	100 100	1.0 µg/ml (CB) 0.25 µg/ml
Levofloxacin	TBD	TBD	249	3 ml	83	100	1.0 µg/ml
Bedaquiline[a]	215406	TBD	170	2 ml	83	100	1.0 µg/ml
Delamanid	N/A	N/A	-	-	-	-	0.06 µg/ml
Amikacin	215350	106586	332	4ml	83	100	1.0 µg/ml
Streptomycin[b]	245123	106028	332	4ml	83	100	1.0 µg/ml
Linezolid	N/A	N/A	-	-	-	-	1.0 µg/ml
Clofazimine	N/A	N/A	-	-	-	-	1.0 µg/ml

[a] Bedaquiline not currently available. For bedaquiline only polystyrene or glass material should be used for the preparation of stock solutions, working solutions and the final plates or tubes used.

[b] Streptomycin catalogue numbers refer to the full SIRE kit

Table 5. Critical concentrations (CC) and clinical breakpoints (CB) for medicines recommended for the treatment of RR-TB and MDR-TB. (Interim CC are highlighted in red)

Group	Medicine	Abbreviation	Critical concentrations (µg/ml) for DST by medium			
			Löwen-stein Jensen[1]	Middle-brook 7H10[1]	Middle-brook 7H11[1]	BACTEC MGIT liquid culture[1]
Group A	Levofloxacin (CC)	LFX[2,3]	**2.0**	1.0	-	1.0
	Moxifloxacin (CC)	MFX[2,3]	**1.0**	0.5	0.5	0.25
	Moxifloxacin (CB)[4]			2.0	-	1.0
	Bedaquiline[5]	BDQ	-	-	**0.25**	**1.0**
	Linezolid[6]	LZD	-	1.0	1.0	1.0
Group B	Clofazimine	CFZ	-	-	-	**1.0**
	Cycloserine TerizidoneTerizidone	CS TZD	- -	- -	- -	- -
Group C	Ethambutol[7]	E	2.0	5.0	7.5	5.0
	Delamanid[8]	DLM	-	-	**0.016**	**0.06**
	Pyrazinamide[9]	PZA	-	-	-	100.0
	Imipenem-cilastatin Meropenem	IMP/CLN MPM	- -	- -	- -	- -
	Amikacin[10] (Or Streptomycin)	AMK (S)	30.0 4.0	2.0 2.0	- 2.0	1.0 1.0
	Ethionamide Prothionamide	ETO PTO	40.0 40.0	5.0 -	10.0 -	5.0 2.5
	Para-aminosalicylic acid	PAS	-	-	-	-

[1] The use of the indirect proportion method is recommended. Other methods using solid media (such as the resistance ratio or absolute concentration) have not been adequately validated for anti-TB agents.

[2] Testing of ofloxacin is not recommended as it is no longer used to treat DR-TB and laboratories should transition to testing the specific fluoroquinolones (levofloxacin and moxifloxacin) used in treatment regimens.

[3] Levofloxacin and moxifloxacin interim CCs for LJ established despite very limited data.

[4] Clinical breakpoint concentration (CB) for 7H10 and MGIT apply to high-dose moxifloxacin (i.e. 800 mg daily).

[5] No evidence is available on safety and effectiveness of BDQ beyond six months; individual patients who require prolonged use of BDQ will need to be managed according to 'off-label' best practices.

[6] Optimal duration of use of LZD is not established. Use for at least 6 months was shown to be highly effective, although toxicity may limit use for extended periods of time.

[7] DST not reliable and reproducible. DST is not recommended.

[8] The position of delamanid will be re-assessed once individual patient data by Otsuka are available for review. No evidence is available on effectiveness and safety of DLM beyond six months; individual patients who require prolonged use of DLM will need to be managed according to 'off-label' best practices.

[9] Pyrazinamide is only counted as an effective agent when DST results confirm susceptibility in a quality-assurred laboratory. Its use with BDQ may be synergistic.

[10] Amikacin and streptomycin are only to be considered if DST results confirm susceptibility and high-quality audiology monitoring for hearing loss can be ensured. Streptomycin is to be considered only if amikacin cannot be used and if DST results confirm susceptibility. Streptomycin resistance is not detectable with 2nd line molecular line probe assays).

Anti-TB agents and critical concentrations for testing

The concentrations of antimicrobial agents used in MGIT DST have been recently reviewed concentrations determined for certain second-line agents in MGIT media and the procedures used to prepare media containing these antimicrobials are given in Table 6. Only those second-line agents with established critical concentrations with reliable and reproducible results should be tested.

Table 6: Availability of pure powders from GDF and other manufacturers

Drug	Description and ingredients	Manufacturer Catalogue No.	GDF Catalogue No	Quantity	Storage
Levofloxacin	>98% HPLC	Sigma- Aldrich (28266-IG)	106560	1g	2-8C
Moxifloxacin	Pure substance	Sigma- Aldrich (32477-50MG)	106314a	50mg	RT
Bedaquiline	Bedaquiline fumarate 12 mg BDQ fumarate salt equivalent to 10 mg BDQ base	Available through free of charge: NIH AIDS Reagent Program (https://www.aidsreagent.org/register.cfm)	Not available	40 mg	2-8C
Linezolid	Pure substance ≥ 98% activity	(1) Sigma (PZ0014-5MG) (2) Caymen Chemical (CAS	Pending	5mg 10mg	RT RT
Clofazimine	Pure substance	Sigma-Aldrich (C8895-1G)	Pending	1.0g	2-8C
Delamanid	Pure substance	Available through: (1) ATCC BEI (https://www.beiresources.org/About/ BEIResources.aspx) Item number for delamanid: NR-51636 (2) Adooq Bioscience (http://adooq.com/delamanid.html) (A12864-10)	Not available	10 mg	2-8C
Amikacin	Amikacin disulfate salt potency: 674-786 µg per mg (as amikacin base)	Sigma-Aldrich (A1774-250MG)	106318b	250mg	2-8C
Streptomycin	Streptomycin sulphate. Potency ≥ 720 µg per mg (as streptomycin base)	Sigma- Aldrich (S6501-5G)	106311c	5.0g	2-8C

Critical concentrations for the new and repurposed anti-TB agents bedaquiline, delamanid, linezolid and clofazimine are included. In addition, revised consensus critical concentrations for fluoroquinolones and amikacin are presented.

Preparing solutions of anti-TB agents

I. Using lyophilized agents

A limited number of lyophilized anti-TB agents are available from the manufacturer. However, when these become available, the procedures given by the manufacturer should be followed, but it is important to ensure that the final test concentrations are the same as those recommended by WHO (Table 7).

II. Using pure drug powders:

Table 5 provides an overview of manufacturers providing the pure powders. In order to prepare and sterilize anti-TB agent solutions make the needed dilutions in such a way that when 0.1 ml (100 µl) is added to 7 ml of MGIT medium the desired test concentration is achieved based on the amount of the medium in millilitres. Guidelines on preparing the recommended critical concentrations are shown in Table 8.

Table 8. Concentrations and solutions needed to prepare second-line anti-TB agents for use with the BACTEC MGIT 960 system

Final concentration of anti-TB agent (µg/ml)	**Stock solution and solvent**	**Subsequent dilutions in sterile distilled water (SDW) (DMSO must be used for clofazimine and bedaquiline)**
Levofloxacin (1.0)	• Dissolve 10 mg LFX in 2.5 ml sterile 0.1 M NaOH[a] – 4000 µg/mL (Solution A) • Dilute 1 mL solution A to a final volume of 5 mL with SDW (Solution B) 800 µg/mL	Add 1.05 mL of solution C to 8.95 mL of SDW[b]. 84 µg/ml (Working solution)
Moxifloxacin (0.25) Critical concentration	• Dissolve 10 mg MFX in 5 ml sterile 0.1 M NaOH[a]. (2000 µg /mL (Solution A) • Dilute 1 mL solution A to a final volume of 10 mL with SDW (Solution B) 200 µg/mL	Add 1.05 mL of solution B to 8.95 mL of SDW[b]. 21 µg/ml (Working solution)
Moxifloxacin (1.0) Clinical breakpoint	• Dissolve 10 mg MFX in 2.5 ml sterile 0.1 M NaOH[a]. 4000 µg /mL (Solution A) • Dilute 1 mL solution A to a final volume of 5 mL with SDW (Solution B) 800 µg/ml	Add 1.05 mL of solution B to 8.95 mL of SDW[b]. 84 µg/ml (Working solution)
Bedaquiline[c] (1.0)	• Dissolve 12 mg BDQ fumarate salts (equivalent to 10 mg BDQ base) in 2.5 ml sterile DMSO. 4000 µg /mL (Solution A) • Dilute 1 mL solution A to final volume of 5 mL with DMSO (Solution B) 800 µg/mL	Add 1.05 mL of solution B to 8.95 mL of DMSO[d]. 84 µg/ml (Working solution)
Linezolid (1.0)	• Dissolve 10 mg LZD in 10 ml SDW. 1000 µg /mL (Solution A) • Dilute 4 mL of Solution A to a final volume of 5 mL with SDW. 800 µg/mL (Solution B)	Add 1.05 mL of solution B to 8.95 mL of SDW. 84 µg/mL (Working solution)
Clofazimine (1.0)	• Dissolve 10 mg clofazimine in 2.5 ml sterile DMSO (Solution A – 4000 µg/mL) • Dilute 1 ml Solution A to a final volume of 5 mL with DMSO – 800 µg/ml (Solution B)	Add 1.05 mL of solution B to 8.95 mL of DMSO[d] 84 µg/ml (Working solution)
Delamanid (0.06)	• Dissolve 10 mg DLM in 2.5 ml sterile DMSO. • Dilute with sterile water to final volume of 10 ml – 1,000 µg/ml (Solution A) • Dilute 1 mL solution A with 9 mL of SDW (Solution B) 100 µg /mL	Add 0.5 mL of solution B to 9.5 mL of SDW 5 µg/mL (Working solution)

Amikacin (1.0)	• Assume potency of 0.71 (varies between lots) • Dissolve 140 mg amikacin in 10 ml sterile distilled water -10,000 µg/ml (Solution A). • Dilute 1 mL solution A with 9 mL of sterile distilled water (Solution B) 1,000 µg/mL • Dilute 4 ml Solution B to a final volume of 5 mL with SDW (Solution C) 800 µg /mL	Add 1.05 mL of solution B to 8.95mL of SDW. 84 µg/ml (Working solution)
Streptomycin (1.0)	• Assume potency of 0.71 (varies between lots) • Dissolve 140 mg streptomycin in 10 ml sterile distilled water -10,000 µg/ml (Solution A). • Dilute 1 mL solution A with 9 mL of sterile distilled water (Solution B) 1,000 µg/mL • Dilute 4 ml Solution B to a final volume of 5 mL with SDW (Solution C) 800 µg/mL	Add 1.05 mL of solution B to 8.95 mL of SDW. 84 µg/ml (Working solution)

NaOH: sodium hydroxide.

[a] Use sterile distilled water.

[b] For 1M NaOH: dissolve 40 g NaOH in 1 litre of distilled or deionized water (or dissolve 4.0 g in 10 ml); dilute 1:10 to achieve 0.1M strength.

[c] For bedaquiline only polystyrene or glass material should be used for the preparation of stock solutions, working solutions and the final plates or tubes used.

[d] Use DMSO as the diluent for clofazimine and bedaquiline instead of distilled water is necessary to avoid precipitation of the drug

Note: The preparation scheme shown in Table 5 may be modified as needed. However, the final concentration of each anti-TB agent should not be altered.

For example:

Moxifloxacin: 1.0µg/ml

Potency: 1000.0 µg/ml

To obtain 10 mg of pure Moxifloxacin the equation is [$10mg \times \frac{1}{1.000} = 10mg$]

Prepare of Stock Solution:

- Dissolve 10 mg MFX in 2.5 ml sterile 0.1 M NaOH[a]. 4000 µg /mL (Solution A)

$$\frac{10\text{mg} \times 1000.0\mu\text{g/ml}}{2.5 \text{ ml Sterile } 0.1\text{M NaOH}} = 4000\mu\text{g/ml}$$

- Dilute 1 mL solution A to a final volume of 5 mL with SDW (Solution B) 800 µg/mL

[Note: Store 0.5 ml per cryotube. Stock solutions of agents may be kept at -70° C ± 10° C for up to 1 year (or until the original expiry date if that occurs before 1 year) without losing any significant activity. Agents stored at –20° C may be slightly less stable; at this temperature they may be stored for up to 6 months (only 3 months for bedaquiline) or until the original expiry date. It is better to store more concentrated solutions than to store diluted solutions.]

Preparation of Working Solution: 84µg/ml

- Add 1.05ml of Solution B to 8.95ml of Sterile Distilled Water to obtain 84µg/ml (Working Solution)
- Store 0.5 ml per cryotube

[Note: Working solutions of agents may be kept at -80° to -20° C for up to 6 months (or until the original expiry date if that occurs before 6 months)]

Weighing anti-TB agents, calculating their potency and storing the solutions

All antimicrobial agents are assayed for standard units of activity (potency); these may differ from agent to agent, and from lot to lot. Thus, a laboratory must standardize its antimicrobial solutions using the information provided by the manufacturer or supplier and the potency of the antimicrobial powder in the lots that are being used.

The antimicrobial powder should be weighed on an analytical balance that has been calibrated. It is advisable to accurately weigh the antimicrobial agent, and then to dissolve the compound in a small volume of appropriate diluent (sterile distilled water, DMSO or NaOH) prior to adjustment with sterile water (or DMSO for bedaquiline and clofazimine) needed to obtain the final concentration.

Solutions should be sterilized using a membrane filter (Note: filtration is not recommended for bedaquiline) (for example, cellulose nitrate or mixed cellulose ester containing nitrate and acetate) with a pore size of 0.22 µm. Paper, asbestos or sintered glass filters, which may absorb appreciable amounts of certain anti-TB agents, must not be used. The first 10–15% of the filtered solution must be discarded because initially some of the agents may be adsorbed into the filter.

Calculations

To calculate the amount of powder needed, first find the specific activity of the agent; then, incorporate the activity factor into the total amount weighed. For example, if the activity of an anti-TB agent were 740 µg/mg, the weighing factor would be: 1000 µg/740 µg = 1.35. Use one of the following formulae for weighing the quantity of an anti-TB agent in a given volume of diluent necessary to achieve the desired stock solution.

$$Weight(mg) = \frac{\text{Volume (ml)} \times \text{Concentration (µg/ml)}}{\text{Potency (µg/mg)}}$$

For an agent with a potency of 904 µg/mg requiring 415 µg/ml stock solution in 50 ml of diluent:

$$\frac{50 \times 415}{904} = 22.9\ mg\ of\ the\ agent$$

The actual procedure for making a stock solution of 415 µg/ml in 50 ml of diluent is shown below.

- In a 100 ml flask add 50 ml diluent. Weigh 22.9 mg of the agent using an analytical balance. Add the powder to the flask and mix well.
- Suppose that for some reason you weigh out a different amount of an agent, say 23.2 mg. In this case you would need to determine the volume of diluent required to have 415 µg/ml, using the formula shown below.

$$Volume(ml) = \frac{\text{Weight (mg)} \times \text{Potency (µg/mg)}}{\text{Concentration (µg/ml)}}$$

- For 23.2 mg of an agent with a potency of 904 µg/mg that needs 415 µg/ml of a stock solution: 23.2 × 904/415 = 50.5 ml diluent.

[Note: If lyophilized drugs are available from a commercial source for DST for second- line agents, follow the manufacturer's recommendations.]

Storing stock solutions of anti-TB agents

Small volumes of the sterile stock solutions of antimicrobials should be dispensed into sterile polypropylene or polyethylene vials appropriate for low-temperature storage, such as cryovials; the vials should be carefully sealed, labelled, dated and stored. Solutions of anti-TB agents should be frozen, preferably at –70°C±10°C. Freezing at –20° C is also acceptable.

Stability varies among agents at different temperatures. Most of the stock solutions of agents may be kept at 70°C±10°C for up to 1 year (or until the original expiry date if that occurs before 1 year) without losing any significant activity. Agents stored at –20° C may be slightly less stable; at this temperature they may be stored for up to 6 months (only 3 months for bedaquiline or until the original expiry date. It is better to store more concentrated solutions than to store diluted solutions.

Frozen solutions should be thawed to room temperature and used immediately. They must not be refrozen. Leftover quantities should be discarded.

Important considerations

- Store pure agents in a cool, dry place, preferably in a desiccator, and refrigerate.
- Use only the pure form of the agents. Impurities will cause unreliable results.
- Check the activity (potency) of an agent and calculate the total weight of the agent required by taking into account the actual active component of the agent. Calculations for the amount of an agent to be weighed may differ because the potency of agents varies from lot to lot.
- Weigh the agent (at least 2000 µg) extremely carefully, using a reliable and calibrated high- quality analytic balance appropriate for weighing small quantities.
- Mix the solution containing the agent extremely carefully and ensure that the agent is completely dissolved before filtering it or making further dilutions.
- All of the agents should be filter sterilized. Use a polycarbonate filter to minimise loss of the agent caused by absorption to the filter. If polycarbonate filters are not available and another kind must be used, discard the first 10-15% of the filtered solutions, and collect the remaining solution for use.
- If using a self-sterilizing solution or an aqueous stock solution, all further dilutions should be made in water, using sterile water and aseptic techniques.
- Add the exact amount of the stock solution to the medium.
- Keep track of the expiry date for each agent and solution. Do not use an agent after its expiry date.
- Record the weights used, and the calculations and dilutions made.

Calculating the dilution factor for the MGIT medium

The dilution factor for the MGIT medium is calculated as follows:

7.0 ml of medium + 0.8 ml of SIRE supplement + 0.5 ml of inoculum = 8.3 ml.

Add 0.1 ml of the solution with the anti-TB agent to 8.3 ml of the medium: this gives a 1:84 dilution of the working solution. This dilution factor should be considered when preparing a working solution of the anti-TB agent.

Adding an anti-TB agent to the medium

To prepare a set of MGIT tubes for DST, take the required number of MGIT tubes and label each tube with the relevant information that will identify the name and concentration of the antimicrobial agent being tested, and the patient. Aseptically add 0.1 ml (100 µl) of the properly dissolved and diluted solution of the agent being tested to each labelled MGIT tube. It is important to always add the test

solution 0.1 ml at a time, and to add the correct amount of each test solution to each tube. Any slight variation in the quantity added to the tubes may affect the results.

Use a well calibrated micropipette to make each addition to the tubes. The same micropipette tip may be used to add the same test solution to several MGIT tubes, but a separate pipette or micropipette tip should be used for each anti- TB agent being tested. Do not add the anti-TB agent solution to the growth control tube. After adding the solution, mix well.

Preparing the mycobacterial inoculum

The DST should be performed using only cultures that have been freshly grown in liquid media or solid media. The growth should not be contaminated by bacteria or mycobacteria other than *M. tuberculosis* (that is, there should not be any nontuberculous mycobacteria). The purity of the culture may be checked. If there is any suspicion of contamination, streak the test suspension on a blood agar plate. If blood agar is not available, use chocolate agar or brain-heart infusion agar. Incubate at 35 °C ± 1 °C for 48 hours, and check for growth. If growth appears, the suspension is contaminated, and the susceptibility test should not be performed. If there is no growth on the blood agar, and the inoculum suspension looks turbid test can be performed.

Rapid tests, both molecular and antigen- based, can be used for definitive differentiation of *M. tuberculosis* from nontuberculous mycobacteria It is important to establish the purity of the culture before performing DST, particularly if contamination is suspected.

Precautions: The handling of cultures, and additions and manipulations, should be done inside a BSC. WHO's recommendations for these procedures or established national guidelines should be followed. Use properly sterilized and quality-controlled reagents, and aseptic techniques throughout the DST procedure.

1. Using inoculum from the MGIT tube

It is important that a positive MGIT tube should be used to set up DST within the recommended time frame for positivity as described below.

- The day that an MGIT tube is positive as determined by the instrument is considered Day 0. Growth is not yet ready for DST.
- The tube should be incubated for at least 1 more day before being used for DST (it may be incubated in a different incubator at 37 °C±1 °C). Day 1 starts after the tube has been incubated for 1 day.
- A positive tube may be used for DST up to and including the fifth day after incubation (that is, on day 1, 2, 3, 4 or 5). A tube that has been positive for more than 5 days should be sub-cultured in a fresh MGIT tube that has been supplemented with MGIT 960 growth supplement; it should then be incubated in the instrument until it is positive. This tube may be used from day 1 until day 5 as described above.
- If growth occurs in a tube on day 1 or day 2, mix the tube well by shaking vigorously or vortexing for 2–3 minutes to break up any clumps. Leave the tube undisturbed for about 5–10 minutes to let larger clumps settle to the bottom. Carefully collect the supernatant (using a sterile transfer pipette placed just below the liquid surface in a clean sterile container and use undiluted to inoculate the tubes with medium containing the anti-TB agent. If growth occurs on day 3, 4 or 5, mix the tube well to break up the clumps, either by hand or with a vortex mixer.
- Leave the tube undisturbed for 5–10 minutes to let the larger clumps settle to the bottom. Note: For pyrazinamide leave the tube undisturbed for 15-20 minutes.
- Transfer (using a sterile transfer pipette placed just below the liquid surface) 1.0 ml of the supernatant to a clean sterile container containing 4.0 ml of sterile saline and mix contents well. Use this 1:5 dilution of the test culture to inoculate the test medium in tubes containing the anti-TB agent.

2. Using inoculum from growth on solid media

It is important to use fresh growth from a solid medium, such as a Lowenstein–Jensen slant; growth is considered fresh if it is used within 15 days of appearing on the medium. Inoculum made from older growth may result in unreliable results.

- Add 4 ml of sterile saline (0.85% sodium chloride) to a short glass bottle with capacity of 15ml with 2–3 mm glass beads (about 8–10 beads).
- Obtain a representative portion of growth by scraping off as many colonies as possible. Be careful not to scrape off any medium because residual medium will give false turbidity readings.
- Transfer the growth to the tube with the normal saline and glass beads. Tighten the cap and vortex the tube for 1-2 minutes to break up any clumps. If a vortex is not available, mix thoroughly by hand to homogenize the suspension. The turbidity of the suspension should be greater than the McFarland number 1.0 standard.
- Let the suspension stand undisturbed for 30 minutes to allow large clumps of bacteria to settle.
- Use a pipette to carefully transfer (aspirate liquid carefully from just below the liquid surface) the supernatant suspension to another sterile tube. Avoid transferring any growth that has settled to the bottom.
- Let this tube stand undisturbed for 15 minutes.
- Use a pipette to carefully remove the supernatant without disturbing the sediment; transfer the supernatant to another sterile tube. The turbidity of this suspension should be higher than the McFarland 0.5 turbidity standard.
- Adjust the turbidity of this suspension to the McFarland 0.5 standard by adding sterile saline and adjust by making a visual comparison. Do not reduce the turbidity below this standard. (See Annex B for information on preparing the McFarland standards.)
- Dilute the above suspension to 1:5 by adding 1.0 ml of the suspension to 4.0 ml of sterile saline. Mix well and use this as the inoculum for tubes with medium containing an anti-TB agent.

Precautions

- Make sure that the culture being tested is pure and free from mycobacterial or bacterial contamination. If there is any doubt, check by making an AFB smear and streaking a loop of the culture on a bacterial medium plate, such as blood agar. Mixed cultures of *M. tuberculosis* and other mycobacteria or contaminating bacteria will give either an X error or a false result showing resistance.
- Always use fresh cultures. Old cultures may have low viability and may give false susceptibility results or other erroneous results.
- The culture suspension should be well dispersed and homogeneous. Inoculum with large clumps may give erroneous results.
- Over-inoculation may give false resistance results or an X400 error.
- Insufficient inoculum may give false susceptibility results or an X200 error.

Inoculation and incubation

Label the number of tubes required based on the number and concentrations of agents being tested for each test culture. Label one tube as "growth control" (that is, without any anti-TB agent) and one for each concentration of each agent being tested.

Adding to and inoculating the MGIT medium

The procedure should be performed in the following manner.

- Aseptically add 0.8 ml of BACTEC 960 SIRE supplement or BBL OADC supplement to each of the MGIT tubes. Mix well.
- Aseptically add 0.1 ml (100 µl) of the anti- TB agent being tested to the appropriately labelled MGIT tube. Mix well.
- The last step should be the inoculation of the test culture. Aseptically add 0.5 ml of the well mixed suspension (inoculum) to each of the tubes containing the anti-TB agent. Do not add inoculum to the control tube.
- For the control, first dilute the test culture suspension to 1:100 by adding 0.1 ml of the test culture suspension to 10.0 ml of sterile saline.
- For PZA DST a 1:10 growth control is prepared by adding 0.5 ml of the test culture suspension to 4.5 ml of sterile saline.
- Mix well by inverting the tube 5–6 times or by using a vortex mixer. Add 0.5 ml of this diluted suspension to the control tube.
- If it is difficult to measure 0.1ml accurately, make two 10-fold dilutions by taking 0.5 ml of the suspension and adding it to 4.5 ml of sterile saline. Each 10-fold dilution should be mixed thoroughly.
- Tighten the caps on all tubes and mix the inoculated broth well by gently inverting the tube several times.

DST set carriers

Place the inoculated tubes in the appropriate carrier. The first tube in a carrier should always be the control tube (that is, the tube that does not contain an anti-TB agent).

Carriers for tubes used in the BACTEC MGIT 960 system are available in the configurations described below.

- 2 tubes: this allows testing of a single concentration of one agent; it comprises a tube for the anti-TB agent and a control tube.
- 3 tubes: this set allows testing of two agents or two different concentrations of the same agent along with a control tube.
- 4 tubes: this set allows testing of three agents or three different concentrations of the same agent along with a control tube.
- 5 tubes: this set allows testing of four agents or four different concentrations of the same agent along with a control tube.

Entering the DST set carrier into the MGIT instrument

The BACTEC MGIT 960 instrument maintains a constant temperature of 37° C ± 1° C inside the cabinet where the tubes are placed for incubation.

To run the susceptibility test, the barcode on the carrier is scanned and then the carrier is placed into the instrument using the entry feature for the susceptibility test. Ensure that the tubes are placed in the correct order in the carrier, following the manufacturer's instructions.

The software is programmed to interpret results for only those set carriers that have been previously entered into the instrument. Default settings normally utilized for testing of first-line anti-TB agents do not apply when testing second-line agents. However, there is an option to enter a carrier as containing an unknown agent or "Unspecified Drug". In this case, the instrument will monitor the

growth and GU values of all the tubes in a set. Once the GU of the control reaches 400 GU or more, the instrument terminates the test and flags it as completed but does not interpret the results. The results will need to be interpreted manually.

Alternatively, some users enter second-line sets as if they were the SIRE agents but code each tube to indicate which agent it actually contains; for example, instead of streptomycin, a tube may actually contain capreomycin or instead of isoniazid, another agent may be used. When the test has been completed, the instrument will provide a printout interpreting the results as if Streptomycin and isoniazid have been used but the user will need to decode the printout by inserting the correct name of the agent. This approach is not recommended owing to the risk of transcription errors at the time of coding or decoding the names of the agents.

Precautions

- There should be a continuous supply of electricity to the MGIT 960 instrument and printer. If there is a risk of interruption, a back-up electricity source should be able to automatically provide power.
- Voltage should be constant and should not fluctuate.

Duration of the test

When the DST set carrier is entered as an unknown agent, the instrument continuously monitors the test set. The protocol terminates once the control reaches 400 GU or more (within 4–13 days). Once the test has been completed, the instrument indicates that results are ready. The carrier is then removed, its barcode is scanned, and the report is printed. The report will show GU values for all of the tubes in the set but will not have interpretations for the results. The EPI Centre Microbiology Data Management System (Becton Dickson) may be useful for DST for second-line agents, including aiding in interpreting results and providing guidance on increasing the incubation period if necessary.

Interpreting and reporting results

Results are interpreted as described below.

- Resistant: a strain is described as resistant when the GU value of the control reaches 400 or more and the GU value of the tube with the agent being tested is 100 or more.
- Susceptible: A strain is described as susceptible when the GU value of the control reaches 400 or more and the GU value of the tube containing the agent being tested is less than 100.
- X errors: The X error message indicates that results are indeterminate when certain conditions occur that may affect the test such as:
    1. X400 error: this error occurs when the GU of the control reaches 400 or greater in less than 4 days. If this occurs, the instrument terminates testing. This error indicates that the test culture is contaminated or has been over inoculated. In such situations, the test should be repeated with pure, actively growing and properly diluted culture that has been confirmed to be *M. tuberculosis* Complex
    2. X200 error: this error occurs if the inoculum contains only a low number of viable organisms. In this case, the control will not have reached the required number of GU within 13 days; this indicates that the culture has been insufficiently inoculated. Since the duration of the protocol is set for 13 days, the instrument will terminate the test at that time. Ensure that fresh, actively growing culture is used, and that the suspension has been carefully prepared.

Important considerations

When the BACTEC MGIT 960 is first introduced to a laboratory, it is advisable to compare results from the MGIT 960 with those from another DST method that is already being used with either solid media or liquid media. It is also important when introducing a new test to ensure that the results of the test are reproducible.

Certain resistant strains grow very slowly in the MGIT medium, and the results may not be achieved within 13 days using standard inoculum. In such cases, in order to achieve reportable results, the amount of inoculum used should be increased by decreasing the dilution of the culture suspension (for example, by using without dilution MGIT tubes that are positive on day 2 or day 3, or by using tubes that are positive on day 4 or day 5 with a 1:2 dilution). When the interpretation is resistance, check the medium visually, and make sure that the test culture is not contaminated (look for turbidity, then put 1 drop of the medium on a blood agar plate or a nutrient agar plate). If the test culture is nontuberculous mycobacteria or is *M. tuberculosis* but it is mixed or contaminated with nontuberculous mycobacteria or other bacteria, then it may falsely be reported as resistant. Make a Ziehl–Neelsen smear and look for the clumps and cord formation typical of the TB complex.

If there are unexpected results or mono- resistance against a test agent it is best to check the purity of the inoculum, and repeat the DST, if necessary, to verify resistance. It is advisable to incorporate a rapid test for identifying *M. tuberculosis* complex. Some rapid tests are given results within 15 minutes (the lateral flow antigen test) to a few hours (molecular tests, specifically, LPA).

Results must be reported as soon as they are available. When results are reported, it is important to include the method used, the agent tested and its concentration. In general, results are reported as "S" (susceptible) or "R" (resistant). However, in some situations, such as when testing moxifloxacin, an agent is tested at different concentrations to establish the level of resistance. In such cases susceptibility may be reported at different test concentrations with the interpretation of S and a low level or high level of resistance.

Chapter 4: Biosafety and Waste Management

Biosafety and Biosecurity

Laboratory biosafety and biosecurity are fundamental biorisk management practices that should be employed in all biological research laboratories.

Introduction

Biosafety in the TB laboratory is complex and has several different components that all must be harmonized to achieve the maximum reduction in risk.

This section will include:

- Safe work practices
- Administration policies, and standard operating policies
- Good laboratory practices, both general and specific
- Safe sputum specimen collection
- Avoiding creation of aerosols through good microbiological technique
- Safety Equipment, understanding the reasons the equipment is in place and why good maintenance and certification is paramount
- Different levels of biosafety and requirements for each level
- The use of disinfectants
- Coping with accidents in the laboratory

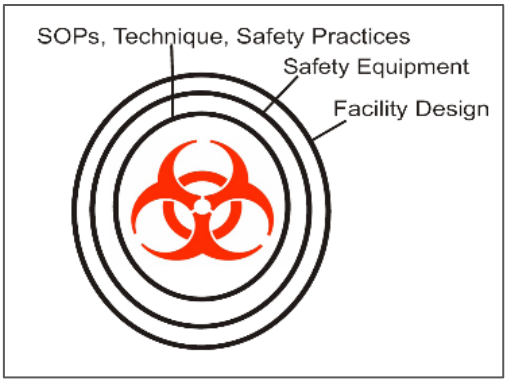
Safe work practices

Safe work practices are the result of implementing administration policies and standard operating procedures, incorporating and ensuring all staff adopts good laboratory practices (general practices for all laboratories) as well as good microbiological practices. Good microbiological practices incorporate certain techniques that assist in avoiding the creation of aerosols.

Special attention must be paid to ensuring safe sputum collection. Safe work practices also involve knowing and adhering to what types of work are appropriate for which areas, such as biosafety levels I, II, and III.

Disinfectants and modes of effective decontamination used in the TB laboratory are also critical to the health and safety of laboratory personnel and the public. Having a practiced spill procedure in place is critical to safe work practices. In regard to biosafety there are three lines of defence in a TB laboratory. Policies need to be put into place that will address all three lines of defence.

In considering the safety risk involved in working with Tuberculosis it is helpful to visualize the infectious agent (M. tuberculosis) with three separate lines of defence. If these are in place and well defined, they can all work in synergy to provide a safer environment for processing specimens, culturing, and manipulating cultures of M. tuberculosis.



- The first level and the one that laboratorians have the most control over is standard operating procedures, techniques used for processing specimens and manipulating cultures, and the safety practices that are employed in everyday work by all laboratory personnel working in the TB lab.
- The second level is safety equipment, such as safety centrifuges and biosafety cabinets, used in the laboratory. Maintaining and annually certifying this
- safety equipment is just as crucial, if not more important than simply having it in the laboratory. If the equipment is not operating properly and providing the expected protection, then the laboratory staff is working under a false sense of security.
- Facility design is the third level, and this involves the placement of the TB laboratory amongst other laboratories in the facility, equipment placement within the TB laboratory, and

incorporating negatives air pressure which works to direct the flow of air in the TB laboratory in order to contain and remove aerosolized tubercule bacilli from the laboratory.

These three levels all work together to protect the laboratory staff, the community and the environment.

Risk

As with every aspect of life, risks can never be eliminated. Every morning we choose to leave home to go to work. We take precautions to ensure that we make it to work safely and reduce our risk, such as wearing a seat belt or a cycling helmet, being aware of our surroundings and trying to take the best route. All these things reduce our risk of being in a serious accident while we are on our way to work, however we all know that accidents could happen at any moment. The same theory applies to working in a TB laboratory. Even though we take precautions to limit our risk, NOTHING can eliminate the safety risk associated with the TB laboratory. But the good news is that good laboratory practices with an emphasis on biosafety can significantly reduce the risk of laboratory-acquired infection. Specialized equipment aids good laboratory practice but does NOT replace it. For example, the fact that a laboratory might have and employ the use of a biosafety cabinet is not an excuse for the laboratory workers to use sloppy microbiological technique.

Laboratory Practices

It is important to adhere to good laboratory practices. In TB lab general laboratory practices apply as well as specialized practices specific for aerosol control.

- Access to Lab must be limited
- No eating, drinking, smoking, or handling of contact lenses or cell phones
- Always wear close toed shoes
- No mouth pipetting, no placing pencils or pens in the mouth
- Institute policies for the safe handling and disposal of sharps
- Hand washing required
- Lab coats are decontaminated and laundered regularly
- Remove gloves before handling laboratory phones, instruments or computers
- A biohazard sign should be posted at the entrance to laboratories performing work on infectious agents.
- All procedures must be performed carefully to minimize the creation of splashes or aerosols.
- Work surfaces should be decontaminated upon completion of each task and after any spill of viable material.
- All cultures, stocks, and other regulated wastes must be decontaminated before disposal by an approved decontamination method such as autoclaving or incineration to ash.

Job Hazard Analysis

- Perform an analysis of hazards for potentially infectious activities performed in the laboratory from specimen collection to specimen processing to culture manipulation.

Safe collection of sputum specimens

- To implement safe sputum specimen collection, one must understand how TB transmission and infection occurs.
- TB patient expels bacilli in small droplets of respiratory secretions (aerosols)
- Secretions quickly evaporate leaving "droplet nuclei" in the air
- Droplet nuclei containing 1-3 bacilli can remain suspended in the air almost indefinitely (This is why directional air flow is so important!)
- Following inhalation, droplet nuclei carrying TB are able to penetrate deep into the lungs
- TB becomes established in susceptible hosts

Avoiding the creation of aerosols in the TB laboratory

The primary risk to TB laboratorians are infectious aerosols containing *M. tuberculosis*. There are several precautions laboratorians can take to avoid the creation of aerosols. The potential for aerosol generation is present when both liquid and TB are involved. Aerosols can be produced by the following activities:

- Pipetting
- Mixing
- Shaking
- Centrifugation
- Grinding
- Vortexing
- Pouring
- Use of squeeze bottles during processing
- Flaming loops or wet slides
- Opening tubes at non-ambient temperatures or pressures

Technologists need to be aware of the processes that can create aerosols so that extra caution can be applied, and steps can be taken to minimize their production.

If proper precautions are not taken or equipment is not used or maintained properly then potential for risk is elevated. These could include:

- Deficiencies in personal protective equipment (PPE) allow aerosols and organisms better access to laboratory personnel.
- This could be when PPE is not available, the available PPE is not effective, or PPE is not used properly.
- Safety equipment may not be in place or not used properly to contain the aerosols produced in the lab.
- Examples could include using centrifuges that do not have sealed buckets or are not disinfected regularly, using safety equipment that is not maintained or certified properly, or if an autoclave or incinerator is not available and waste is disposed of improperly.
- Laboratory facility not designed to remove suspended droplet nuclei.
- No directional air flow in the laboratory.
- Biohazard spills not handled correctly.
- This hazard is increased when laboratory personnel are not trained on how to cope with a biohazard spill. Accidents DO happen. We can not always control these occurrences. But what we can control is how we react when accidents do happen and how we prepare for it to happen.

When performing TB specimen and culture work in a BSL 2 or 3 laboratory keep the following points in mind to minimize the production of aerosols:

- Always open and manipulate cultures in a certified biosafety cabinet.
- Use disposable loops or incinerators to sterilize loops
- Work over absorbent material soaked in disinfectant
- Use centrifuge safety cups and open them in the BSC
- Never express all the liquid in the transfer pipette (leave the last bit inside the tip)
- Use caution when disrupting bubbles in tubes of liquid media
- Never vortex an open tube, always ensure that they are securely capped

Safety Equipment

The bio-safety cabinet (BSC) functions to keep the operator safe from aerosols by controlling the airflow into and within the cabinet. It directs the flow of air inside the cabinet to prevent the aerosols from escaping from the BSC. Microbes, aerosols and other small particles are drawn through the grates at the front and back and are trapped in a high efficiency particulate air (HEPA) filter. The cabinet then exhausts the filtered air.

- A biosafety cabinet is NECESSARY for processing specimens, opening cultures and inoculating drug susceptibility testing (DST) and anytime when high concentrations of TB are being handled.
- The following work should be performed in a BSC:
    - Homogenisation and decontamination of specimens
    - Opening centrifuge cups
    - Decanting processed specimens
    - Inoculating media
    - Inoculating and adding reagents to biochemical test media
    - Preparing smears from cultures
    - Diagnostic test procedures
    - Inoculation of drug susceptibility testing media
- AFB direct smear preparation does not require a BSC only if specimen is deactivated before smear preparation.
- Centrifuges used to process TB specimens must have safety cups that are correctly used
- Safety cups protect laboratory staff by providing containment of any aerosols that are produced during centrifugation, especially if tubes break.
- Safety cups must be opened inside the BSC
- Safety cups have a silicone coated rubber O-ring that provides an airtight seal
- These seals must be routinely coated with silicone so seals will remain air-tight (after washing and decontamination)

Personal protective equipment is the safety equipment that each individual laboratorian wears to protect herself/himself. In a TB laboratory this equipment includes:

- Respirators
- Gloves
- Lab Coats/Gowns
- Safety goggles

Summary points

- TB can be transmitted by aerosols emitted by infected persons or generated in the laboratory
- The three inter-related levels of biosafety
    - Good laboratory practice
    - Safety equipment
    - Facility design and layout
- Job hazard analysis performed
- Safety committee as advocate for staff safety
- BSC and other equipment must be used properly to ensure safety
    - Equipment checked annually for electrical safety
- PPE must be worn properly to be effective
    - Fit test staff for N95 respirators
- Level 2 PPE differs from Level 3 PPE
- Ensure all waste is decontaminated (autoclaved) before disposal and all surfaces are chemically decontaminated daily
- Ensure spill kit is on hand and ALL personnel who have access to the laboratory are trained to react in the event of a spill
- Ensure all chemicals are handled and stored properly

SAFETY

- **Skills**: Possessing the skills, technique, and knowledge to work safely in a TB culture laboratory, by making every effort to minimize aerosol production and use equipment to contain aerosols that are produced.
- **Accountability**: Now that we possess the knowledge and the skills to work safely, we must hold ourselves and each other accountable for following the safety guideline and practices of our laboratories.

OR

- **Advocacy:** We must be advocates for biosafety in our own laboratories. We need to request that proper safety equipment is available and certified for TB laboratory work.
- **Facility**: Should be designed to keep traffic away from areas with infectious work practices and directional air flow should be created to remove and dilute droplet nuclei that become suspended in the air.
- **Equipment**: In a TB culture laboratory, basic safety equipment is a must, such as bio-safety cabinets and safety centrifuges
- **Training**: All new employees and laboratory staff from laboratory managers to cleaning staff need to have trained on the risks and dangers involved in working in the TB laboratory. All current employees need to have skills refreshed once a year
- **YOU**: You are the key to having safer laboratories. You have the choice to work safely. If safety equipment is available but not used properly it will not provide the intended protection. **Do not forget that the careful and cautious technique that we decide to exercise while handling TB is the most effective safety measure available.** The protection available to all of us in the TB laboratory begins with you.

Waste-management

Waste-management procedures must comply with all pertinent local or national requirements and regulations. Waste is anything that is to be discarded. The overriding principle in minimizing risks from waste is that all infectious materials should be decontaminated, incinerated, prepared to be buried or autoclaved. Discard bags should be used to segregate waste. Most glassware, instruments and laboratory clothing will be reused or recycled.

The principal questions to be asked before any objects or materials are removed from a laboratory are:

- Have the objects or materials been effectively decontaminated or disinfected using proper procedures?
- If not, have they been packaged in a closed container or bag for immediate on-site incineration or autoclaving?
- Does the disposal of the decontaminated material involve any additional potential hazards or risks, biological or otherwise, to those who carry out the disposal procedures or who might come into contact with the items outside the facility?

Incineration is useful for disposing of laboratory waste, regardless of whether it has been decontaminated. Incinerating infectious materials is an alternative to autoclaving only if the laboratory manager can ensure that proper incineration procedures are followed.

Incineration

To incinerate hazardous waste properly requires an efficient means of controlling the temperature, and a secondary burning chamber. Many incinerators, especially those with a single combustion chamber, are unsatisfactory for dealing with infectious materials or plastics. If this type is used, such materials may not be completely destroyed, and the effluent from the chimney may pollute the atmosphere with microorganisms, toxic chemicals and smoke. However, there are many satisfactory configurations for combustion chambers. Ideally the temperature in the primary chamber should be at least 800°C, and in the secondary chamber at least 1000°C. In order to achieve the required temperatures, the incinerators must be properly designed, operated and maintained.

Materials for incineration, even if they have been decontaminated, should be transported to the incinerator in bags, preferably plastic. Attendants should receive proper instruction in loading the incinerator and controlling the temperature. The efficient operation of an incinerator depends on having the right mix of materials in the waste being incinerated.

There are concerns about the possible negative environmental effects of incinerators, and efforts continue to make incinerators more environmentally friendly and energy efficient. Autoclaves provide an alternative to incineration.

Autoclaving

Separate autoclaves should be used to sterilize solutions or glassware (clean materials), and to decontaminate infectious materials.

The following materials are suitable for autoclaving:

- instruments, glassware, media or solutions for sterile use in the general diagnostic TB laboratory
- mycobacterial cultures for waste disposal
- all infectious materials from TB-containment laboratories where mycobacterial culture is performed.

The time, temperature and pressure should be recorded each time the autoclave is run to monitor whether it is functioning properly. Biological indicators should be used regularly to validate the ability of the autoclave to achieve sterilization.

Disinfection

The killing action of disinfectants depends on the population of organisms to be killed, the concentration used, the duration of contact, and the presence of organic debris.

Proprietary disinfectants recommended as suitable for use in TB laboratories are those containing phenols, chlorine or alcohol. These are usually selected depending on the material to be disinfected.

- **Phenol**

Phenol should be used at a concentration of 5% in water. However, inhalation and dermal exposure to phenol is highly irritating to the skin, eyes and mucous membranes. Ingestion of phenol is considered to be toxic. Because of its toxicity and odor, phenol derivatives are generally used in place of phenol.

Phenol solutions are used for decontaminating equipment and single-use items prior to disposal.

- **Chlorine**

Chlorine is widely available. Sodium hypochlorite solutions (domestic bleach) contain 50 g/l available chlorine and should therefore be diluted to 1:50 or 1:10 in water to obtain final concentrations of 1 g/l or 5 g/l. Bleach, either in stock or in solution, must be stored in a well ventilated, dark area. In good storage conditions, the 50g/l solution may last as long as 3 months; diluted solutions should be prepared daily.

Bleach can be used as a general-purpose disinfectant and for soaking contaminated metal-free materials; because it is highly alkaline, it can corrode metal.

- **Alcohol**

Alcohols, ethanol (denatured ethanol, methylated spirits) or isopropyl alcohol are used at a 70% solution. Alcohols are volatile and flammable and must not be used near open flames. Solutions should be stored in proper containers to avoid evaporation. Bottles with alcohol-containing solutions must be clearly labelled so they are not autoclaved.

A solution of 70% alcohol can be used on laboratory benches and BSCs for routine decontamination. A major advantage of aqueous solutions of alcohols is that they do not leave any residue on treated items. When hands become contaminated, a rinse with 70% ethanol or isopropyl alcohol followed by thorough washing with soap and water is effective.

- **Peracetic acid**

Peracetic acid is characterized by rapid action against all microorganisms. Special advantages of peracetic acid are that it lacks harmful decomposition products, enhances removal of organic material, and leaves no residue. Working solutions (2% concentration) are stable for 48 hours after preparation.

Disposal procedures for contaminated materials

A system for identifying and separating infectious materials and their containers should be adopted. Categories may include:

- uncontaminated (non-infectious) waste that can be reused, recycled or disposed of in the same way as general household waste
- contaminated (infectious) sharps, such as broken glass, syringes and slides
- contaminated infectious material to be disposed of by burying, incinerating or autoclaving.

1. Broken glass and glass slides

Broken slides and used slides must be disposed of in a sharps container. Containers for sharps disposal must be puncture-proof, have a fitted lid, and must not be filled to capacity. When they are three quarters full, they should be placed in containers for infectious waste and incinerated. Containers for sharps disposal must not be discarded in a landfill unless they have been incinerated or autoclaved. Used slides must not be reused.

2. Contaminated or potentially infectious materials for disposal

All positive TB cultures must be autoclaved before disposal. An autoclave should be available close to or in the laboratory where TB culture is performed. All contaminated (that is, potentially infectious) materials except sharps should be placed in disposable plastic bags before being transported for

incineration. If possible, materials from TB laboratories should not be discarded in a landfill even after decontamination.

Discard containers, or pans or jars that are unbreakable (for example, plastic), should be placed at every workstation. Appropriate disinfectants effective against *M. tuberculosis* must be used; waste materials must remain in contact with the disinfectant (that is, they must not be protected by air bubbles) for the appropriate time, depending on the disinfectant used. Discard containers should be decontaminated and washed before reuse.

In laboratories where the risk of infection with TB is low, plastic sputum containers, cartridges used for molecular analysis (e.g., Xpert MTB/RIF cartridges), and wooden applicator sticks should be removed from the laboratory in sealed disposal bags and incinerated.

Chapter 5: Quality Assurance

Quality Assurance

Quality Assurance is planned and systematic activities to provide confidence that an organization fulfils requirements for quality. A comprehensive and systematic QA programme should be implemented to enable laboratories to achieve and maintain high levels of accuracy and proficiency in testing, to ensure the reliability and reproducibility of results, and thus to inspire confidence in clinicians and patients who are users of the laboratory's services

Key QA activities:

a. Training and competence assessment

b. Instrument verification

c. Equipment maintenance

d. Method validation

e. Quality control (QC)

f. Lot testing (also known as incoming quality control or new batch testing)

g. External quality assessment (EQA)

h. Quality indicator monitoring

i. Continuous quality improvement (QI)

Quality control

It is important to perform quality control of DST periodically. The best practice is to run a quality control strain with each batch being tested. *M. tuberculosis* H37Rv can be used for quality control because it is susceptible to all anti-TB agents. It can be useful to include testing a resistant strain in each batch even if the strain is highly resistant that may allow for the detection major errors in the preparation of drug stock solutions.

Quality control testing

Quality control strain: *M. tuberculosis* H37Rv (American Type Culture Collection 27294) is recommended for use in quality control testing.

If the American Type Culture Collection reference strain cannot be obtained, a well characterized strain derived from a patient's isolate that is completely susceptible to first-line anti-TB agents may be used instead. It is preferable to use a strain that has been fully sequenced and shown to have a wild-type pattern for all known genes associated with TB drug resistance.

After a standardized suspension has been made, it should be frozen at -70° C ±10° C. It may be stored for up to 1 year with no significant decrease in viable counts.

1. Preparing the culture suspension

- Subculture the reference strain on several Lowenstein–Jensen slants.
- Incubate the slants at 37° C ± 1° C.
- Observe growth visually. Growth should appear within 10-15 days of sub-culturing. As soon as there is good, confluent, and pure growth, use it to make the suspension. The growth should be used as soon as possible (that is, within the 10-15 days). Older cultures do not give reliable results.
- Remove growth from the slant by carefully scraping the colonies from it with a sterile loop or a sterile spatula made from wooden applicator sticks. Be extremely careful not to scrape off any culture medium, which will give false measurements of turbidity.
- Transfer the growth into Tube A, a sterile glass tube with a screw cap that contains 4ml of sterile Middlebrook 7H9 broth and 1-2 mm glass beads (about 6-10 beads); the glass beads will help to break up any clumps. Sterile normal saline or sterile deionized water may also be used.

- Vortex the tube for 1-2 minutes. Make sure the suspension is well dispersed and has turbidity greater than the McFarland number 1 standard.
- Let the suspension stand undisturbed for 30 minutes.
- Using a pipette, carefully transfer the supernatant to Tube B, another sterile glass tube with a screw cap. Avoid transferring any sediment. This suspension should have a higher turbidity than the McFarland number 1 standard.
- Let Tube B stand undisturbed for 15 minutes.
- Carefully transfer the supernatant from Tube B into Tube C, another sterile glass tube with a screw cap; do not transfer any sediment.
- Visually adjust the turbidity of the suspension in Tube C to McFarland Turbidity Standard number 1.0 or 0.5, whichever is needed, by adding Middlebrook 7H9 broth, sterile saline, or deionized water; mix well. If the suspension is too turbid, transfer some of the suspension to another sterile tube and adjust the turbidity. This will be the working suspension for quality control testing. This suspension may be frozen in small aliquots (1-2 ml) in appropriate tubes or vials at –70° C ± 10° C. The frozen suspension may be used for up to 1 year. Once thawed, it should not be refrozen.

2. Validating a test procedure

Whenever a new procedure is introduced to a laboratory – such as performing DST using liquid medium for first-line or second-line anti- TB agents – it is critical to establish that the new procedure gives reliable results; it is also critical to establish that the results are in concordance with results obtained using another method that has already been well established and thoroughly tested in the laboratory. The procedure described below is recommended for validation testing.

2.1 Procedure

2.1.1 Comparing a new method with an established method:

- Select 10–15 cultures, one which should be H37Rv. Cultures should be selected from clinical isolates, some of which are completely susceptible to all anti-TB agents and some of which are known to be resistant to the second- line agents being tested.
- If cultures from the supranational regional laboratory used in the most recent round are available, use these instead of laboratory isolates because these cultures will have been tested by several laboratories, and judicial results will already have been established and be available.
- If information on resistance to second-line agents is not available, select cultures from patients whose disease was resistant to isoniazid, rifampicin, and perhaps other first-line and second-line agents.
- Prepare fresh subcultures as described earlier. Following the recommended standard techniques for DST, use the selected cultures to validate the testing of the second-line agents. Test the cultures using the new procedure as well as with the laboratory's established DST procedure for second-line agents.
- The tests using the reference method and the new method should be set up at the same time using the same culture suspension.
- The concentrations of the agents used in the tests should be those that are recommended for each procedure; test concentrations may vary according to which procedure is used.

2.1.2 Expected results

- If you are comparing DST results from two methods and the results are found to be discordant, the test should be repeated using both methods simultaneously.
- There should be more than 90% agreement (preferably 95% agreement) between the results obtained using the new method and results obtained using the established method (that is, the reference method). The quality control strain (H37Rv) should be tested along with each batch of samples and should show complete susceptibility to all agents being tested.
- If a laboratory does not have an established reference method, then validation may be performed by comparing results from the new test with results from previous DST obtained

from other sources for the same agent. In this case, rigorous testing for reproducibility may be an option; the procedure for reproducibility testing is described in the next section.

- DST results should be able to clearly differentiate between susceptible and resistant strains and should help physicians prescribe more effective anti-TB therapy.

3. Reproducibility testing

It is important to establish that the results obtained by testing with a new procedure are reproducible – that is, the same result will be reported if the test is repeated several times. This kind of testing establishes the reproducibility of a test procedure as performed by a specific technician. If more than one technician in a laboratory performs DST, it is important to establish that there is no variation in the techniques used by the different technicians.

3.1 Procedure

- Select about 5-7 cultures, one of which should be H37Rv, 2-3 of which should be susceptible and 2-3 of which should be resistant to the agents being tested.
- Set up susceptibility testing at least five times on all the cultures.
- Repeat testing should be done on different days.
- The standard procedures outlined in this manual should be followed for DST for all cultures, that is, testing should be performed on all cultures in one batch under the same conditions and using the same procedures for all tests.
- Standardized frozen suspensions may be used for repeat testing, but it is preferable to make a suspension of fresh culture each time so that the reproducibility of suspension preparation is also assessed. For DST using liquid media, fresh growth in MGIT medium should be used as described.

3.2 Expected results

- Results from all repeated tests of a culture should be in complete agreement; if they are, then the procedures are reproducible in the laboratory that ran the tests.
- If there is any discrepancy in the repeat testing, then there are variations in the procedures, and corrective measures should be taken.
- If DST is performed by more than one technician in a laboratory, reproducibility among technicians must be evaluated with the target of achieving complete agreement of results.

The following steps are required to maintain Quality control

1. Laboratory QC Schedule

Table: Laboratory QC Schedule

Daily or with each patient run	Weekly or with each patient run	Monthly	New lot # or batch #
Refrigerator, freezer, incubator, rooms, and centrifuge temperatures	Positive and negative controls for MGIT and LJ cultures	Internal quality assessment to improve microscopy results	Positive and negative controls for new staining reagents for AFB smears
Positive and negative controls for AFB smears	Reference strain for drug susceptibility testing	MGIT Time to detection QC for MTB reference strain	Sterility and performance testing of culture media – MGIT, LJ, 7H9 Broth, BAP
MGIT Maintenance (See Section 10: Liquid Culture – MGIT)	Positive, negative, and reagent controls for identification kits	Complete Monthly Data Monitors form	Reference strain testing for new lots of MGIT SIRE, PZA kits and other anti-TB drugs or drug kits
		MGIT QC Report (See Section 10: Liquid Culture – MGIT)	Positive, negative, and reagent controls for new lots of identification kits

2. Quality Control Activities

2.1 Media QC

2.1.1. McFarland Turbidity Standards

Many QC procedures involve preparing dilutions from a standardized suspension of organisms using a turbidity standard, so that the number of bacteria present will be within a given range. According to CLSI guideline M24-A, each McFarland turbidity standard is equivalent to the turbidity of a bacterial concentration.

For example,

- McFarland No. 0.5 turbidity standard is equal to the turbidity of approximately 1.5 x 108 CFU/ml
- McFarland No 1.0 standard equals approximately 3 x108 CFU/ml

The standard is compared visually or using a densitometer/spectrophotometer to a suspension of bacteria in sterile 7H9 broth and adjusted by adding more broth if too heavy, or more bacteria if too light. However, the latter must be done after making a single cell suspension, i.e., breaking up clumps with beads, allowing clumps to settle, and transferring the supernatant to a new tube. An inoculum that is too heavy or too light will adversely affect the results, so it is important to adjust inoculums as closely as possible to the standard.

2.2.1. Laboratory-Prepared Media

Lab-prepared media must be thoroughly quality controlled. It is recommended to make small batches of media (e.g., every four weeks or more frequently) to ensure freshness and quality. 1-3% of each new batch of medium must be incubated to test for sterility and 1-3% must be tested for performance characteristics (ability to support a certain amount of growth in a specified time of incubation). Baseline

criteria for optimal growth with standardized inoculum and incubation periods should be established from results of several well-prepared batches.

The time to detection of growth, and number and size of colonies within a specified time of incubation should be critically evaluated. If a newly prepared batch of medium, when tested, yields result outside the established range, it must be considered unsatisfactory. Colony counts 20% above or below the established range may be considered unacceptable; however, it is preferable to establish lab-specific criteria (that is, determine mean CFU and standard deviation of several well-prepared batches).

Required procedures for Sterility and Performance QC of each media type are detailed below. Media can be released for routine laboratory use after passing all QC checks for sterility and performance.

12.5.1.1. QC Protocol for LJ Medium

Sterility Check

Frequency: Each new batch of prepared medium.

Controls:

- 1-3% of LJ tubes from a batch (for example: for a batch of 100 tubes, select 2 tubes) b. Incubate for 14 days at 37°C (±1°C).
- Acceptable Results: No growth on any tube. Visual inspection confirms proper color, texture, and homogeneity of medium.

Corrective Actions:

- If all tubes are contaminated, notify supervisor immediately, discard entire batch, and prepare new media.
- I f one tube is contaminated, repeat exercise with at least 10 additional tubes.
- If >1 tube is contaminated upon repeat testing, notify supervisor immediately and discard entire batch.
- Investigate and resolve all problems, and then prepare new media.

Documentation: Record results on the Reagent/Media QC form – Appendix E. If contamination is seen, prepare an Appendix K form to document the corrective action.

Performance QC

Frequency: each new batch of prepared medium.

- Controls: 1-3% of LJ tubes from a batch, tested with 10^{-2}, 10^{-3} and 10^{-4} dilutions of M. tuberculosis (H37Rv or H37Ra) in 7H9 broth (e.g., testing 2% of a batch of 100 tubes of media would include 6 tubes in total, 2 tubes inoculated with each of 3 working dilutions). See procedure below.
- Acceptable Results: Growth on all tubes is consistent with M. tuberculosis and within 20% of the laboratory's own established reference ranges for each dilution.

Corrective Actions:

- If tubes show no growth, notify supervisor immediately, discard entire batch, and prepare new media.
- If growth still not in acceptable range after repeat testing, notify supervisor immediately and discard entire batch.
- Investigate and resolve problems, then prepare new media.
- If colony counts are lower than the acceptable range, check the preparation of the MTB suspension, especially if prepared from a frozen stock. Loss of viability is a consideration when freezing low concentrations of MTB.
- Documentation: Record results on the Reagent/Media QC form – Appendix E. If QC results are not acceptable, prepare an Appendix K form to document the corrective action.

Preparation of MTB Culture Suspension and Working Dilution

- Subculture a M. tuberculosis QC strain (H37Rv or H37Ra) onto several LJ slants.
- Incubate the tubes at 37°C (±1°C) and observe growth visually.
- U se colonies showing good, confluent, and pure growth within 10-15 days of first appearance. Old cultures do not give reliable results.
- Remove growth from the slant by carefully scraping the colonies off the slant with a sterile loop or sterile wooden applicator stick. 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 6-10 glass beads (2 mm diameter), and vortex well.
- Let tube sit for 30 minutes for clumped organisms to settle.
- Transfer the supernatant fluid to another sterile tube (avoid transferring any of the sediment) and let the suspension stand for another 15 minutes.
- Transfer the supernatant to a new tube and adjust turbidity of this suspension to a McFarland No.1 standard using 7H9 broth.
- Prepare 3 serial 10-fold dilutions of the adjusted solution (10^{-2}, 10^{-3}, and 10^{-4}) in 7H9 broth.
- Prepare 20-50 cryotubes for each dilution. Label with strain type, dilution, and expiration date (six months after preparation).
- Aliquot 1.5 ml of each dilution to the appropriate cryotubes and freeze at –70°C (±10°C), up to six months.

Inoculation and Incubation

- Remove one aliquot of each dilution (10^{-2}, 10^{-3} and 10^{-4}) from the freezer.
- While thawing, bring LJ medium to room temperature.
- With a micropipettor and sterile aerosol resistant tips (ART) tips, mix the working solution with the pipettor 2-3 times to ensure even distribution of MTB.
- Inoculate LJ tubes with 200 µl of each dilution.
- Incubate at 37°C (±1°C), reading weekly for 21-30 days.

12.5.1.2. Commercially Prepared Media

Commercial media are thoroughly tested by the manufacturer and require either less QC testing or are categorized as generally "exempt" from user quality control according to the US Clinical and Laboratory Standards Institute (CLSI).

Recommendations for New Shipments of Media

Upon receipt of each new media shipment, check the following:

- Manufacturer's QC records are provided - these records must be retained with the lab's QC records.
- Expiration dates - notify the vendor of recurring short expiry dates.
- Tubes or plates are not damaged or cracked.
- The medium is not contaminated or changed in its appearance, e.g., color change with LJ media, blood agar haemolyzed, etc.
- Agar is not detached from the sides of the tube/plate, frozen or softened.
- Tubes/plates are sufficiently and equally filled.
- Media is not excessively moist or dehydrated.
- If any problems are found, notify supervisor, contact vendor immediately, and withhold media from patient use until issues are resolved.

QC of Commercial Media Inventory

It is important to monitor the performance of commercial media closely during use, and if necessary, perform complete quality control to ensure the recovery of isolates is satisfactory and as expected.

- QC of Commercial LJ Media
  Commercial LJ media has been demonstrated to deteriorate over time, and its ability to support growth can be compromised. Therefore, commercially sourced LJ media that has been stored in the lab ≥6 weeks should be tested according to the sterility/performance checks.
- QC of Commercial MGIT Medium
  Becton Dickinson (BD), the manufacturer of MGIT reagents, recommends QC testing of BACTEC MGIT medium and MGIT 960 Growth Supplement upon receipt (before putting into routine use), to ensure that the performance characteristics of the medium, once supplemented with the OADC/PANTA mixture, are acceptable. At a minimum, the Sponsor requires the QC procedure outlined below for new lots/shipments of MGIT culture medium using an M. tuberculosis strain. Refer to the MGIT culture media package insert for further instructions on QC procedures with non-tuberculous mycobacterial strains.

QC Protocol for BACTEC MGIT 960 Culture Medium and MGIT 960 Growth Supplement Kit

- Frequency: Each new lot or new shipment of 960 media or growth supplement.
- Controls: Dilutions of M. tuberculosis (H37Rv or H37Ra) in 7H9 broth.
- Acceptable results: MGIT tube will fluoresce positive (TTD) in 6 to 10 days
- Corrective actions:
  - If the TTD is not within the specified range, repeat the test.
  - If test still does not give satisfactory results upon repeat, notify supervisor immediately, and check the viability of the inoculum, age of the culture, if stored frozen, and other procedures.
  - If all procedures are within established specifications, contact Technical Services at BD Diagnostic Systems for assistance and withhold use of lot.
- Documentation: Record results on the Reagent/Media QC Form – Appendix E. If QC results are not acceptable, prepare an Appendix K form to document the corrective action.

Preparation of Culture Suspension

- Subculture the MTB strain onto several LJ slants.
- Incubate the tubes at 37°C (±1°C) and observe growth visually.
- Use colonies showing good, confluent, and pure growth within 10-15 days of first appearance. Younger or older cultures may not give reliable results.
- Remove growth from the slant by carefully scraping the colonies off the slant with a sterile loop or sterile wooden applicator stick.

→ 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, 2 mm diameter), which help to break up clumps (Tube A).
- Vortex Tube A for at least 1-2 minutes, making sure the suspension is well dispensed and very turbid.

→ Turbidity should be greater than McFarland No. 1 standard.

- Let the suspension stand undisturbed for 20 minutes.
- Using a transfer pipette, carefully transfer the supernatant from Tube A to another sterile screw cap glass tube (Tube B).

→ A void pipetting any sediment.

- Let Tube B stand undisturbed for 15 minutes.
- Carefully transfer the supernatant from Tube B into another screw cap glass tube (Tube C) without taking any sediment.
- Adjust the turbidity of the suspension in Tube C to a McFarland No. 0.5 standard by adding more 7H9 broth. Mix well.

→ If the suspension is too turbid, transfer some of the suspension to another sterile tube and adjust the turbidity to a McFarland No. 0.5 standard.

- Tube C is the stock suspension for QC testing.

→ This suspension may be frozen in small aliquots (~1.5 ml) in cryotubes at -70 to -80°C (±10°C).
→ The frozen suspensions may be used for up to six months.
→ Once thawed, do not refreeze.

- Ideally, ensure that new frozen stock passes testing indicated below before using in routine QC procedures.

Preparation of Dilutions

- Remove one aliquot of the stock suspension from the freezer and allow to thaw.
- Alternatively, prepare a fresh 0.5 McFarland suspension, using colonies within 1015 days of first appearance from LJ media. Carefully adjust the inoculum with a spectrophotometer or visually with a Wickerham card.
- Dilute the stock suspension (freshly prepared or frozen) 1:5 by transferring 1.0 ml of suspension to 4.0 ml of sterile water or saline. Mix well (Tube 1).
- Dilute 1:10 by adding 0.5 ml of suspension from Tube 1 into 4.5 ml of sterile water or saline (Tube 2).
- Mix well and then dilute 1:10 again by adding 0.5 ml from Tube 2 to 4.5 ml of sterile water or saline (Tube 3). Mix well.
- The final dilution of Tube 3 is 1:500.

Inoculation and Incubation

- Supplement MGIT medium with Growth Supplement and PANTA as specified in Section 10: Liquid Culture – Mycobacteria Growth Indicator Tube (MGIT).
- Inoculate 0.5 ml from Tube 3 to a MGIT tube. Mix well.
- Enter the inoculated tube in the MGIT 960 instrument.
- Remove the tube when indicated positive by the instrument.
- Retrieve data for time to detection from the MGIT printout.

3 Reagent Quality Control

Periodic quality control of all reagents is critical for ensuring confidence in laboratory results. For the clinical trial, the Sponsor requires the following reagent quality control procedures for staining reagents, sputum digestion/decontamination reagents, immunochromatographic identification tests and extraction buffer, and drug susceptibility reagents. In addition, record reagent name, batch number, date prepared, and expiry date on all reagent containers.

3.1 Acid-fast Stains: Fluorescent and Ziehl-Neelsen Methods

- Frequency: Each batch of patient tests and each new batch of in-house prepared reagents, or each new lot/new shipment of commercial reagents.
- Controls: Smears with known positive M. tuberculosis (H37Rv or H37Ra) and negative (non-acid-fast bacteria) control organisms.
- Acceptable results: Correct results as expected for positive and negative controls.
  Positive controls must demonstrate the presence of acid-fast bacilli.
  Negative controls must be clearly negative with no acid-fast bacilli present.

- Corrective Actions:
    - If either control result is unacceptable, do not report patient results.
    - Repeat controls; if acceptable, repeat patient tests.
    - If results still unacceptable, notify supervisor immediately and prepare new reagents and/or new controls, as applicable, to resolve issue.
    - When QC results are acceptable, repeat patient tests and report results.
- Documentation: Record results for new staining reagents on the Reagent/Media QC Form For each batch of smears, record results on the appropriate Daily AFB Staining QC Form If QC results are not acceptable, prepare a form to document the corrective action.

Preparation of Smears

Prepare a batch of positive and negative control smears in advance, heat-fix, and store unstained in a closed container in a dry area until used. It is preferable to use a seeded sputum sediment to prepare these controls, but if not available, a suspension of M. tuberculosis (H37Rv or H37Ra) for the positive control and Escherichia coli (or another non-acid-fast bacillus) for the negative control should be used. The E. coli suspension may be prepared from the stock culture.

3.2 Sputum Digestion/Decontamination Reagents

For each new batch of the NALC, NaOH, and Na-citrate reagents, record preparation details. Quality control for these reagents is performed as part of Quality Monitoring of Sputum Processing, below. Additional QC for the phosphate buffer is performed as follows:

- Frequency: Each new batch of phosphate buffer.
- Controls: pH paper or pH meter.
- Acceptable results: pH for phosphate buffer is 6.8.
- Corrective Actions: If pH for buffer cannot be adjusted to 6.8, discard batch, notify supervisor immediately, and prepare new batch using new reagent powder, if necessary.
- Documentation: Record reagent details and pH on Reagent/Media QC Form. If QC results are not acceptable, prepare an Appendix K form to document the corrective action.

3.3 Immunochromatographic Identification Tests

- Frequency:
- Each new lot or shipment of kits and each new prepared lot of extraction buffer.
- Weekly, or with each batch of patient tests, if testing is performed less frequently.
- Controls: Internal reagent control in device.
- Positive control: Culture of M. tuberculosis reference strain (H37Rv or H37Ra) in MGIT broth.
- Negative control: Culture of a MOTT strain (e.g., a well characterized strain of M. avium complex) in MGIT broth or broth from an uninoculated MGIT tube.
- Acceptable results: Correct results as expected for all controls.
- Internal control line is visible.
- M. tuberculosis must result in a positive test.
- MOTT strain or uninoculated broth must result in a negative test.
- Corrective actions:
    - If any control result is unacceptable, do not report patient tests.
    - Repeat test with new controls; if acceptable, repeat patient tests.
    - If repeat results still unacceptable, notify supervisor immediately and investigate potential causes for failure.
    - After investigation is complete and QC is acceptable, repeat patient tests and report results.
- Documentation: Record results for new extraction buffer; Record results for new kits on MTB Identification QC Form. If QC results are not acceptable, prepare form to document the corrective action.
- Perform QC tests immediately before testing the patient batch and resolve issues, if applicable.

3.4 Drug Susceptibility Testing Quality Control

- Frequency:
  Upon receipt of a new lot or new shipment of MGIT 960 SIRE kit, PZA kit, or PZA tubes.
  Upon receipt of a new lot, new shipment, or newly prepared batch of stock solutions of antibiotic powders for second-line drugs.
  Weekly or with each batch of patient testing.
- Controls: A pan-susceptible M. tuberculosis H37Rv or H37Ra strain
  Acceptable results: Susceptible results for all drugs within the defined time protocol;
  e.g., SIRE and second-line drugs within 4-13 days; PZA within 4-21 days.
- Corrective Actions: If the control results show any resistance, do not report patient results for that particular drug.
  Thoroughly review cause for unacceptable results and repeat QC test; if acceptable, repeat patient tests.
  If repeat results still unacceptable, notify supervisor immediately; prepare new drugs and/or a new control, as applicable, to resolve issue.
  If unacceptable results persist with SIRE or PZA kit, consult BD Technical Services for assistance.
  When QC results are acceptable, repeat patient tests and report results.
- Documentation: Record results for new antibiotics and weekly testing on the DST QC Form. If QC results are not acceptable, prepare a form to document the corrective action.

<u>Inoculum Preparation</u>

The inoculum must consist of a pure culture of M. tuberculosis (H37Rv or H37Ra) and be tested following the same procedures as for patient isolates. The inoculum can be prepared from one of three options:

- A freshly grown culture of MTB in MGIT medium following the guidelines
- A fresh, pure subculture of MTB growing on LJ. Using an Inoculum from Positive LJ Culture.
- QC Protocol for BACTEC MGIT 960 Culture Medium and MGIT 960 Growth Supplement above.

Remove one aliquot of the organism suspension from the freezer and allow to thaw.

Dilute 1 ml of the suspension in sterile saline or 7H9 broth (1:5 dilution).

Use this adjusted suspension as the inoculum for drug tests and growth control.

<u>Set-up of DST Quality Control</u>

Follow instructions Growth Control Tube Preparation and Inoculation for the drugs being tested. Important considerations when preparing the DST QC are:

→ Proper reconstitution of lyophilized drugs.

→ Proper preparation of drug powder suspensions.

→ Proper dilution of the QC organism for the Growth Control and drug tubes.

Monitor QC drugs until signalled complete by the instrument and interpret the same as for patient isolates.

Quality Monitoring Activities

Monitoring of critical practices that may affect the outcome of clinical trial results is required for the duration of the study. These practices include:

1. Temperature monitoring
2. Equipment maintenance
3. Regular monitoring of specimen processing
4. MGIT time to detection (TTD) monthly exercise
5. Contamination rate assessment
6. Supervisor review of QC activities monthly

In addition, though not required, the following quality monitoring activities are strongly recommended and will serve to strengthen laboratory results:

1. Review of AFB examination competency
2. Analysis of laboratory data

1 Temperature Monitoring

1.1 Thermometers

Internal thermometers must be used for all equipment monitoring. All thermometers used in assessing equipment temperatures must be calibrated against a standardized/certified thermometer (US Bureau of Standards or equivalent) before putting into use and annually thereafter, either inhouse or through a commercially available service. Identify thermometers by a numbering system and maintain documentation of their calibration in a lab worksheet or logbook.

Table: Equipment Temperature Ranges

Equipment	Temperature Ranges (°C)
Refrigerators[a]	2-8
Freezer (Ultralow)	–70 to -80 ±10
Incubators	37±1
Refrigerated centrifuge	4-12
MGIT 960	37±1[b]
Room Temperature[c]	Varies

a. Temperature of the refrigerator where sputum specimens are stored until they are sent to the laboratory must be monitored daily.

b. Internal instrument temperature reading should be ± 1.5°C of manual reading.

c. Range determined by requirements of reagents or instrument housed in the particular room, e.g., MGIT room: 19-30°C; room containing unrefrigerated centrifuge: ≤ 20°C.

1.2 Temperature Reading/Recording

- Frequency:
    a) Daily reading/recording of temperatures is required, preferably in the early morning before work commences. Twice daily readings are recommended as equipment may malfunction during working hours, which may not be detected until the following day.
    b) Weekends/holidays may be excluded if no staff are available to monitor the equipment during those times, check temperatures immediately the beginning of the next working day.
- Acceptable results: Temperatures are within the defined range for the specific piece of equipment/environment.
- Corrective actions: If the temperature reading is not acceptable:
    a) Adjust temperature control and monitor until correct range is achieved; if temperature has not returned to normal within the next working day, notify supervisor immediately to decide on further corrective action.
    b) If equipment is not functioning, notify supervisor immediately so service can be instituted. Relocate specimens, reagents, etc., as applicable, to a functioning piece of equipment/environment with proper temperature range.
- Documentation:
    a) Record temperatures on Equipment Temperature Record Form.
    b) Document minor adjustments and corrective actions.
    c) Document all major unacceptable results, e.g., equipment failure, corrective actions and resolutions on a form.

2 Equipment Cleaning/Maintenance

2.1 Equipment cleaning

Keeping laboratory equipment clean and performing recommended routine maintenance are essential for accurate performance of laboratory tests and maintaining the longevity of equipment. Suggested equipment cleaning activities are given in the table below, however the manufacturers' specific recommendations and procedures should be followed. Maintain documentation for the performance of routine cleaning in a lab worksheet or logbook.

2.2 Equipment Maintenance

Routine, in-house maintenance must be carried out at the time intervals recommended by the manufacturer of the equipment, and a schedule developed for performing the applicable maintenance. Maintenance may be performed by in-house technical personnel and/or a vendor service contract Maintain documentation of all routine maintenance in a lab worksheet or logbook.

A suggested maintenance schedule performed in-house or by outside technical personnel, as applicable, is shown below. Semi-annual or annual maintenance is required for all equipment in this list. In addition, if large equipment is moved/ relocated, it must be re-certified or serviced prior to using.

Table: Minimum Equipment Maintenance Schedule

Equipment	Maintenance	Semi-annually	Annually
Autoclave	Maintenance by autoclave technician	recommended	required
Micropipettors/ Pipette-Aid	Calibration	recommended	required
Analytical balance	Calibration	recommended	required
Biosafety cabinet	Certification	–	required
Centrifuge	Calibration of speed, timer, temperature	recommended	required
Microscopes	Inspection, cleaning, and lubrication by service provider	recommended	required
Air conditioners	Clean condensers, fans and blower motor; verify mechanics and check filter	recommended	required
Freezer -70 to -80°C	Clean condensers; verify door gaskets	recommended	required
	Clean fan and blower motor; routine maintenance	recommended	required
Refrigerators	Clean condensers and fans; routine maintenance	recommended	required
Incubator	Check door gasket seal, heating and cooling elements, electronic components; routine maintenance	recommended	required
MGIT	Calibration tube check, thorough cleaning of relevant components	required	–

For further details on equipment maintenance and cleaning, refer to: Maintenance Manual for Laboratory Equipment, 2nd Edition. World Health Organization. Geneva, Switzerland. 2008.

Equipment Cleaning Schedule

Equipment	Cleaning	Daily	Weekly	Monthly	Other
Bio-safety cabinet	Spray work surface thoroughly with tuberculocidal disinfectant; let stand 3 minutes. Wipe dry with absorbent towel. if indicated, follow with 70% alcohol to remove disinfectant residue that may harm BSC surface. Wipe equipment stored in the BSC with disinfectant-soaked absorbent towel.	AM & PM and following each task			
	Thorough interior and exterior cleaning with appropriate disinfectant(s)				at least once / Year or as needed for any spill of infectious material
	Clean UV light with 70% alcohol				Every 1-2 weeks
Centrifuge	Spray interior walls of centrifuge with disinfectant (70% alcohol) and let stand 3 minutes. Wipe dry with absorbent towel. Clean exterior surfaces with mild detergent, rinse and dry	X			
	Remove carriers and soak in warm, soapy water. Rinse thoroughly and place upside down to drain/dry		X		
Counters	Spray thoroughly with appropriate tuberculocidal disinfectant; let stand 3 minutes. Wipe dry with absorbent towel. if indicated, follow with 70% alcohol to remove disinfectant residue	AM & PM			
-70 to -80 freezer	Clean fan cover and remove ice buildup			X	
	Defrost and clean interior				when needed
Micropipettors/ pipette-Aid	Wipe exterior with 70% alcohol	Before / after each procedure use			
pH meter	Replace electrode immersion fluid (pH 7.0 standard). Clean electrode tip				As needed
Refrigerator	Wipe interior surface and shelves with damp towel soaked in mild detergent; rinse and dry			X	
Incubator	Wipe interior surface and shelves with damp towel soaked in mild detergent, rinse and dry. Follow with 70% alcohol spray. Autoclave shelves if applicable.			X	
Slide warmer	Wipe surface with a damp towel soaked in mild detergent; rinse and dry		X		
Vortex Mixer	Wipe surface with a damp towel soaked in mild detergent; rinse and dry		X		
Analytical balance	Clean pan surface with brush.	X			

3 Monitoring of Sputum Processing

Careful attention to technique when processing specimens is essential to preventing cross-contamination from a heavily acid-fast positive specimen to other, possibly negative, samples. Running positive and negative controls on a regular basis assesses techniques and assures that all aspects of the culture process (manual and instrumented), from sputum processing to isolation, are performing properly.

- Frequency: Once per week, or with each patient batch.
- Controls: Place controls at the end of the batch of patient tests, with the positive control before the negative control. Process both control specimens in the same manner as patient specimens and perform routine microscopy, MGIT, and LJ culture.

    a) Positive control – 4 ml 7H9 Broth, or a known negative sputum specimen, inoculated with 700µl of a 10–2 dilution of a McFarland No. 0.5 suspension of M. tuberculosis (H37Rv or H37Ra). Suspension should be prepared using 10–15 days old growth on LJ.
    b) Negative control: 4 ml 7H9 broth.
- Acceptable results:

    a) Positive control
        i. Positive fluorescent smear.
        ii. Growth in MGIT; TTD should be comparable with similar tests.
        iii. Growth on LJ culture; colony counts should be comparable with similar tests.

    b) Negative control
        i. Negative fluorescent smear.
        ii. No growth in MGIT or on LJ culture.
- Corrective actions: If QC results are unexpected, notify supervisor immediately and have technician repeat exercise with next processing batch.

    a) If growth is detected on either media from the negative control, either cross-contamination has occurred or there is a problem with sterility of the processing reagents. Notify the Sponsor immediately if cross-contamination of MTB is suspected.

        i. Observe the technician who processed these specimens for techniques known to contribute to contamination, e.g., poor organization of tubes, splashing when adding reagents, opening caps too soon after vortexing, etc.
        ii. Visually check all reagents used; subculture to BAP if contamination is suspected.
        iii. Record patient samples processed at this time and monitor closely for expected results.

    b) If no growth occurs on either media from the positive control, check age of isolate used and diluting technique.

    c) If TTDs and/or colony counts on LJ vary widely, investigate possible cause for deviations.
- Documentation: Record the microscopy, MGIT and LJ culture results on the Weekly MGIT/LJ Culture QC Form. If QC results are not acceptable, prepare a form to document the corrective action.

4 MGIT 960 Time to Detection Monthly Exercise

An important control measure of the MGIT culture system is to evaluate the consistency (reproducibility) of the TTD for known quantities of M. tuberculosis organisms. This exercise assesses consistency of technical performance in preparing MTB suspensions according to a turbidity standard, diluting MTB suspensions, and pipetting skills.

- Frequency: Once per month, and performed by alternating technicians, if appropriate.
- Controls: One dilution of M. tuberculosis (H37Rv or H37Ra) in 7H9 broth. It is highly recommended that a frozen stock, no older than six months, be used to prepare the dilution.
- Acceptable results:
    a) The TTD must fall within the 6-10day range as referenced in the FIND MGIT Procedure Manual.
    b) Month to month consistency in the lab's established TTD range for the 1:500 dilution.
- Corrective actions: TTD results will be closely monitored by comparing each monthly TTD result to the laboratory's own ongoing range of results and to a global range established by the Sponsor laboratory network. If expected results vary by more than ± 2 standard deviations within the laboratory's own established range:
    a) Review data and all procedures with technician performing the exercise and repeat test under observation if necessary.
    b) If TTD is significantly decreased, check culture for contamination and, if contaminated, repeat exercise with fresh culture.
    c) If TTD is significantly increased, check viability of culture and age of culture (if using frozen stocks).
- Documentation:
    a) Record results on the MGIT TTD Worksheet
    b) If QC results are not acceptable, prepare an Appendix form to document the corrective actions.
    c) Each month upon test completion, provide completed Appendix L form, along with applicable MGIT printouts and any Appendix K forms, to the Sponsor. File copy of documents in laboratory QC binder.

<u>Preparation of Culture Suspension</u>

Follow instructions in QC Protocol for BACTEC MGIT 960 Culture Medium and MGIT 960 Growth Supplement Kit to prepare the culture suspension.

<u>Preparation of 1:500 Dilution</u>

NOTE: It is highly recommended to prepare the dilution from a frozen stock suspension.

1. Remove one aliquot of the stock suspension from the freezer and thaw. Alternatively, prepare a fresh McFarland No. 0.5 suspension using colonies within 10-15 days of first appearance on LJ media. Carefully adjust the inoculum with a spectrophotometer or visually with a Wickerham card.
2. Dilute the stock suspension (freshly prepared or frozen) 1:5 by transferring 1.0 ml of suspension to 4.0 ml of sterile water or saline. Mix well (Tube 1).
3. Dilute 1:10 two more times by adding 0.5 ml of suspension from 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 water or saline (Tube 3).

Tube 3 will be used for the inoculation and incubation of the MGIT tube. This tube represents a 1:500 dilution, representing about log105 organisms

Inoculation and Incubation

1. Supplement MGIT medium with Growth Supplement and PANTA as Liquid Culture – Mycobacteria Growth Indicator Tube (MGIT).
2. Inoculate one MGIT tube with 0.5 ml of the 1:500 dilution (Tube 3).
3. Enter the inoculated tube in the MGIT 960 instrument. Take the tube out when indicated positive by the instrument. Retrieve data for time to detection.

5 Contamination Rate Assessment

A monthly assessment of the rates of contamination in MGIT and LJ cultures demonstrates trends of increasing or decreasing contamination and should be monitored.

- Frequency: Once a month
- Results: Contamination of liquid and solid cultures occurs:
    a) When specimens are inadequately decontaminated because specimens are heavily mucoid or have been improperly stored during transport encouraging bacterial overgrowth.
    b) Some highly resistant bacterial species are unaffected by decontamination.
- Corrective Actions: Reasonable rates of contamination are unavoidable.
    a) If MGIT contamination rate is >10%, notify the Sponsor immediately for guidance and troubleshooting instructions.
    b) If LJ contamination rate is >6%, notify the Sponsor immediately for guidance and troubleshooting instructions.
- Documentation: Report findings on Monthly Data Monitors Form.

Procedure

1. Count the total number of MGIT cultures reported in the month and the number of contaminated cultures reported in that month. Calculate the percentage (%) contaminated (number contaminated ÷ number reported × 100 = % contamination).
   NOTE: Using this method, the cultures processed in any given month may not correlate exactly with the cultures reported in that same month, but statistics over time will provide consistent indicators for the overall performance of lab procedures.
2. Repeat Step 1 for LJ cultures.
3. Repeat steps 1 and 2, separating patient-collected specimens and hospital/clinic collected specimens for both MGIT and LJ cultures.
4. Record the % contaminated for each medium and compare results with data from the previous month and the lab's average.
5. Calculate the on-going cumulative mean rate for each medium.

6 AFB Smear Review for Technical Competency

Periodic smear review is recommended to assess and improve technician proficiency and assure consistency among examiners.

- Frequency: Once a month
- Acceptable results:
    a) Negative smears should be resulted as negative by all technicians.
    b) Positive smears should not vary by more than one quantification level.
- Corrective actions:
    a) Review discrepant results with technician(s) and re-examine smears that are not in agreement.
    b) Provide additional training as indicated to improve performance.
- Documentation: Save results in QC binder and technicians' personnel fi les, if appropriate.

Procedure

1. Select 10 slides from those read in the previous month and that represent a range from negative to scanty, 1+, 2+, and 3+.

2. Ask technicians to read and record results in a blinded manner.
3. Review results for consistency and any discrepancies among technicians.

7 Analysis of Laboratory Data

It is strongly recommended that laboratory data be recorded and analysed to help assure that specimen processing procedures, as well as mycobacterial isolation rates, are performing properly. After initially analysing the records for three to six months, an overall average or normal trend for an individual laboratory workload can be determined.

If there is significant change or deviation from normal results in any of the parameters, all procedures must be reviewed, and corrective measures instituted.

- Frequency: Each parameter should be calculated monthly.

Culture Positivity Rate

- Procedure:

1. Count the number of positive MGIT cultures that are smear positive. Count the total number of positive MGIT cultures. Use these values to calculate the MGIT isolation rate from smear-positive specimens (smear positive ÷ total culture positive reported in the month x 100).
2. Count the number of positive MGIT cultures that are smear negative. Count the total number of positive MGIT cultures. Use these values to calculate the MGIT culture positivity from smear negative specimens (smear negative ÷ total culture positive reported in the month x 100).
3. Repeat Steps a. and b. for LJ cultures.
4. Record isolation rates and compare results with data from the previous month and the lab's average.
5. Calculate the cumulative mean positivity rate for each medium.

- Results:

1. 90% of smear positive specimens should be culture positive.
2. 50% of culture positive specimens may be from smear-negative specimens.
3. Rates should be relatively consistent for each medium. Due to the higher sensitivity of liquid medium, the isolation rate on LJ may be slightly lower, especially if smears show very few AFB.

- Corrective Actions:

1. If there is a significant decrease in overall culture positivity (isolation) rate for both media types, review decontamination procedures.
2. If there is a significant decrease in only one media type, check the growth performance, following media QC guidelines above.
3. If the LJ isolation rate differs by >20% of the MGIT isolation rate for the same set of specimens, perform QC procedures for the applicable lot of LJ (commercial or homemade), following the guidelines in Protocol for LJ Medium.
4. If there is a significant change in smear positivity/negativity, unrelated to the visit intervals examined, review all microscopy procedures and discuss with personnel.

- Documentation:

1. Record data on the Monthly Data Monitors Forms
2. If any corrective measures are necessary, prepare an Appendix form to document the activities.

Isolation Rate of MTB and MOTT

- Procedure:

a) Count the number of MTB isolates and calculate the percentage using the total MGIT cultures reported in a given month (MTB culture ÷ total culture positive x 100).
b) Count the number of MOTT isolates and calculate the percentage using the total MGIT

cultures reported in a given month (MOTT culture ÷ total culture positive x 100).

c) Repeat steps a. and b. for LJ cultures.
d) Record isolation rates for both species and compare results with data from the previous month and lab's average.
e) Calculate the cumulative mean positivity rate for each mycobacterial species.

- Results: Isolation of MTB complex organisms in relation to other mycobacterial species should remain fairly constant over time. If there is a sudden increase in isolation of MOTT, it may be due to increase in numbers of follow-up specimens from subjects colonized with MOTT (a common observation). However, this may also indicate the presence of an environmental contaminant or cross-contamination event.
- Corrective Actions:
  If there is a significant increase in MOTT isolation, seemingly unrelated to individual patient colonization, thoroughly review all procedures (e.g., safety, cleaning, specimen processing, culture media, etc.), using supervisor observation whenever necessary.
- Documentation:

a) Record data on the Monthly Data Monitors Forms or other lab-specific comparable document.
b) If any corrective measures are necessary, prepare form to document the activities.

<u>Time to Detection</u>

- Procedure:
  a. Calculate the time to detection for MGIT cultures from smear positive and smear negative specimens that are determined to be MTB.
  b. Repeat step a. for LJ cultures.
  c. Record results for both media and compare results with data from the previous month and lab's average.
  d. Calculate the cumulative average TTDs for each medium type.
- Results: If the monthly average TTD is <20% of the lab's established average (without an increase in contamination rate or isolation of rapidly growing mycobacteria), this likely indicates that processing has improved. If the monthly average TTD >20% of the lab's established range, processing of specimens may be too harsh, or centrifuge speed/time may be less than optimal.
- Corrective Actions:
  a. If there is a significant increase in TTD (unrelated to visit interval), review processing procedures and discuss with personnel, using supervisor observation if necessary.
  b. Although an improvement in processing is generally considered beneficial, significant decreases in TTD may also prompt review of applicable procedures.
  c. Review settings and maintenance records for centrifuge
- Documentation:
  a. Record data on the Monthly Data Monitors Forms

  b. If any corrective measures are necessary, prepare an Appendix form to document the activities.

Quality Improvement

Continuous efforts to improve the overall quality of the laboratory, by improving service, function, workflow, personnel, and customer satisfaction are necessary and require the input and support of all personnel. Regular staff meetings should be held to share and discuss quality issues found with QC and QM, to elicit suggestions for improvement, and encourage input from staff on any issues of concern.

The Sponsor requires the documentation of efforts to improve the quality of the lab. When Quality Control tests fail to give the proper results and/or when deviations from baseline data are observed, the use of a standardized form, must document the action taken to correct the problem. The form includes the following components:

- Form Number - number assigned to each form to allow easy fi ling and retrieval. Often the date is incorporated into this number.
- Description of the problem/improvement - The date and a short description are written here.
- Investigation into the root cause of the problem - Oftentimes, there is a bigger underlying problem that causes a QC failure or deviation to occur. The issue should be analysed, and the root cause written in this section.
- Corrective action to fi x the current problem - The first action taken to resolve the immediate issue is written here, along with the expected date of resolution.
- Preventative action required to eliminate root cause of problem - This action often differs from the action taken to fi x the immediate problem (mentioned above) and should address the root cause of the problem.
- Person(s) responsible for monitoring the effectiveness of action - This section should explain how the effectiveness of the previously mentioned action is evaluated.
- All persons involved in the incident, e.g., the person identifying the issue, the QA delegate, and the lab supervisor, should review the form and sign the document, attesting that s/ he has been notified of the issue and agree to the actions taken.

Training and competence assessment

Training materials have been developed and are freely available for most WHO recommended TB diagnostics, including smear microscopy (light and fluorescence), solid and liquid culture, DST, LPA, and Xpert MTB/RIF

Instrument verification

Instruments should be evaluated as being “fit for purpose” through verification with known positive and negative material prior to commencing testing of clinical specimens, and after calibration or repair of instruments.

Method validation

All tests used in the laboratory must be validated for their intended use.

Quality control

Quality control (QC) monitors activities related to the examination, i.e., analytical phase of testing. The goal of QC is to detect, evaluate, and correct errors due to test system failure, environmental conditions, or operator performance before patient results are reported

Lot testing

QC testing should be performed on new kits or lots of reagents prior to their use for testing patient samples to ensure that they perform as expected

External quality assessment

External quality assessment (assurance) is defined as follows: "Inter-laboratory comparisons and other performance evaluations that may extend throughout all phases of the testing cycle, including interpretation of results; determination of individual and collective laboratory performance characteristics of examination procedures by means of inter-laboratory comparison.

EQA for TB laboratories may include the following components:

• On-site supervision

• Proficiency testing

• Blinded re-checking

While all laboratories should ensure that all tests are part of an EQA programme, monitoring performance using laboratory quality indicators (also known as performance indicators) is the most effective way to assure the quality of the laboratory results and identify areas for improvement. Quality indicator monitoring should always be implemented in conjunction with an EQA programme. See the next section for more information on quality indicators.

Key Performance Indication:

i) General quality indicators

The following set of quality indicators apply to all technologies and should be collected, analysed on a monthly basis, and disaggregated according to tests. These indicators are provided as a guide, and laboratories should review and set locally appropriate targets.

Table General quality indicators

INDICATOR	TARGET
Number of tests performed, by type of test	–
Service interruptions	No interruptions
(a) Stock outs	No stock outs leading to service interruption
(b) Equipment down time	No equipment downtime leading to service interruption
Turnaround time (TAT)	90% of results meet test-specific TAT
Test statistics (quality indicator) report	100% reports completed by defined due date
EQA results	>90% EQA panels are passed
QC results	>90% QC results meet expected criteria
Specimen rejection	<1% specimens rejected
Customer satisfaction	>80% surveyed customers are satisfied
Technician productivity	Report average number of tests performed per month per technician

a Where resources allow, additional secondary indicators may be collected by some laboratories, such as volume and quality of sputum specimens. This may be important for certain tests (e.g., >1ml sputum is required for Xpert MTB/RIF test). Specimen rejection criteria related to quality of specimen, or incompletely labelled or leaking specimens are applied in some laboratories

ii) Test-specific quality indicators

This section provides recommended quality indicators for each WHO-approved method, which are in addition to the general quality indicators listed in Table 5. Targets provided in the tables below are intended as a guide, and laboratories should determine their own targets. These targets, and especially isolation rates, will vary based on local situation, patient population tested, and other relevant factors. Deviations from the usual rates should be investigated.

Culture

Table 3 lists quality indicators recommended for culture. They should be collected and analyzed on a monthly basis in addition to the general quality indicators. Indicators should be disaggregated by type of culture medium if more than one type is used. For laboratories processing a range of specimen types for MTB culture further disaggregation is recommended.

Table 3 Quality indicators for culture

INDICATOR	DESCRIPTION	TARGET	REFERENCE
Number and proportion of diagnostic specimens (new and relapse) that were culture positive (MTB and NTM combined)	Number of diagnostic specimens that were culture positive for MTB or NTM / Number of diagnostic specimens processed for culture	15–20%	Siddiqi SH, and Rüsch-Gerdes S. *MGIT procedure manual*. Geneva, FIND, 2006.
Number and proportion of diagnostic specimens (new and relapse) that were MTB positive	Number of diagnostic specimens culture positive for MTB / Number of diagnostic specimens processed for culture	10–15%	
Number and proportion of diagnostic AFB smear positive specimens (new and relapse) that were culture positive for MTB	Number of AFB smear positive specimens culture positive for MTB / Number of smear positive diagnostic specimens processed for culture	95–98% (liquid) 85–90% (solid)	
Number and proportion of diagnostic AFB smear negative specimens that were culture positive for MTB	Number of AFB smear negative specimens culture positive for MTB / Number of smear negative diagnostic specimens processed for culture	20–30%	
Number and proportion of contaminated cultures leading to uninterpretable results a	Number of inoculated culture tubes or plates discarded due to contamination / Total number of inoculated tubes or plates inoculated for culture	3–5% (solid) 8–10% (liquid)	
Laboratory turnaround time	Time between receipt of specimens for culture at the laboratory and result reporting (mean, range and 90th centile)	Solid culture: On average, 3 weeks for smear-positive samples and 4–8 weeks for smear negative samples Liquid culture: 8–10 days for smear positive samples and 2–6 weeks for smear negative samples	

a For solid culture, some results may be interpretable in the presence of low-level contamination. Some laboratories may also reprocess contaminated cultures and the results of the repeat testing may be reportable.

Phenotypic DST

Table 4 lists quality indicators recommended for use with phenotypic DST methods. These indicators should be collected and analysed on a monthly basis in addition to the general quality indicators. Other secondary indicators may be collected on a less frequent basis (e.g., quarterly), such as the number and proportion of unusual drug resistance patterns.

Table 4 Quality indicators for DST

INDICATOR	DESCRIPTION	TARGET	REFERENCE
Number and proportion of isolates with mono-resistance and multidrug resistance to all combinations of drugs tested (e.g., isoniazid monoresistance, rifampicin mono resistance, MDR)	Number of isolates resistant to single or multiple drug combination/ Total number of isolates tested	Dependent on population tested and country drug resistance prevalence and patterns	Siddiqi SH, and Rüsch-Gerdes S. *MGIT procedure manual.* Geneva, FIND, 2006.
Number and proportion of isolates inoculated for DST that were discarded due to contamination	Number of isolates discarded due to contamination / Total number of isolates inoculated for DST	<3%	
Number and proportion of isolates inoculated for DST that were uninterpretable due to lack of growth of control (drug-free) tubes/plates	Number of isolates discarded due to lack of growth on drug-free media / Total number of isolates inoculated for DST	<3%	
Laboratory turnaround time	Time between inoculation of DST and result reporting (mean, range and 90th centile)	Solid media: 3–4 weeks Liquid media: 2–3 weeks	
Total DST turnaround time including time for primary culture to produce inoculum	Solid media: 8–16 weeks Liquid media: 4–6 weeks		

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