Mycobacteriology Laboratory Sourcebook For Harmonization and Support of Tuberculosis (TB) Clinical Trials

Version 1.0

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Summary

The Mycobacteriology Laboratory Sourcebook for Harmonization and Support of Tuberculosis (TB) Clinical Trials was developed to ensure high quality results and comparability of data across laboratories participating in TB clinical trials sponsored by the AIDS Clinical Trials Group (ACTG) and International Maternal Pediatric Adolescent AIDS Clinical Trials (IMPAACT) Networks. The essential technical components of mycobacteriology laboratory procedures that can affect the microbiology endpoints of the study, comparability and quality of results, and participant safety are described. These are referred to as Key Elements. The Sourcebook also discusses how test results are used in the context of clinical trials. All the routine procedures performed with respiratory specimens, including collection and transport, are included with a focus on the Key Elements and important technical points. TB laboratory specialists with experience in TB drug trials, and who are a part of the ACTG and IMPAACT Networks, were involved in defining the Key Elements and important technical points. The Sourcebook was developed as a reference for all laboratorians participating in TB clinical studies; however, it will also be informative for clinical staff involved in studies and those developing study protocols. Checklists consisting of the Key Elements and other important technical points for each of the laboratory procedures are included to assist the laboratories in performing self-assessments and auditors in assessing procedures for quality elements.

Disclaimer

The Sourcebook mentions specific commercial products and assays. Such instances do not constitute endorsement of these. While every effort is made to ensure accuracy, readers are advised that new methods and techniques may come into practice. The recommendations contained in these guidelines do not indicate an exclusive course of action, or serve as a standard of laboratory practice. Variations, taking individual circumstances into account, may be appropriate. **For DAIDS Network protocols, protocol specific information (e.g. appendix, MOP) supersedes this document**.

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For Harmonization and Support of TB Clinical Trials

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1 List of Abbreviations and Acronyms

Table 1-1. List of Abbreviations and Acronyms	
AC	Amplification Control
ACTG	AIDS Clinical Trials Group
AFB	Acid-fast bacilli
AG	Aminoglycoside
AIDS	Acquired Immune Deficiency Syndrome
AST	Antimycobacterial Susceptibility Testing
ATCC	American Type Culture Collection
AUC	Area Under the Curve
BAP	Blood Agar Plate
BCG	Bacillus Calmette-Guerin
BHI	Brain-heart infusion
BSC	Biosafety cabinet
BSL	Biosafety Level
CAP	College of American Pathologists
CC	Conjugate Control
CDC	Centers for Disease Control and Prevention
CDISC	Clinical Data Interchange Standards Consortium
CFU	Colony Forming Units
CI	Confidence Interval
CO_2	Carbon dioxide
CP	Cyclic peptide
CRF	Case Report Form
Ct values	Cycle threshold values
DAIDS	Division of AIDS
DMID	Division of Microbiology and Infectious Diseases
DNA	Deoxyribonucleic acid
DST	Drug Susceptibility Testing
EBA	Early Bactericidal Activity
EMB	Ethambutol
EQA	External Quality Assessment
FIND	Foundation for Innovative New Diagnostics
FLQ	Fluoroquinolone
FM	Fluorescence Microscopy
GCLP	Good Clinical Laboratory Practices
GCP	Good Clinical Practice
GLI	Global Laboratory Initiative
GU	Growth Unit

Table 1-1. List of Abbreviations and Acronyms	
Hain AS	Hain Lifesciences GenoType® Additional Species
Hain CM	Hain Lifesciences GenoType® Common Mycobacteria
HANC	HIV/AIDS Network Coordination
HIV	Human Immunodeficiency Virus
ID	Identification
IMPAACT	International Maternal Pediatric Adolescent AIDS Clinical Trials
INH	Isoniazid
INSTAND	Institute for Standardization and Documentation in the Medical Laboratory
ISO	International Organization for Standardization
IUATLD	International Union Against Tuberculosis and Lung Disease; referred to as The Union
LED	Light Emitting Diode
LJ	Lowenstein-Jensen
LPA	Line Probe Assay
LPC	Laboratory Processing Chart
MDR TB	Multi-drug resistant tuberculosis; defined as resistant to isoniazid and rifampin
MGIT	Mycobacteria Growth Indicator Tube
MIC	Minimum Inhibitory Concentration
MOP	Manual of Operating Procedures
MOTT	Mycobacteria other than tuberculosis, see also NTM
MPT64	24-kilodalton secreted protein specific to the *Mycobacterium tuberculosis* complex
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex (MTBC)
NALC	N-Acetyl-L-Cysteine
NaOH	Sodium Hydroxide
NTM	Nontuberculous mycobacteria; also referred to as mycobacteria other than tuberculosis (MOTT)
OADC	Oleic Acid, Albumin Bovine, Dextrose, Catalase
PAL	Protocol Analyte List
PANTA	Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim, Azlocillin
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PID	Participant Identification
PNB	p-Nitrobenzoic acid
PPE	Personal protective equipment
pSMILE	Patient Safety Monitoring in International Laboratories
PI	Principal Investigator
PT	Proficiency testing
PZA	Pyrazinamide
QA	Quality Assurance

Table 1-1. List of Abbreviations and Acronyms	
QC	Quality Control
QI	Quality Indicators
QM	Quality Monitoring
RCF	Relative Centrifugal Force
RIF	Rifampicin or Rifampin
RT-PCR	Real Time Polymerase Chain Reaction
SANAS	South African National Accreditation System
SCC	Sputum Culture Conversion
SOE	Schedule of Events (same as table of laboratory assessments)
SOP	Standard operating procedure
SPC	Sample Processing Control
TAT	Turnaround time
TB	Tuberculosis
TBTC	Tuberculosis Clinical Trials Consortium
TSA	Tryptic Soy Agar
TSG	Transformative Science Group
TTD	Time to detection, as reported by MGIT instrument; also referred to as time to positivity (TTP)
WHO	World Health Organization
XDR TB	Extensively drug resistant tuberculosis; defined as resistance to isoniazid, rifampin, any fluoroquinolone, and any injectable second-line drug (aminoglycoside, cyclic peptide)
ZN	Ziehl-Neelsen

2 Introduction

2.1 Role of the Mycobacteriology Laboratory

The success of any clinical trial is dependent on pre-determined endpoints and a statistical analysis plan. Mycobacteriology laboratories generate key data that are used for endpoint determinations for each participant. For this reason, **every participant specimen and every mycobacteriology test performed in the laboratory could affect the success or failure of the clinical trial.** The role of the laboratory is therefore critical to the success of the clinical trial. Table 2-1 shows examples of primary and secondary endpoints for tuberculosis (TB) drug trials and the corresponding critical mycobacteriology tests, from which results are used to determine study endpoints (1).

Table 2-1. Examples of Primary and Secondary Endpoints for TB Drug Trials

Primary Endpoint	**Critical Mycobacteriology Test**
Proportion of participants who are sputum culture-negative at completion of 8 weeks of treatment (solid and liquid culture considered separately)	Liquid and solid culture
Time to stable sputum culture conversion (solid and liquid culture considered separately)	Liquid and solid culture
TB disease-free survival at 12 months after study treatment assignment	Liquid and solid culture
Speed of decline of sputum viable *Mycobacterium tuberculosis* (MTB) by colony forming units (CFUs)	Quantitative sputum culture
Proportion of participants with grade 3 or higher adverse events during study drug treatment	None
Secondary Endpoint	**Critical Mycobacteriology Test**
Speed of decline of sputum viable MTB by mycobacteria growth indicator tube (MGIT) culture time to detection (TTD)	MGIT culture
Study drug Area Under the Curve (AUC)/Minimum Inhibitory Concentration (MIC) providing shortest time to sputum culture conversion (solid and liquid culture considered separately)	Liquid and solid culture MIC determination
Time to first negative sputum culture	Liquid and solid culture
TB disease-free survival at 18 months after study treatment assignment	Liquid and solid culture

2.2 What Tuberculosis Tests are Performed?

As microbiology endpoints depend on maximum recovery of MTB, the specimen processing and culture procedures are the backbone of TB testing performed by the laboratory. Smear microscopy and molecular tests for rapid detection of MTB and drug resistance are performed to determine if individuals are eligible to participate in the study. Phenotypic drug susceptibility testing (DST) is performed to ensure the safety for participants enrolled in the study (see section 12.1.1).

The laboratory tests to be performed are described in the study protocol (section 3). Often a specific assay or test method is delineated and required. Further specifications and expansion of testing details appear

in other study documents, such as the Schedule of Events (SOE), Laboratory Processing Charts (LPCs), and Manual of Operating Procedures (MOP) (section 3).

2.3 Why Harmonize TB Testing?

TB clinical trials are performed at multiple sites in different geographical settings. TB laboratories worldwide use a variety of methods for diagnosing TB. Many procedures are not automated and can be affected by the individuals performing them (e.g., smear reading, sputum processing). These variations can affect microbiology endpoints, comparability of results, and quality of results for participant safety. To minimize variations and achieve high quality, comparable testing results, the procedures used across the laboratories participating in the clinical trial must be harmonized. The intention of this Sourcebook is to provide a resource for mycobacteriology laboratories participating in TB clinical trials to ensure that procedures are appropriately harmonized, thus allowing comparability of clinical trial data.

2.4 Harmonization Based on Key Elements

The harmonization process is based on the concept of Key Elements. Key Elements in TB laboratory procedures are those that have the greatest impact on microbiology endpoints of clinical trials, allow for comparison of results among all trial sites (or from one study to another), and provide accurate test results to ensure the safety of trial participants. Key Elements have been identified for all TB laboratory procedures performed with respiratory specimens, including collection and transport.

2.5 Key Elements for Pediatric Studies

TB laboratories will usually perform technical procedures such as NALC-NaOH decontamination, smear microscopy, Xpert® MTB/RIF, culture, and DST the same way for an adult or a child. However, due to the paucibacillary (e.g., smear-negative) nature of pediatric TB, microbiological confirmation is typically only available in 30% of cases. Furthermore, since children do not produce sputum easily for diagnostic purposes, a variety of specimen types other than expectorated sputum are collected, including gastric aspirates and induced sputum. Key Elements of pediatric specimens are addressed in the specimen collection and specimen processing sections (sections 6 and 7, respectively).

2.6 Approach to Harmonization

Since mycobacteriology laboratories use validated standard operating procedures (SOPs) and participate in trials sponsored by different networks, efforts were directed toward harmonizing Key Elements, rather than generating new SOPs. The proposed approach to harmonization includes: (1) identifying Key Elements in mycobacteriology laboratory procedures that have the greatest impact on data quality; and (2) harmonizing those elements across all laboratories in both networks. Therefore, laboratory procedures should be reviewed for the presence or absence of these necessary Key Elements, and incorporated as necessary. The AIDS Clinical Trails Group (ACTG) and Tuberculosis Clinical Trials Consortium (TBTC) have endorsed the Key Elements, and **they are now required for participation in clinical trials sponsored by these networks**. The first version of the Key Elements was implemented in the Division of Microbiology

and Infectious Diseases (DMID) 13-0057/TBTC Study 32, and the second version in the TBTC Study 31/ACTG 5349. It is anticipated that the Key Elements will be used in all future TB clinical trials, thereby creating a means for harmonization of mycobacteriology data across all trial networks.

2.7 The Mycobacteriology Laboratory Sourcebook

The Sourcebook is directed towards harmonization of laboratories participating in clinical trials and focuses on the Key Elements for TB laboratory procedures. In addition, other important technical points for each test procedure have been defined. Although these Important Technical points do not directly affect the microbiology endpoints, comparability of results among laboratories, or participant safety, they are important in the overall performance of the test. While not required, incorporation of these technical points into each laboratory SOP is strongly recommended.

The Sourcebook consists of individual sections for each laboratory test, including biosafety, quality assurance (QA), and storage of specimens and isolates. There are also sections on specimen collection, transport, and receipt in the laboratory. For the main procedures, each section includes the following:

- Background on the historical use of the method/assay, how the test results are used, and why certain test methods are required, preferred, or optional, all in the context of clinical trials
- List of Key Elements related to the procedure with discussion on how they impact the quality, reproducibility, and comparability of results, and participant safety
- Reporting terminology for study data
- Quality controls (QCs) related to the performance of the procedure

The final section (section 17) includes a series of checklists comprised of Key Elements and important technical points for each of the laboratory procedures. The checklists do not contain all the detailed steps found in complete SOPs. If more information is needed, laboratories are invited to use published and established references (2-7). Training modules are also available (8-11).

It is assumed that laboratories using this Sourcebook are already working according to Division of AIDS (DAIDS) Guidelines for Good Clinical Laboratory Practice (GCLP) standards (12). QA and biosafety references should also be used for further details (see sections 4 and 5).

While the Sourcebook is a useful reference for harmonization of most TB laboratory procedures, it does not include the following:

- Key Elements and important technical points for collection of extra-pulmonary specimens
- Processing procedures for extra-pulmonary specimens
- Specialized procedures such as quantitative cultures, determination of minimal inhibitory concentrations, strain typing, and DNA sequencing
- Collection and processing of other specimen types (serum, urine) for TB biomarkers
- Immunological methods used to diagnose TB infection such as Interferon Gamma Release Assays

The Sourcebook is intended for laboratorians participating in TB clinical studies conducted by the ACTG and IMPAACT networks, as well as other trial networks and sponsors. Additionally, it will be a valuable reference for those involved in developing and reviewing study protocols. The checklists, in particular, will be useful for the review of TB laboratory procedures to ensure Key Elements and important technical points are being performed.

2.7.1 Checklist Versions

The Key Elements and important technical points have become evident through the experience of TB laboratories participating in TB drug trials and analyses of mycobacteriology data generated in these trials. TB laboratory specialists with experience in TB drug trials and who are a part of the ACTG and IMPAACT Networks were involved in developing the checklists.

Checklists and supporting technical information for the routine TB laboratory procedures were initially developed by the ACTG TB Transformative Science Group (TSG) Core Laboratory Team. These documents were called guides, and the guides were piloted in a small group of network laboratories. The Key Elements were distributed in January 2016 to all the network laboratories in the context of TBTC Study 31/ACTG 5349 (13). All guides, accompanying checklists, and Key Elements have been revised and combined in this Sourcebook.

It is the responsibility of the laboratories to ensure they have the latest version of the checklists, which can be found on the HIV AIDS Network Coordination (HANC) website: https://www.hanc.info/labs/labresources/procedures/Pages/actgImpaactLabManual.aspx.

3 Guide to Reviewing TB Clinical Trial Study Protocols

3.1 Introduction

The guide was developed to assist in the review of tuberculosis (TB) clinical study protocols at an early stage of development. The Sourcebook includes the guide for the following reasons: (1) understanding how TB laboratory testing specified in the study protocol relates to the study design; (2) an appreciation of the process of translating the microbiology aspects of the study protocol to documents which designate the laboratory's responsibilities in the study; and (3) as a source of information for TB laboratory specialists who may be called upon to serve on a study protocol team or create TB laboratory-associated study documents.

Mycobacteriology test results and testing methodologies are important components of TB clinical trials. The extent and nature of the TB testing depends on the type of study and the outcomes measured. This guide was developed primarily for therapeutic studies (e.g., TB drug trials), of which there are different types: Early Bactericidal Activity (EBA), Phase II, Phase III/Registration, and Phase IV (post-marketing). However, the guide can also be used for the review of protocols for prevention (treatment of TB infection and vaccine) trials.

3.2 Study Protocol Review

The purpose of performing a mycobacteriology review of a study protocol is several-fold. One is to ensure that the specimens collected, collection schedule, test methods, and test results are appropriate for the study design and outcomes. Another is to ensure that all microbiology aspects are clearly described so that these details can be accurately translated into TB laboratory study protocol-related documents, such as the TB laboratory guide, laboratory case report forms (CRFs), TB laboratory Schedule of Events (SOE) table, laboratory section of the Manual of Operation Procedures (MOP), Laboratory Processing Charts (LPCs), and study-specific training.

If the mycobacteriology is not sufficiently described, a TB laboratory specialist may need to discuss this with the Study Principal Investigator (PI) or Chair. A microbiology specialist (TB laboratory expert) should be included on every study protocol team, thus enabling mycobacteriology guidance while the protocol is being developed.

3.2.1 Sections of the Study Protocol to Review

Initially, the entire protocol should be read to understand the nature of the trial and to put the TB laboratory testing into perspective. To focus on the mycobacteriology a more thorough review of the TB laboratory testing must be conducted. Although study protocols are generally organized in the same way, the laboratory testing descriptions and specifications can appear in many different places in the protocol. The following is a list of headings for sections where you will find mycobacteriology testing methods, specimen collection specifications and frequency, and analyses of test results. Different headings may be used for a section describing the same information. Such examples are listed here:

- Schema/Protocol Summary/Synopsis and Trial Flow Chart
- Objectives
- Inclusion criteria
- Reasons for discontinuation/late exclusion
- Study schedule/Treatment plan-schedule of assessments
- Laboratory evaluations/Management of participant (TB laboratory testing)
- Trial endpoints including Mycobacteriology
- Appendix – SOE/Schedule of Procedures and Evaluations

After examining these sections in the study protocol, it is advisable to search the document for the following words/terms to ensure nothing has been missed:

analysis	*laboratory test*	*sample*	*strain*
culture	*microbiology*	*smear*	*susceptibility*
endpoint evaluations	*molecular*	*specimen*	*treatment failure*
isolate	*mycobacteriology*	*sputum*	*treatment outcome*
laboratory	*resistance*	*storage*	*typing*

3.2.2 Study Protocol Review Form

To identify all the mycobacteriology aspects of the clinical study, a series of questions have been developed; and are outlined in the **Study Protocol Review Form** (see Appendix 18.1). The first section of the review form includes pertinent study-related information, and identifies the study, PIs/Chairs, study objectives, and intended study population. The second section outlines the specific questions to address during review of the study protocol. These questions help the reviewer go through the protocol to ensure all microbiological aspects are covered and clear. The questions and items help in systematically reviewing the study protocol. During this process, notes should be made about aspects that need clarification or are missing. This should be indicated in section 3 of Appendix 18.1 and discussed with the study protocol team.

3.2.3 Minimum Specifications for TB Laboratory Testing in the Study Protocol

Some study protocols describe the TB microbiology aspects in great detail, while other protocols do not, which might cause problems when implementing the study. The laboratory testing requirements will vary depending on the type of study; however, the minimum specifications for TB laboratory testing as described in Table 3-1 should be included the study protocol.

Table 3-1. TB Laboratory Testing: Minimum Specifications in Study Protocol
Collection and transport of sputum specimens
▪ Type of specimen (early morning, spot) ▪ Number of specimens per time interval ▪ Frequency of collection (time intervals/visit days) ▪ If sputum induction should be performed or optional
Sputum smear microscopy
▪ Stain method (fluorescence or Ziehl-Neelsen (ZN))

Table 3-1. TB Laboratory Testing: Minimum Specifications in Study Protocol
▪ Sputum smear method (direct, concentrated)
Molecular detection of INH, RIF, and fluoroquinolone (FLQ) resistance directly in sputum specimen
▪ Specific assay not necessary ▪ When new molecular detection assays are available, there may be a preference of one over the other, at which time it would be important to specify assay
Sputum culture on solid medium
▪ Media type or state it is optional
Sputum culture in liquid medium
▪ Specific liquid culture system not necessary unless the time to detection (TTD) values will be analyzed, in which case mycobacteria growth indicator tube (MGIT) culture system should be specified
Identification of growth on solid or liquid culture
▪ Specific method/assay not necessary, provided a rapid and highly specific method/assay is used ▪ Time intervals/visit days to perform identification ▪ Culture media to be used if solid and liquid cultures are performed
Phenotypic DST
▪ Which drugs to be tested ▪ If the minimum inhibitory concentrations (MIC) are to be determined, range of drug concentrations to be tested ▪ Time intervals/visit when isolates are to be tested ▪ Specific phenotypic method not necessary, provided an external quality assessment (EQA) of the drug susceptibility testing (DST) method is routinely performed

3.3 TB Laboratory Study Related Documents

Once the study protocol is finalized, specific documents will be created specifically for the laboratory, and may include one or more of the following components, but at a minimum, an SOE and operational guide for the laboratory are required.

- TB laboratory guide
- TB laboratory manual
- TB laboratory SOE table
- Laboratory section of the MOP
- LPCs
- Protocol Analyte List (PAL)

A TB laboratory guide usually includes an SOE, all TB laboratory-related information normally found in the MOP and LPCs, in addition to instructions on completing the laboratory CRFs and submitting quality indicators (QI) data. It does not have routine SOPs; however, specialized procedures would be included if required. The TBTC Mycobacteriology Reference Manual developed for TBTC Study 31/ACTG 5349 is an excellent example (14).

In addition to what normally would be in a laboratory guide, a TB laboratory manual includes all the SOPs that will be used for the study. Other details such as quality assurance (QA) and quality control (QC) testing, instructions for preparing media and laboratory solutions, and examples of various source

documents will also be included. Thus, these laboratory manuals are comprehensive documents; such examples are those developed for pharmaceutical supported trials and the TB Alliance studies (3, 15-17).

The TB laboratory SOE table (also referred to as table of laboratory assessments) is a concise presentation of what TB tests and procedures must be performed at time points throughout the study. Footnotes are used to describe pertinent details (e.g., eligibility criteria related to TB test results, volume of specimen, what test method). An example is found on page 6 of the TB Alliance NC-005 Microbiology Manual (16).

The MOP is an operational manual for all aspects of the study. The MOP usually has a laboratory section that summarizes TB laboratory testing to be done and provides SOPs for specimen collection and for storage/shipping of specimens and/or isolates, as well instructions for laboratory CRF completion. Thus, MOPs may contain both detailed procedures and generic guidance.

LPCs provide guidance for specimen collection, transport, and storage (see also sections 6 and 16). If a TB laboratory guide is not prepared for the study, an SOE with a comprehensive set of footnotes must be included in the LPC or in the MOP.

The PAL is a protocol specific testing plan, which lists all protocol specific tests and storage/shipping requirements. Information is requested from the laboratory on the instruments, methods/kits/reagents that will be used for each protocol analyte (TB test); the primary laboratories performing the testing; EQA participation; and appropriate testing backups (instruments and/or laboratories). PALs are available to participating site laboratories once the protocol has been published as a version 1.0 document. The ACTG Laboratory Science Group and the IMPAACT Laboratory Center require that laboratories submit a protocol specific testing plan -- a PAL for each protocol for which they will be participating, and submit updated versions whenever instrumentation, testing methods or testing backup plans change.

4 Biosafety

4.1 Background Information

The World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC) have classified *Mycobacterium tuberculosis* (MTB) amongst biosafety level (BSL) 3 pathogens, thus the safe handling of mycobacteriology specimens is critical to avoid laboratory-acquired infections and potential cross-contamination of clinical specimens. Both the CDC (18) and WHO (19, 20) have developed biosafety guidelines based on risk assessments for the handling of clinical specimens potentially infected with MTB. To ensure the safety of staff, at minimum, handling of clinical specimens potentially infected with MTB (including microscopic examination of smears, specimen processing, primary cultures, and nucleic acid amplification assays) must be carried out in a BSL-2 containment laboratory with safety equipment and BSL-3 work practices in place. Manipulating positive cultures of MTB and drug susceptibility testing (DST) should be done in a BSL-3 containment laboratory, if available. Manipulation of all clinical specimens and cultures suspected or known to contain MTB must be carried out in a Class II biosafety cabinet (BSC) with strict adherence to biosafety practices, including use of appropriate personal protective equipment (PPE), tuberculocidal disinfectants, and waste management.

MTB is primarily spread through airborne transmission; thus, many aerosol-generating microbiological procedures can contribute to the spread of MTB. To avoid exposure to MTB, special precautions must be taken when handling containers with clinical specimens, performing centrifugation; centrifuging; pipetting; mechanical homogenizing; and sonicating, heating, boiling, and working with bacteriological loops, as these can contribute to formation of aerosolized droplets of MTB. More information is available in the above references.

4.2 Biosafety Checklist

A biosafety checklist was not developed for the Sourcebook since there is an existing Patient Safety Monitoring in International Laboratories (pSMILE) Laboratory Biosafety Inspection Checklist for TB laboratories (21). The checklist covers all standard and special microbiology practices, safety equipment, and laboratory facilities that are applicable to laboratories performing TB testing.

5 Quality Assurance

5.1 Background Information

5.1.1 Introduction

Quality assurance (QA) is a system targeting continuous improvement of laboratory results and services. It is based on components such as quality control (QC), external quality assessment (EQA), quality monitoring (QM), and validation. All tuberculosis (TB) laboratories carry out some form of QA. Those being accredited by an accrediting agency, including the College of American Pathologists (CAP), International Organization for Standardization (ISO), and South African National Accreditation System (SANAS), are required to have a more comprehensive QA program in place. The examination and monitoring of multiple parameters involved in laboratory testing are also guided by Good Clinical Laboratory Practice (GCLP) standards (12), which are required for all laboratories participating in clinical research. Adherence to GCLP standards helps assure regulatory authorities that the data produced in clinical trials are a true reflection of the laboratory results obtained during the study.

5.1.2 QA Activities from Specimen Collection to Results Reporting

Just as QA is a critical component of laboratory testing for diagnostic services, it is also a critical component of TB testing in clinical trials. In TB drug trials, isolating *Mycobacterium tuberculosis* (MTB) is the most important TB test performed, because these results are used to determine sputum culture conversion (SCC). SCC is the most common measure of efficacy in Phase II/III TB trials. To ensure accuracy and consistency in the isolation of MTB (and a reliable study endpoint), it is essential that each specimen is handled (from time of collection until received in the laboratory), processed, cultured, and results recorded per the laboratory's standard operating procedures (SOPs). Each of these steps is examined frequently via QA activities, including:

- Specimen transport time is monitored by checking the time of collection with the time of receipt, and the cooler box, if used, is checked to see if the ice packs are cold (section 6)
- Recovery of MTB is monitored through the use of a positive control in the sputum processing procedure (sections 5.1.3 and 7.3)
- Performance of the media (both solid and liquid) is monitored by QC testing the media with known quantities of MTB (section 5.1.3)
- Accuracy of the MTB identification test is monitored through the use of positive and negative controls (section 11.3)
- Proficiency of culture accuracy and technical competency are monitored by testing mock sputum specimens in a proficiency testing (PT) panel (section 5.1.4)
- Culture results are reviewed by two persons before reporting (section 5.1.5)
- Number of cultures contaminated (the consequence of which is lost culture results) is examined monthly (section 5.1.5.1)

Thus, to ensure high quality culture results, multiple, diverse QA activities are required.

5.1.3 Quality Control

QC testing consists of monitoring test performance, media quality, reagent activity, and test accuracy. QC testing is performed at two stages: before placing new lots of reagents, media, kits, etc. into use, and when testing participant specimens/cultures/isolates. Including QC samples in routine testing is referred to as *internal QC testing* (or internal controls). Furthermore, some commercial assays have controls incorporated in the test, which also may be called internal or test controls. Note that monitoring test performance before placing new lots into use is not a substitute for testing controls in parallel with patient specimens/cultures/isolates.

The use of a viable MTB suspension as a positive control is sometimes not recommended, since it could be a source of cross-contamination (22, 23). However, in the settings of clinical drug trials, positive controls must be included (tested in parallel with clinical specimens) to determine the quality of the processing procedure and culture performance. Furthermore, there are often safety concerns when using multi-drug resistant (MDR) strains as controls for drug susceptibility testing (DST). In laboratories where MDR strains are tested, reference strains or clinical isolates with drug resistance should be used in QC testing new lots of drugs/kits. Options to use mono-resistant strains to test drugs such as isoniazid (INH), rifampin (RIF), and fluoroquinolones (FLQ) should be considered.

A summary of essential QC testing for TB laboratories appears in Table 5-1. QC composition, placement in the run, and testing frequency are often subjects of debate. These differ depending on the test but can also differ from laboratory to laboratory for the same test. A discussion of these details can be found in the individual sections of the Sourcebook and various references (3, 7, 22, 24-26).

Table 5-1. Summary of QC Testing

Test Evaluated	QC Testing
Specimen processing	*Before putting into use:* Verifying pH of each new lot of phosphate buffer.
	Internal QC testing: Processing of positive and negative controls with patient specimens and setting up cultures with resuspended pellets (see 7.3).
Smear microscopy	*Before putting into use:* Staining and reading of positive and negative control slides before putting stain into use. Control slides are the same as those used when testing patient specimens.
	Internal QC testing: Staining and reading of positive and negative control slides at least daily or with every batch of patient specimens (see 8.3).
	Smear review for technical competency: Ten slides from the previous month ranging from negative to scanty, 1+, 2+, and 3+ are selected. Technicians read and record results in a blinded manner. These results are reviewed for consistency and discrepancies.

Table 5-1. Summary of QC Testing	
Test Evaluated	**QC Testing**
MGIT culture	*Before putting into use:* Visually examine the mycobacteria growth indicator tube (MGIT) media, growth supplement, and PANTA before use to ensure these have not been contaminated or changed appearance during storage or transport and tubes are not cracked/damaged.
	Testing performance characteristics before putting MGIT media into use: MGIT media supplemented with OADC/PANTA mixture tested with suspensions of MTB H37Rv (ATCC 27294™), *M. kansasii* (ATCC 12478™), and *M. fortuitum* (ATCC 6841™) prepared as described in the Foundation for Innovative New Diagnostics (FIND) MGIT Manual (24) to check the growth rates per time to detection (TTD). Alternative strains can be used, e.g., MTB H37Ra (ATCC 25177™) and clinical isolates of slow and rapid growing non-tuberculosis mycobacteria (NTM).
Solid media culture *(media prepared by the laboratory)*	*Sterility testing before putting any solid media into use:* 1-3 % of batch is incubated for 14 days to rule out bacterial/fungal contamination.
	Testing performance characteristics before putting mycobacterial media into use: ▪ Positive control –three dilutions (10^{-2}, 10^{-3}, 10^{-4}) of McFarland No. 1 suspension of MTB H37Rv; inoculate 200 µL of each suspension; 10^{-4} suspension critical to ensure isolating low numbers of MTB; test both non-selective and selective media; alternatively, MTB H37Ra (ATCC 25177™) can be used. ▪ Negative control – non-AFB organism (e.g., *Staphylococcus sp.*), to check inhibitory effectiveness of selective media (e.g., 7H11S or LJ-PACT); test only selective media.
	Testing performance characteristics before putting blood agar plate (BAP), tryptic soy agar (TSA), brain heart infusion (BHI), or chocolate agar into use: Positive control – McFarland No. 1 standard of *Escherichia coli* or *Staphylococcus aureus* (clinical isolate or reference strain) to show growth of typical colonies within 48 hours
Solid media culture *(media purchased commercially)*	*Before putting into use:* Visually examine media before use to ensure tubes/plates are not cracked/damaged and medium has not been contaminated or changed appearance during storage or transport.
	Sterility testing before putting media into use: Solid media stored ≥ 1 month, check for sterility and growth performance, as for in-house prepared media.
MPT64 antigen identification (ID)	*Before putting into use:* Testing new lot numbers with negative and positive controls before putting kits into use. Composition of controls is the same when testing in parallel with patient specimens (section 11.2).
	Internal QC testing: In addition to the internal reagent control in the device, test negative and positive controls with each batch of cultures or at least weekly (see 11.3).

Table 5-1. Summary of QC Testing	
Test Evaluated	**QC Testing**
MGIT DST	*Before putting into use:* Testing new lots of MGIT medium, growth supplement, and drugs with a drug-susceptible QC strain of MTB before putting into use. MTB H37Rv is the preferred reference strain. If drug-resistant isolates are routinely tested, include a clinical isolate known to be MDR-TB (resistant to INH and RIF). *If second-line drugs are tested, options are to include:* ▪ Clinical isolates mono-resistant to FLQ and AG; or ▪ Clinical isolates MDR+FLQ resistant and MDR+AG/CP resistant (pre-XDR-TB isolates with resistance to FLQ or AG/CP).
	Internal QC testing: Include a drug-susceptible QC strain of MTB at least once per week or with each batch of isolates (see 12.3).
Solid media proportion DST	*Before putting into use:* Testing new lots of medium and drugs with a drug-susceptible QC strain of MTB before putting into use. MTB H37Rv is the preferred reference strain. If second-line drugs are tested, include isolates with resistance to FLQ and AG/CP as described above for MGIT DST.
	Internal QC testing: Include a drug-susceptible QC strain of MTB at least once per week or with each batch of isolates as described above for MGIT DST (see 13.3).
MTBDR*plus*, MTBDR*sl*	*Testing each new lot of kits before putting into use:* ▪ Positive control – an in-house prepared control (e.g., resuspended pellet of the specimen processing positive control) (section 14.3). Or, INTROL™ External Run Controls used with Xpert (section 15.3). There are no INTROL™ External Run Controls for the FLQ and AG/CP probes in the MTBDR*sl* assay. ▪ Negative control – molecular grade or sterile water. Or if INTROL™ External Run Controls are used for positive controls, use the INTROL™ negative control.
	Internal QC testing: In addition to the internal controls present in the device, test negative and positive controls with each batch of specimens or at least once weekly. Positive – in-house prepared control; negative – molecular grade or sterile water (see 14.3).
Xpert® MTB/RIF	*Before putting into use:* Testing each new lot of cartridges before putting into use and annual calibration (section 15.3): INTROL™ External Run Control (catalog # TBWT-04) as MTB-positive control and RIF-susceptible control (can also be used as INH-susceptible control). INTROL™ External Run Control (catalog # TBMDR1-04) as RIF-resistant positive control (can also be used as INH-resistant control). INTROL™ External Run Control (catalog # TBNEG-04) as negative control.

Table 5-1. Summary of QC Testing	
Test Evaluated	**QC Testing**
	Internal QC testing: Internal controls (Sputum Processing Control and Probe Check Control) are incorporated in the assay to check test performance each time an assay is performed.

5.1.4 External Quality Assessment (EQA)

The purpose of EQA is to assess technical competency as well as test accuracy and reproducibility. EQA is sometimes referred to as PT. Various EQA programs exist for TB laboratories; including the CAP (https://estore.cap.org) and the INSTAND (https://www.instand-ev.de/en.html). Typically, the PT panels consist of unstained slides for smear and sputum specimens (artificial sputum spiked with mycobacteria) for culture, identification, and DST. Panels of MTB isolates for DST are available from the World Health Organization (WHO) TB Supranational Reference Laboratory Network (https://sites.google.com/site/srtblaboratories/home).

Molecular tests can also be assessed using the simulated sputum specimens from CAP and INSTAND. More recently, a specific molecular EQA program has been developed for GeneXpert and line probe assays (LPAs) using dried cultured spots (http://smartspotq.com/). The SmartSpot Quality program is endorsed by the AIDS Clinical Trials Group (ACTG) and International Maternal Pediatric Adolescent AIDS Clinical Trials (IMPAACT) networks. Although DNA (deoxyribonucleic acid) panels are available (QC for Molecular Diagnostics, UK; UK National External Quality Assessment Service (UKNEQAS) for Microbiology) and practical for biosafety aspects, testing such samples prevents quality assessment of DNA extraction; thus, they are not recommended for EQA of molecular tests.

It is essential that laboratories participating in clinical trials perform EQA of all the TB tests performed for the trials. Most clinical trials networks mandate that laboratories subscribe to EQA programs. In the ACTG/IMPAACT networks, satisfactory results must be obtained to maintain approval status for participating in clinical trials. Performance by the individual laboratories in EQA programs is monitored by Patient Safety Monitoring in International Laboratories (pSMILE) and the networks.

5.1.5 Quality Monitoring

QM is a part of the overall quality management program in diagnostic service laboratories. QM is equally important in laboratories that participate in clinical research. QM encompasses a range of activities which are the same whether performing routine service or clinical research. These include, but are not limited to: monitoring critical parameters of equipment (e.g., temperature, centrifugation speed, carbon dioxide level, and biosafety cabinet (BSC) airflow), specimen storage and transport conditions before receipt in the laboratory, and quality indicators (QI). Cleaning and maintaining the equipment is essential for optimal test performance and accurate test results. Additionally, reviewing test results for accuracy before reporting is another quality check.

5.1.5.1 Maintaining and Reviewing Study Data

Accurate and complete documentation of all mycobacteriology data is essential for the scientific integrity of the study. Before laboratory results are reported to the clinicians or study coordinators, results on the local report form must be checked with those on the source document(s). Likewise, results on the study report form or laboratory case report form (CRF) are compared with those on the source document(s) before submitting the results to the study sponsor. Source documents are the original worksheets/logs/registers where results and observations are entered or recorded. For example, source documents for a positive MGIT culture result will include:

- MGIT instrument off-loaded report
- Ziehl-Neelsen (ZN) smear log
- BAP culture log
- ID test worksheet

All of the above source documents must be reviewed to ensure the correct interpretation of the final culture result, and that no transcription errors occurred. Since final test results are often based on interpretation of several results (e.g., MGIT culture, Hain LPAs), the recording of data on local and study report forms and review with source documents is recommended to be performed by a laboratory staff member. Additionally, before sending out the report or uploading to the study database, results must be reviewed by a second person. This aligns with the Good Clinical Practice (GCP) standard of verification of data transferred from paper source of results (source documents) to laboratory information system or study CRF (27). Names or initials of persons reviewing/reporting the results and the corresponding date should be on the study report form (27).

Timely reporting of study data assists in the management of the study. Results from screening tests (i.e., smear microscopy and Xpert MTB/RIF), allow participants to be screened for study eligibility in a timely manner. Other study data should be reported as soon as they are available to facilitate data management by the study sponsor.

Since laboratory documents are subject to review by auditors and regulatory inspectors, they must be filed in a timely manner using an organized system to facilitate easy retrieval and review, while protecting the confidentiality of all participants. If corrections to data recorded on laboratory documents need to be made, a single line is drawn through the incorrect data, initialed, and dated per GCP guidelines.

5.1.5.2 Quality Indicators in the Context of TB Trials

QI data are those TB test results which, when analyzed in a particular manner, indicate the quality of those results. Examples of QI data are listed in Table 5-2. QI data are collected monthly, based on test results reported during the month. Over time, cumulative data are used to establish baselines or normal values for the patient population(s) being tested by the laboratory. When a laboratory participates in a clinical trial, the monthly data can vary significantly depending on the TB testing requirements, screening and enrollment rate, and at what visit/time point participants are in the study timelines. The proportion of

baseline/diagnostic specimens (those from untreated individuals) and follow-up specimens (those from individuals receiving treatment) received differs from month to month and year to year.

The total number of specimens received and the frequency of smear-positive and smear-negative specimens, as well as MTB-positive and MTB-negative cultures observed by the laboratory, fluctuates considerably. Correlation between sputum smear and culture is different between baseline/diagnostic and follow-up specimens, and differs among individuals throughout the treatment period. Likewise, culture contamination rates can vary between baseline and subsequent time intervals throughout the study. Furthermore, not all the standard tests (sputum microscopy, culture on solid media, MGIT culture, identification of culture, and DST) are performed on all the sputum specimens submitted, as the testing required is study-specific. Therefore, establishing baselines and monitoring QI data are challenging for the laboratories that receive mainly study specimens. Monitoring QI data for a particular study is difficult in any laboratory when the number of study specimens received each month is low. Some of the parameters recommended as QI data of TB testing are not informative or have to be examined in a different manner to be meaningful or useful QI data. Laboratories participating in clinical trials must therefore adapt the QI data targeting routine diagnostic activities (28, 29) to their setting as much as possible (e.g., by analyzing results separately according to baseline and follow-up specimens). Also, when the number of test results is low, it may be prudent to examine the monthly data from all specimens (routine and study) to obtain reliable and meaningful QI data.

The source of QI data varies from laboratory-to-laboratory, but typically worksheets, log books, registers, and laboratory information systems are examined for pertinent patient and QC results. The format for reporting QI data to a study sponsor will also vary. Please see (https://www.hanc.info/labs/labresources/procedures/Pages/actgImpaactLabManual.aspx) for an example of a QI report form.

Table 5-2. Examples of Quality Indicators

Test or Procedure	Examples of Quality Indicators
Specimen collection, storage, and transport	▪ Percentage of specimens with volume less than the minimum requirement or specimens rejected due to insufficient volume ▪ Percentage of specimens transported after 1 hour of collection without using a cooler box with ice packs
Smear microscopy	▪ Percentage of smears reported as positive (separated by baseline and follow-up specimens) ▪ Percentage of smear results reported within target turnaround time (TAT)

Table 5-2. Examples of Quality Indicators	
Test or Procedure	**Examples of Quality Indicators**
MGIT culture	▪ Percentage of MGIT cultures reported as *Mycobacterium tuberculosis* complex (MTBC) (separated by baseline and follow-up specimens) ▪ Percentage of MGIT cultures reported as MTBC and contaminated (separated by baseline and follow-up specimens) ▪ Percentage of MGIT cultures reported as contaminated (separated by baseline and follow-up specimens) ▪ Percentage of MGIT cultures reported as non-tuberculosis mycobacteria (NTM) ▪ Correlation between smear-positive diagnostic specimens and acid fast bacilli (AFB) positive cultures ▪ Percentage of smear-negative diagnostic specimens resulting in MTBC-positive cultures ▪ Mean/median and range of MGIT TTD for sputum processing positive controls ▪ Percentage of MGIT results reported within target TAT
Solid media culture	▪ Percentage of positive solid media cultures reported as MTBC ▪ Percentage of solid media cultures reported as contaminated ▪ Percentage of positive solid media cultures reported as NTM ▪ Correlation between smear-positive diagnostic specimens and AFB-positive cultures ▪ Correlation between solid media cultures and MGIT cultures ▪ Percentage of smear-negative diagnostic specimens resulting in *M. tuberculosis* complex-positive cultures ▪ Percentage of solid media culture results reported within target TAT
DST	▪ Percentage of MGIT DST with X200 errors ▪ Percentage of MGIT DST with X400 errors ▪ Percentage of successful DST repeats with X200 errors ▪ Percentage of successful DST repeats with X400 errors ▪ Percentage of MGIT DSTs reported within the target time
Molecular tests (Hain LPAs, Cepheid Xpert MTB/RIF)	▪ Percentage of indeterminate/invalid results for MTB ▪ Percentage of indeterminate/invalid results for INH ▪ Percentage of indeterminate/invalid results for RIF ▪ Percentage of indeterminate/invalid results for FLQ ▪ Percentage of successful repeats with indeterminate/invalid specimens ▪ Percentage of molecular test results reported within the target time ▪ Mean Ct (cycle threshold) values of Sample Processing Control (SPC) (Xpert) ▪ Percentage of molecular test results reported within target TAT

5.1.5.3 Culture Contamination Rates

The study sponsor will specify the QI data to be reported. Typically, QI data that allow early identification of deviations (e.g., proportion of contaminated cultures or proportion of inconclusive/indeterminate results) are required to be reported monthly. This enables such problems to be addressed as soon as possible instead of during data verification and analysis.

Of all the possible QI data, culture contamination rate is the single most commonly monitored QI data in clinical trials. This is due to culture results being lost for analysis, especially if MTB is not detected in the presence of contaminant growth. Culture contamination is defined as any growth other than mycobacterial. An example for reporting MGIT culture contamination from TBTC Study 31/ACTG 5349 is shown in **Table 5-3**. Results are examined for cultures contaminated without MTB (i.e., those discarded due to contamination). The same is collected for solid media cultures; however, an example is not shown. Contamination rates are also discussed in sections 7.3.2 and 9.1.6.

Table 5-3. Quality Indicator Report – MGIT culture contamination

Indicator	Numerator	Denominator	Laboratory Result
MGIT culture contamination rate, baseline specimens	Number of baseline MGIT cultures discarded this month due to contamination: _____	Number of baseline MGIT cultures reported this month: _____	(Numerator/Denominator) x 100 = _____%
MGIT culture contamination rate, follow-up specimens	Number of follow-up MGIT cultures discarded this month due to contamination: _____	Number of follow-up MGIT cultures reported this month: _____	(Numerator/Denominator) x 100 = _____%
Overall MGIT culture contamination rate	Number of MGIT cultures discarded this month due to contamination: _____	Number of MGIT cultures reported this month: _____	(Numerator/Denominator) x 100 = _____%

5.1.6 Validation

A study may require a specific test/method that the laboratory has not previously performed or established. The laboratory must confirm that it can properly perform the test/method before providing results from this test/method. For standard methods and commercial kits, it is only necessary to validate that the method works in the laboratory as expected. Note that laboratory-developed methods would not be required in a clinical trial and non-standardized methods may not require validation, especially if there is not a comparative method or result. Validation is based on the comparison of the new method/test results with results from a standard, verified method/test yielding the same type of results. The comparative method may be carried out in the local laboratory or results from this method may be available to the local laboratory. In either case, the comparative testing is performed on the same specimen/isolates.

Before carrying out the validation the laboratory must have a procedure describing all pertinent details (e.g., specimen/isolate source, type, specifications, and number; the reference method; number of replicative tests; number of persons to perform testing; testing timeframe; expected or acceptable results). The technologists performing the validation should do the testing in a blinded manner (i.e., results from the comparative method are not known to those performing the method being validated). The acceptable percent agreement between the new and comparative test/method must be determined before conducting the validation. It is not possible to recommend one procedure for validating all

tests/methods. Guidance is available from pSMILE (30).

5.2 Quality Assurance Checklist

See section 17.1.

6 Specimen Collection, Transport, and Laboratory Receipt

6.1 Background Information

6.1.1 Introduction

Although laboratory staff is not involved in the collection of respiratory specimens, specimen collection and transport is an important part of the pre-analytical testing phase. If culture contamination rates are unacceptable, the laboratory staff may have to review specimen collection, on-site storage, and transport with the clinical research team and/or collection staff. Thus it is important for the laboratory to have knowledge of the collection procedures and understanding how the Key Elements of these relate to the microbiology endpoints of clinical trials. Proper handling of specimens once received in the laboratory is included in this section as it is also an important part of the pre-analytical testing phase.

6.1.2 Infection Control Measures

During specimen collection, infection control measures should be observed (20). Refer to the AIDS Clinical Trials Group (ACTG) and International Maternal Pediatric Adolescent AIDS Clinical Trials (IMPAACT) infection control standard operating procedure (SOP) for more information (31).

6.1.3 Specimen Collection SOPs

An ACTG and IMPAACT SOP for collection of expectorated sputum is available online (32). Collection SOPs for other specimen types will be developed in the future. Until then, studies should use their Manual of Operating Procedures (MOP) or Laboratory Processing Charts (LPCs) to guide sites on specific specimen collection procedures. References for gastric aspirate collection as well as sputum induction are also available and listed in Table 6-1.

Table 6-1. Specimen Collection References

Specimen Type	References
Sputum (expectorated)	(32)
Sputum (induced)	(33) p.122 (34) p.83
Gastric aspirate	(33) p.120 (35)

The protocol or study-related documents (MOP, laboratory guide, etc.) should include the following study-specific aspects of specimen collection:

- Which participants will have sputum collected
- Number of specimens required per participant per visit
- Type of sputum specimens: spot versus early morning
- Time of collection: early morning, during the day, overnight
- If multiple specimens are collected at one visit, specify minimum time between specimens

- Schedule of specimen collection: baseline, week 2, week 4, etc.
- Site of collection: home, clinic/hospital
- If expectorated sputum collected at home is unsatisfactory in quality (saliva, spit), insufficient in quantity (< 1 mL), or transported from home to clinic after a delay (> 3 hours), specify that another specimen should be collected at the clinic
- If the participant does not produce expectorated sputum, specify if sputum induction is required or if collection can be obtained at another time

6.1.4 Collection and Neutralization of Gastric Aspirates

Gastric aspirates are collected in young children who cannot spontaneously expectorate a sputum specimen for tuberculosis (TB) microbiology investigations. Respiratory secretions swallowed by the child during the night are collected early in the morning after fasting. The procedure is preferably performed early in the morning in the ward as an inpatient; however, the technique is also performed in outpatients when the child comes for a study visit. A minimum fasting period of at least 4 hours before early morning gastric aspirate is necessary. Children over 1 year of age can cope with a full overnight fast (6-8 hours), and this is preferable over a shorter fast (4 hours). Some also perform the procedure in daytime after a fast of at least 4 hours although most references recommend early morning. Efforts should be made to collect the gastric content by aspiration first, as lavage introduces dilution, though there is little data comparing both methods. Most references recommend to collect at least 5 to 10 mL (23, 33, 35, 36) although others recommend larger volumes (50 mL) (37). If adequate volumes are not obtained, lavage can be performed using sterile water or saline.

Neutralization of gastric aspirates with bicarbonates is recommended by many organizations, including the World Health Organization (WHO) and the American Society of Microbiology. One recent study (38) has cast some doubt on this practice, finding a significant reduction in culture yield on neutralized gastric specimens. However, more research is needed to confirm these findings before changing practices, since acidity is known to be detrimental to mycobacteria.

Recommended procedures to neutralize gastric aspirate vary from one reference to another; the formulation and percentage of bicarbonates vary, as well as the quantity added to the specimen. In practice, some sites prefer to use the powder form prepared by the laboratory and dispensed in the specimen container. Other sites use the liquid form. Some sites send the specimen as quickly as possible to the laboratory where the specimen is neutralized at reception. Some verify the pH is neutral. There are no recently published data saying that one formulation is superior to the others. Options for neutralization of gastric aspirates are listed in Table 6-2.

Table 6-2. Options to Neutralize Gastric Aspirate Specimens	
Option	**References**
Place the specimen in a sterile container with 100 mg of sodium carbonate.	(23) (4) (36)
Add an equal volume of sodium bicarbonate solution to the specimen (8% sodium bicarbonate solution).	(33)
To prepare the bicarbonate solution for neutralization, dissolve 2.5 grams sodium bicarbonate in 100 mL deionized water. Filter the solution through a 45 µm filter. Add approximately 1.5 mL to each specimen and measure the pH. Adjust the pH of the specimen until it is neutralized.	(35)
For each 35-50 mL of gastric washings, use 1.5 mL of 40% anhydrous disodium phosphate or two pH 7.4 buffer capsules or tablets.	(39)

6.1.5 Appearance of Sputum

Although the appearance of the sputum is usually recorded by TB laboratories and can be an indicator of specimen quality, it is not a Key Element like the volume of sputum collected. Typically, sputum specimens are classified as "salivary," "mucous," "purulent," or "mucopurulent" based on macroscopic examination, which is a subjective interpretation. Often studies require the appearance to be reported; however, this parameter is rarely examined. If the participant has an unusually high number of contaminated cultures or culture results are erratic during the course of treatment, the classification/appearance in addition to the volume of these specimens may be considered as a means of assessing quality. The primary objective in examining specimen appearance is to identify those that are saliva and minimize the number of saliva specimens submitted, as the *Mycobacterium tuberculosis* (MTB) yield is low from these specimens. If the sputum is water like, encourage the participant to submit a new specimen (i.e. exudative material brought up from the lungs after a deep, productive cough).

6.1.6 Transport and Laboratory Receipt

One of the key elements of specimen collection, transport and receipt is that specimens must be transported to the laboratory in a cool box (2-8^{o}C) as soon as possible after collection. Monitoring temperature in the cool box with a thermometer is not usually required unless long distances or delays in transport time are involved. The laboratory should check that the ice packs and specimens are cold and document this at specimen reception.

Specimens may be left at an unattended window or a general laboratory receiving area and not receive proper attention. Often the arrival time in the laboratory does not coincide with the processing time. If the specimen is at room temperature more than 1 hour, there is a risk of contaminants (bacteria or fungi) growing and compromising the quality of the culture (23). More information on TB specimen transport is available elsewhere (36).

6.2 Key Elements of Specimen Collection, Transport, and Laboratory Receipt

Table 6-3. Key Elements of Specimen Collection, Transport, and Laboratory Receipt

Procedure	Key Element	Effect	Impact
Expectorated and induced sputum collection	Participant is to rinse mouth with boiled/sterile/bottled or distilled water prior to sputum collection.	Specimen Quality	Microbiology endpoints
Expectorated and induced sputum collection	Collect at least 3-5 mL of sputum. If larger volumes cannot be obtained, a minimum of 1 mL is acceptable but not optimal to recover MTB. If not possible to collect at least 1 mL expectorated sputum, the study protocol or MOP must specify if sputum induction is required, or if another specimen must be obtained at another time.	Specimen Quality	Microbiology endpoints
Gastric aspirate collection	Collect at least 5-10 mL. Larger volumes are preferred. A minimum of 1 mL is acceptable but not optimal to recover MTB. See section 6.1.4 for details.	Specimen Quality	Microbiology endpoints
Gastric aspirate collection	Collect gastric aspirate after a minimum fasting period of at least 4 hours in infants. Early morning collection is preferred. See section 6.1.4 for details.	Specimen Quality	Microbiology endpoints
Gastric aspirate collection	Gastric aspirate must be pH neutralized as soon as possible after aspiration, unless the laboratory can neutralize or process the specimen within 4 hours of collection. See section 6.1.4 for details.	Specimen Quality	Microbiology endpoints
Storage until transport	Store respiratory specimens in a refrigerator or cool box (2-8^{o}C) if not transported to the laboratory within 1 hour of collection to ensure the specimen is maintained on cold chain.	Specimen Integrity	Microbiology endpoints
Transport	Transport specimens to the laboratory in a cool box (2-8^{o}C) as soon as possible after collection. Respiratory specimens must be delivered to the laboratory as soon as possible and within 24 hours of collection; however, delays up to 3 days in transport from the clinic to the laboratory may be allowable if the transport distance is long, the specimen is kept at 2-8^{o}C, and the extended transit time is agreed upon by the study/protocol team (32).	Specimen Integrity	Microbiology endpoints
Laboratory specimen receipt	Store respiratory specimens in a refrigerator or cool box (2-8^{o}C) if not processed within 1 hour of receipt at the laboratory.	Specimen Integrity	Microbiology endpoints

Table 6-3. Key Elements of Specimen Collection, Transport, and Laboratory Receipt			
Laboratory specimen receipt	If gastric aspirate was not pH neutralized at the site of collection (in the clinic), the laboratory must neutralize the specimen within 4 hours of collection, or process it. See section 6.1.4 for details.	Specimen Integrity	Microbiology endpoints

6.3 Specimen Collection, Transport, and Laboratory Receipt Checklist

See section 17.2.

7 Specimen Processing

7.1 Background Information

7.1.1 Introduction

This section focuses on processing respiratory specimens for acid-fast bacilli (AFB) smear, *Mycobacterium tuberculosis* (MTB) culture, and drug susceptibility testing (DST) (phenotypic or genotypic). Respiratory specimens included here are the most commonly used in tuberculosis (TB) drug trials (i.e., expectorated sputum, induced sputum, and, more specifically in children, gastric aspirates). The N-acetyl L-cysteine (NALC)-sodium hydroxide (NaOH) method is widely used and validated with the BACTEC™ Mycobacteria Growth Indicator Tube (MGIT) TB System. Since MGIT culture is used in all TB drug trials, the NALC-NaOH method is the standard.

A separate standard operating procedure (SOP) has been developed for sputum processing for storage and TB biomarker research (LTC SOP 074 v1.0) (40). It describes the procedure to process and store sputum specimens until they are shipped to a reference laboratory or a formal repository for future TB research investigations. This processing differs from the routine processing for smear and culture.

7.1.2 Processing Reagents

Reagents used in processing respiratory specimens can be prepared in house or purchased. If a commercial kit is used, manufacturer instructions must be followed. Reagents and specimens should be brought to room temperature before processing to optimize activity of NALC. Specimens can be slowly mixed on a platform stirrer during the decontamination step to optimize NALC digestion, which in turn optimizes NaOH decontamination.

Specimens must be decontaminated with a final NaOH concentration of 1.0 to 1.5%, as 1.5% is the maximum allowed with the MGIT system. The final concentration refers to the concentration of the NaOH after adding an equal volume of NALC-NaOH solution to the specimen (24). Details on how to calculate the final concentration can be found in the Global Laboratory Initiative (GLI) Manual (3). Sterile cylinders, serological pipettes, and/or disposable sterile transfer pipettes must be used in preparing processing reagents and adding reagents to specimens.

7.1.3 Volume of Specimen to Process

Recommended specimen processing volumes are as follows:

- For volumes of specimen ≤ 10 mL, an equal volume of NALC-NaOH must be added to the specimen.
- For volumes of specimen > 10 mL, centrifuge at 3000 *x g* for 15-20 minutes in a sterile screw cap 50-mL centrifuge tube, decant the supernatant, resuspend the sediment in 2 to 5 mL of sterile distilled water, and proceed as for sputum (5).

7.1.4 Volume of Resuspended Pellet

After the centrifugation step, the pellet is resuspended with phosphate buffered saline (PBS, pH 6.8). The amount of buffer used often depends on what the resuspended pellet will be used for. Various references specify different volumes. Some references (7, 24) indicate to add 1 to 2 mL of PBS (pH 6.8) to the sediment, while others (3) indicate to resuspend the sediment with the help of a pipette or vortex mixer to a final volume of 2.5 mL.

If the suspension is to be used for smear and culture in MGIT and on solid media, 1 mL is a sufficient volume. If the suspension is to be used for a smear and DNA extraction, 1 mL is also sufficient. MTBDR*plus* and MTBDRs*l* instructions indicate the pellet should be resuspended in a maximum volume of 1 to 1.5 mL. When 1 mL of PBS is often not enough volume to thoroughly suspend the pellet, it is preferable to use more. This is particularly evident when processing larger volume specimens from individuals with moderate to advanced TB (e.g., pellets can be about 1 mL). If additional tests will be performed, extra culture media inoculated, or concentrated specimens are to be saved, a higher volume (> 2 mL) could be required. In such situations, the study-specific laboratory documents will specify the final volume. However, using larger volumes introduces a dilution factor, which can be a problem in the case of paucibacillary disease, such as pediatric specimens.

The important point is to add PBS to a final specified volume (i.e., adding an unspecified amount of buffer to the pellet so the final volume of the suspension is equivalent to the specified volume or within the acceptable range). Occasionally, there will be a large pellet and adding PBS to a final specified volume does not adequately suspend the pellet. In such cases, additional PBS must be added and the final volume of the resuspended pellet noted on the processing worksheet.

Resuspended pellet volume is a Key Element which is further discussed in section 7.2.

7.1.5 Time from Processing to Setting Up Cultures

It is important to set up cultures immediately following the suspension of the decontaminated, concentrated specimen for the following reasons:

- Provides rapid culture results which confirm an individual has TB
- Optimizes recovery, especially when the bacillary load in the sputum is low
- Avoids laboratory errors that can occur when labeling and setting up cultures at a later time

If there is no time to prepare smears and set up cultures, the processing should be postponed. Ideally, specimens should be processed on the day of receipt, but a delay of 1 to 3 days is acceptable when specimens are received late in the day, especially on Fridays. If the delay in processing will be 3 days, the time from collection to receipt must not be more than 2 days (i.e., maximum allowable time from collection to processing is 5 days).

7.2 Key Elements of Specimen Processing

Of the seven Key Elements of specimen processing listed in Table 7-1, six of these could potentially impact the microbiology endpoints of TB drug trials. Specimen processing must be performed per these Key Elements to: (1) optimize isolation of MTB, especially when the sputum is converting from MTB culture-positive to negative; and (2) minimize bacterial and fungal contaminants in the culture.

Negative cultures due to excessive exposure to NaOH (killing of MTB) or inadequate concentration/sedimentation of MTB in the specimen are erroneous results. Non-sterile processing reagents may lead to contaminated cultures. The consequence of contaminant overgrowth is lost culture results and lost data points. In either situation, all analyses related to sputum culture conversion may be adversely affected.

Two variable characteristics of respiratory specimens that affect test outcomes are the amount of MTB they contain, and the volume collected. To capture all the MTB, the entire specimen volume up to 10 mL is processed, optimizing the yield of MTB. To avoid diluting out low numbers of MTB and compromising recovery in culture, a specified volume or limited volume range of PBS is used to resuspend sputum pellets. Resuspending the sputum pellets to approximately the same final volume provides an element of consistency among all specimens, i.e., the MTB in a specimen is contained within a defined, consistent volume. Consistency in this quantitative step of the processing procedure enables culture results to be compared among all sites participating in the trial. The study sponsor may require a specific final volume of the resuspended pellet. If not, the final volume should be between 1.5 and 2.0 mL.

Table 7-1. Key Elements of Respiratory Specimens Processing Procedure

Key Element	Effect	Impact
Use all respiratory specimen up to 10 mL in processing	Isolation of MTB	Microbiology endpoints
Decontaminate respiratory specimen with a final NaOH concentration of 1.0-1.5%	Isolation of MTB	Microbiology endpoints
Decontaminate respiratory specimen for 15-20 minutes prior to adding PBS (pH 6.8)	Isolation of MTB	Microbiology endpoints
Centrifuge specimen with a relative centrifugal force (RCF) of 3000 x *g* for at least 15 minutes; a refrigerated centrifuge is preferred	Isolation of MTB	Microbiology endpoints
Resuspend the digested, decontaminated specimen to a final volume of 1.5-2.0 mL with PBS (pH 6.8); if used for only smear and molecular testing, to a final volume of 1.0-1.5 mL with PBS	Standardization of suspension used for testing	Comparability of results
Set up cultures immediately following the suspension of the decontaminated, concentrated specimen	Isolation of MTB Speed of confirming TB diagnosis and DST results	Microbiology endpoints Participant enrollment and safety

Table 7-1. Key Elements of Respiratory Specimens Processing Procedure		
Include positive and negative control at least once each day that specimen processing is performed	Isolation of MTB Detect cross-contamination and contaminated reagents	Microbiology endpoints

7.3 Specimen Processing Internal Quality Controls

7.3.1 Description

The rationale for including controls in specimen processing is described in section 7.2. If sputum specimens from individuals are used as control material, the source should be sustainable to ensure consistency and availability. Large batches of control samples should be prepared, dispensed in desirable volumes for one-time use, and stored in a -70°C freezer. More information is available in the Global Laboratory Initiative (GLI) Mycobacteriology Laboratory Manual (3). Artificial sputum options are also described elsewhere (41).

Table 7-2. Specimen Processing Internal Quality Controls	
Element	**Description**
Positive control	The positive processing control measures the extent of NaOH killing of MTB. When subjected to smear and culture, these results are monitored to ensure the extent of killing does not deviate from the norm. An ideal positive control yields an MGIT time to detection (TTD) of 6-10 days, which is equivalent to a 3+ AFB smear, 10^6 CFU/mL, or 1:500 dilution of 0.5 McFarland standard. Positive control options: ▪ Suspension of MTB H37Rv or MTB H37Ra ▪ AFB-positive sputum specimen from a known TB case, homogenized or digested ▪ Sputum specimen to which MTB H37Rv or MTB H37Ra has been added ▪ Artificial sputum sample to which MTB H37Rv or MTB H37Ra has been added

Table 7-2. Specimen Processing Internal Quality Controls	
Element	**Description**
Negative control	The two main purposes of the negative processing control are to: (1) detect cross-contamination events; and (2) check the sterility of the processing reagents. Thus, a preferred negative control does not contain any bacteria. Some laboratories use a negative control with a non-AFB organism to evaluate the extent of NaOH killing of contaminants. To accomplish this objective, this control (a suspension of non-AFB organism typically found in sputum, e.g., *Staphylococcus sp.*, *Streptococcus sp.*) should not be used as the negative control, but can be included as another control. However, there are more effective ways to assess decontamination (e.g., monitoring culture contamination rates) (see sections 5.1.5.3 and 9.1.6) and inoculating the blood agar plate (BAP) with processed specimen. Also, multiple negative controls can be used if cross-contamination is of concern. Negative control options: ▪ Middlebrook 7H9 broth or sterile water (different source from that used to prepare processing reagents) ▪ AFB-negative sputum specimen from a known negative patient, homogenized or digested and autoclaved ▪ Artificial sputum sample to which no bacteria have been added
Placement of controls in processing batch	Positive control should be in the first position (at the beginning of the run), as longest NaOH exposure is in the first tube position. Negative control should be in the last position (at the end of the run), to optimize the ability to detect carryover from MTB-positive specimens in the batch. If multiple negative controls are used, place throughout the run (e.g., front, middle, end).
Frequency of testing	Both controls should be included in at least one processing batch/run daily. Frequency depends on the workload volume, number of technicians processing, the quality control (QC) results, and quality indicators (QI) data.

7.3.2 Monitoring Contamination Rates

Contamination and contamination rates are also discussed in sections 5.1.5.3 and 9.1.6.

7.4 Specimen Processing Checklist

See section 17.3.

8 Smear Microscopy

8.1 Background Information

8.1.1 Introduction

This section describes Key Elements and important technical points for acid-fast bacilli (AFB) microscopy of smears made from sputum specimens as well as from solid and liquid media cultures. It also pertains to the carbol fuchsin and fluorochrome staining procedures for the detection of AFB and addresses preparation of smears, staining of slides, reading of slides, and reporting of results. The components of the overall procedure for smear microscopy may appear in more than one standard operating procedure (SOP).

Various references describe in detail the technical steps of the smear preparation and staining (2-7); the references listed are provided as examples. Although several differences exist between these references (e.g., stains, time of staining) (42), the most important aspects for each laboratory are to: (1) use one of these references without modifying it; (2) apply all Key Elements and technical steps in the SOP, with special attention to the use of internal quality controls (QCs), the reading of smears, and the reporting of smears; and (3) participate in an external quality assessment (EQA) program for smear microscopy.

8.1.2 Direct Versus Concentrated Smears

For diagnostic purposes, a smear made directly from a clinical specimen (unconcentrated) is discouraged because it lacks the sensitivity of a concentrated smear (4, 7, 22), and can be more difficult to read unless the background is cleared with the use of hypochlorite (43). However, in laboratories with extremely high volumes of smears, the more practical approach is to perform direct smears, rely on the collection of multiple sputum specimens from the individual to increase detection of AFB. In the context of tuberculosis (TB) clinical trials, the smear is used to determine if the individual has a high amount of *M. tuberculosis* (MTB) in their lungs (high bacillary load), which is required for drug efficacy assessment. As sputum culture conversion is a primary endpoint for TB clinical trials, and culture results become known only weeks after enrollment/randomization, clinical trials enroll subjects who are smear-positive and more likely to have a high enough bacillary load to produce a positive culture. This ensures that a response to treatment will be detectable. Individuals selected for screening usually have moderate to advanced disease, thus generally having relatively high bacillary loads and are more likely to have AFB-positive smears on both direct and concentrated specimens at baseline/screening. Therefore, either method can be used. Many laboratories prefer to prepare a concentrated smear as other screening tests also require a concentrated specimen. Although a study sponsor may indicate a direct smear be performed at screening, this is usually negotiable and concentrated smears can be done instead. In studies where participants are expected to have paucibacillary TB, such as pediatric studies, a concentrated smear is preferred. In studies where the bacillary load or smear result is not an enrollment criterion, smear preparation is irrelevant and unlikely to be specified.

8.1.3 Carbol Fuchsin Versus Fluorochrome Staining Methods

Both carbol fuchsin stains (e.g., Ziehl-Neelsen [ZN], Kinyoun) and fluorochrome stains (e.g., auramine, auramine/rhodamine) can be used to detect AFB. The fluorochrome stains are recommended for the examination of clinical specimens because of their increased sensitivity, ease of reading and speed, and ability to be examined at a lower magnification than the carbol fuchsin stains (4). In the context of a clinical trial, fluorescent staining of sputum specimens is the preferred staining method. Carbol fuchsin stains are recommended for detecting AFB in positive cultures, as the morphology and staining characteristics appear more distinct (7). Although the ZN/Kinyoun smear is used to confirm the presence of AFB, non-acid-fast bacteria or fungi can be seen, providing evidence of cultures being contaminated.

Fluorochrome-stained slides may be directly restained with the carbol fuchsin stain after immersion oil is removed with xylene (4). However, confirmation of fluorescence microscopy (FM) low-positive smears by restaining with carbol fuchsin stain should not be done (2) or reported (3). There is no added value in confirming fluorochrome-positive smears of respiratory specimens by carbol fuchsin staining when the staff is experienced in performing FM (44). A fluorochrome-positive/ZN-negative result cannot be used to confidently exclude *M. tuberculosis* complex (MTBC), nor can it be used to confirm the presence of nontuberculous mycobacteria (NTM) (44).

8.1.4 Fixing and Inactivating Smears

Smears need to be fixed to ensure the liquid culture media, liquid suspension of bacterial colonies, and/or biological material remains on the slide. There are various fixation methods, but the method used must also inactivate/kill MTB in the specimen (45). Preferable methods are heating with an electric slide warmer or exposure to phenol or alcohol, as these can be accomplished inside a biosafety cabinet (BSC). The smears must air dry completely prior to fixing, especially when removing from the BSC to fix in a drying oven. Whichever method is used, details (i.e., time, temperature, concentration) specified in the reference must be strictly followed.

8.1.5 LED Microscopy

Light-emitting diode (LED) microscopy is 5% (95% CI, 0-11%) more sensitive and 1% (95% CI, -0.7-3%) more specific than conventional FM (46). Furthermore, the LED light source is more reliable than the fluorescent light (47). It is strongly recommended that conventional FM be replaced by LED microscopy in all settings where FM is currently used (46).

8.1.6 Reading and Reporting of Fluorochrome Smears

When using a fluorescent microscope, scan the entire smear with the 20X objective lens (with 10X eyepiece for a total of 200X magnification). A 25X objective lens (250X magnification) can also be used. Smears must be examined in a consistent way to ensure a representative area of the smear is reported. At least one length of the smear must be examined before reporting a negative result. Using the 20X objective lens (or 25X), one 2 cm length is equivalent to 30 fields, which is sufficient to report a negative

result. Occasionally, use the 40X objective lens (or 45X) to see more detailed bacterial morphology at a 400X (or 450X) magnification. Confirming morphology at higher magnification avoids a false-positive report due to fluorescing debris (2, 3).

Due to historical inaccuracy, the FM reporting scale for positive smears has been revised; the actual field observed is larger, and therefore more AFB are visible per field than previously calculated (2). Low scanty positives, 1-4 AFB in one length at 200X magnification, or 1-2 AFB in one length at 400X magnification, should be confirmed by either: (a) viewing additional fields; (b) having another technician check the AFB morphology; (c) making another smear from the same specimen, or (d) collecting another sputum specimen (2).

Table 8-1. Fluorescence microscopy grading and reporting scale [adapted from WHO/The Union (2)]

What You See (200X)	What You See (400X)	What to Record	What to Report
No AFB in one length	No AFB in one length	No AFB or negative	No AFB observed
1-4 AFB in one length	1-2 AFB in one length	Confirmation required* Final result after repeat	Scanty
5-49 AFB in one length	3-24 AFB in one length	Scanty	Scanty
3-24 AFB in one field	1-6 AFB in one field	1+	1+
25-250 AFB in one field	7-60 AFB in one field	2+	2+
> 250 AFB in one field	> 60 AFB in one field	3+	3+

*Confirmation required by another technician's reading, or another smear from the same specimen is stained and read.

Reading practices vary among laboratories. Some have a second person review slides before reporting all positive and 10% of all negatives, which is a worthwhile practice if sufficient staff are available. Note all scanty results must be confirmed by another reader as cited in **Table 8-1**. If re-reading positive and a proportion of negative slides is not possible, performing a monthly internal quality assessment as described in **Table 5-1** is highly recommended.

8.1.7 Reading and Reporting of Carbol Fuchsin Smears

Using a brightfield microscope, ZN smears are examined with the 100X oil objective (10X eye piece for a total of 1000X magnification). The recommended reporting scale for carbol fuchsin stains is shown in Table 8-2.

Since ZN is performed on positive cultures (liquid or solid) to confirm the presence of AFB, grading/quantification is not necessary. The ZN result (positive or negative) must be recorded on a laboratory worksheet (e.g., microscopy, mycobacteria growth indicator tube (MGIT), solid media culture.)

If the laboratory has not established the FM method, carbol fuchsin staining may be used for sputum smears, if approved by the study protocol team of the network or study sponsor.

Table 8-2. ZN Microscopy reporting scale [adapted from WHO/The Union (2)]		
What You See	**What to Record**	**What to Report**
No AFB in 100 fields	No AFB or negative	No AFB observed
1-9 AFB in 100 fields	Record exact number of bacilli	Scanty
10-99 AFB in 100 fields	1+	1+
1-10 AFB per field, check 50 fields	2+	2+
More than 10 AFB per field, check 20 fields	3+	3+

8.1.8 Smear Microscopy Turnaround Time

The smear microscopy turnaround time (TAT) is covered in the Sourcebook because it is important to report these results quickly to facilitate enrolment of participants in the study. Smear results should be available to the study team within 48 hours of receipt of the sputum specimen in the laboratory. TATs are standard quality indicators (QI), and the recommended TAT for sputum microscopy by the World Health Organization (WHO) and other organizations is 24 hours from receipt (22, 29). However, 48 hours is acceptable in the context of clinical trials where the TB diagnosis is most often already known.

8.2 Key Elements of Smear Microscopy

Internal QCs are used to monitor the quality of smear results. In the context of clinical trials, producing reliable smear results is important because smear positivity is one of the eligibility criteria for study participation. A false-positive result could mean an individual is enrolled in the study, randomized to a treatment arm, and has to be withdrawn from the study later, when culture results are available.

The WHO and International Union Against TB and Lung Disease (The Union) scale is considered the standard grading scale (2). Harmonization is achieved when using the same grading scale. Thus, when all laboratories report smear results according to the WHO/UNION scale, results can be compared among all sites participating in the trial. See Table 8-3 for a summary.

Table 8-3. Key Elements of Smear Microscopy		
Key Element	**Effect**	**Impact**
Include positive and negative control smears with every batch of patient slides	Ability to evaluate the quality of smear microscopy results	Enrollment in the study
Report results according to WHO/Union grading scale as per Global Laboratory Initiative (GLI) Sputum Microscopy Handbook	Standardization of quantitative smear results	Comparability of results

8.3 Smear Microscopy Internal Quality Controls

QC testing of new lots of staining reagents is described in section 5.1.3.

Table 8-4. Smear Microscopy Internal Quality Controls

Element	Description
Positive control	An ideal positive control is easy to count and in the 1+ range. Positive control options: ▪ Suspension of MTB H37Rv or MTB H37Ra ▪ AFB-positive sputum specimen from a known TB case, homogenized or digested ▪ Sputum specimen to which MTB H37Rv or MTB H37Ra has been added ▪ Artificial sputum sample to which MTB H37Rv or MTB H37Ra has been added
Negative control	An ideal negative control contains a non-AFB organism typically found in sputum. Negative control options: ▪ Suspension of non-AFB organism typically found in sputum (e.g., *Staphylococcus sp.*, *Streptococcus sp.*) ▪ AFB-negative sputum specimen from a known TB case, homogenized or digested and autoclaved ▪ Artificial sputum sample to which no bacteria have been added ▪ Artificial sputum sample to which *Staphylococcus sp.* or *Streptococcus sp.* has been added
Placement of controls in processing batch	Where to place the control slides in the batch is not relevant when staining and reading slides.
Frequency of testing	The important points are to use these slides as a quality check each time a batch of slides are stained and read, and to record results of the control smears.

8.4 Smear Microscopy Checklist

See section 17.4.

9 Mycobacteria Growth Indicator Tube (MGIT) Culture

9.1 Background Information

9.1.1 MGIT Culture – The Preferred Liquid Culture System

Historically, egg-based solid media were used for the culture of sputum specimens in tuberculosis (TB) clinical trials; however, with the potential to decrease the turnaround time (TAT) for culture results, the BACTEC™ 460 TB system was considered a useful adjunct to solid media cultures. With the advent of the BACTEC™ MGIT™ automated mycobacterial detection system MGIT culture was rapidly adopted in clinical trials, since the instrument specifies the time to detection (TTD) of positive cultures, which is indicative of the amount of viable *Mycobacterium tuberculosis* (MTB) in the liquid culture. The benefits of using the MGIT culture system are: (1) as a commercial system, the instrument, media, growth supplements, antibiotic cocktail, and procedure are all standardized; (2) results are available sooner than those from solid media cultures; and (3) MTB can be detected in contaminated cultures (by acid- fact bacilli (AFB) stain and sensitive identification methods unaffected by contaminant growth), thus often avoiding the loss of culture results. The MGIT culture system is preferred for TB clinical trials primarily because it was the first commercially available liquid culture system for mycobacteria, and has been universally adopted by laboratories worldwide.

9.1.2 Working Up MGIT Instrument Positive Cultures

The overarching goal of the laboratory in TB drug trials is to maximize the recovery of MTB. Thus, the efforts to identify MTB even in the presence of contaminants are critical. Using an organized and comprehensive approach in working up MGIT instrument-positive cultures optimizes recovery and identification of MTB. Algorithms have been developed to address scenarios such as contaminated cultures, MTB cultures mixed with contaminants, cultures with non-tuberculosis mycobacteria (NTM), and early positive cultures with negative confirmatory tests. These algorithms are depicted in flow charts (section 9.5). The algorithms are in line with those published by the Global Laboratory Initiative (GLI) (3), though; minor differences in the reporting of results may be noted. Following the algorithms is a Key Element of the MGIT culture (section 9.4).

9.1.3 Identification Tests

For most TB clinical trials, it is not necessary to identify mycobacteria to the species level; identification (ID) of MTB complex (MTBC) is sufficient. Some trials might require further identification (e.g., ID of *M. bovis* Bacillus Calmette-Guerin (BCG) in a vaccine trial).

Many TB drug trials currently require the use of lateral flow assays for MPT64 antigen to confirm the presence of MTB in a positive MGIT culture. These provide rapid and accurate results in contrast to the p-Nitrobenzoic (PNB) inhibition test, which is the common culture ID test in diagnostic settings. Sophisticated technologies such as high-pressure liquid chromatography and mass spectrometry are used in some reference labs but are not practical for daily testing to confirm MTB. Some laboratories routinely

use a molecular method, such as the Hain line probe assay (LPA) MTBDR*plus*, as this enables rapid and simultaneous determination of isoniazid (INH) and rifampin (RIF) susceptibility. In such situations, the use of the MTBDR*plus* as the primary ID method should be discussed with the study sponsor/network. The important point is that the work-up of positive MGIT cultures, including ID, should be done in real time. Thus, if molecular tests are used for ID, they must not be performed in one to two batches per week.

9.1.3.1 MPT64 Antigen Tests

If a lateral flow assay for an MPT64 antigen test is negative on an AFB-positive MGIT culture, it cannot be assumed to be NTM. Both the Gen-Probe AccuProbe MTBC identification test (48), as well as the MPT64 antigen test (49, 50) have been reported as being negative on initial testing, and then positive after further incubation. Some cultures have a sufficient number of MTB to yield a positive Ziehl-Neelsen (ZN) stain (AFB are seen on the smear); however, the density is not high enough to give a positive MPT64 antigen test or AccuProbe test. Neither of these are amplification tests. Thus, a negative MPT64 antigen test must be repeated after re-incubation, and if still negative, an amplification-based identification test, such as the MTBDR*plus,* must be performed. In TB drug trials, it is crucial to correctly identify the presence or absence of MTB in every specimen as this could make the difference in determining efficacy of a drug or drug regimen. Refer to section 11 for more details.

9.1.3.2 Molecular Assays for Identification

As described above, the work-up of positive MGIT cultures may require a molecular test to provide more sensitivity. As seen in Flow Charts 1 and 2 (section 9.5), there are two options for identification of ZN-positive MGIT cultures, depending on what tests are available locally. Either an MTB/NTM amplification test (Hain GenoType® Mycobacterium Common Mycobacteria [Hain CM]) or an MTB amplification test (Hain MTBC, Hain MTBDR*plus*, or Xpert MTB/RIF) can be used. The various Hain LPAs available are listed in Table 9-1. More details on the Hain LPAs and Xpert® MTB/RIF can be found in sections 14 and 15, respectively.

Table 9-1. Available Hain GenoType® Line Probe Assays

Name	**Mycobacterium Genus Control Included**	**MTBC Probes Included**
Hain GenoType® MTBDR*plus* (detects MTBC and its resistance to INH and RIF)	No	Yes
Hain GenoType® MTBDR*sl* version 1 (detects MTBC and its resistance to FLQ, AG/CP, and ethambutol)	No	Yes
Hain GenoType® MTBDR*sl* version 2 (detects MTBC and its resistance to FLQ and AG/CP)	No	Yes
Hain GenoType® CM (simultaneous identification of MTBC and 14 of the most common NTM species)	Yes	Yes
Hain GenoType® AS (simultaneous identification of 16 additional NTM species)	Yes	No
Hain GenoType® MTBC (differentiates species of MTBC)	No	Yes

9.1.4 Viability

There may be a concern that a positive molecular test on an AFB-positive MGIT culture is a false-positive result due to the detection of nonviable MTB, since molecular tests can detect DNA from viable and dead organisms. However, for the ZN smear to be positive, at least 10^5 to 10^6 CFU/mL must be present and replication would have to occur in the culture to achieve this number of organisms. Thus, positive molecular test results from an AFB-positive MGIT culture are considered reliable (i.e., the MTB detected are viable). This may not be the case for a molecular test done directly on the specimen.

9.1.5 Presumptive Identification of Liquid Cultures

In some TB drug trials, such as early bactericidal activity (EBA) studies, specimens are collected frequently (approximately 8 specimens in 14 days). With these multiple cultures, it is not necessary to repeat the MPT64 antigen identification test on each positive culture. If the MGIT culture is ZN-positive and not contaminated, a presumptive identification can be reported (e.g., "Positive for acid-fast bacilli"). This detail should be specified in the study-specific guidance documentation.

9.1.6 Contaminated Cultures and Mixed Cultures

Before culture in the laboratory, sputum specimens are digested and decontaminated. This procedure theoretically eradicates contaminants such as normal flora and colonizing bacteria and fungi of the oral and upper respiratory tracts, while not seriously affecting the viability of the mycobacteria (25). Although some TB laboratory resources list possible contaminants as either NTM, fungi, bacteria, and yeast (26, 51); others refer to contamination as only bacteria or fungi (24, 25). The latter definition is used in the flow charts. A "mixed culture" is defined as more than one species of mycobacteria (e.g., culture with NTM and MTB or culture with two NTM) (7, 24). However, this term is not used for reporting (section 9.1.10.3).

For ZN-negative cultures found to be contaminated (e.g., blood agar plate (BAP) positive) in less than 7 days of being set up, there is a provision to allow the MTB, if present, more time to grow and be detected by the MGIT instrument. Thus contaminated MGIT cultures with a TTD less than 7 days are reincubated an additional 14 days and ZN stained again.

For all instrument-positive MGIT cultures, or whenever growth is suspected, a BAP should be inoculated. Alternative media include chocolate agar, tryptic soy agar (TSA) or brain-heart infusion (BHI) agar. Incubate these subcultures at 35±1°C and observe them after 24 to 48 hours. However, one must wait for at least 48 hours of incubation before proceeding to the next steps in the flow charts. If the BAP culture shows no growth after 48 hours of incubation, incubate the plate another 24 hours, for a total of 72 hours. Note that not all contaminants grow on BAP.

Although the Foundation for Innovative New Diagnostics (FIND) MGIT Manual mentions that a Gram stain and a ZN stain must be performed if contaminating growth appears (24), many TB laboratories do not use the Gram stain to confirm contamination. Thus, the Gram stain is optional. To save cost and technician time, some laboratories do not subculture positive MGIT cultures to rule out contaminants. Note that not

all contaminants are detected on Gram and ZN stains. Thus, subculture to an acceptable agar medium is required. If growth of bacteria/fungi is observed on BAP and/or non-AFB are seen on the ZN smear, the MGIT culture is considered "contaminated."

Decontamination of a contaminated MGIT culture is not routinely performed. If the culture is MTB-positive and a phenotypic drug susceptibility testing (DST) or isolate storage is required, the MGIT culture must be decontaminated to obtain a pure culture. It is preferable to decontaminate the MGIT culture rather than reprocess the sputum sediment (resuspended pellet), as there are more MTB in the culture than in the sputum sediment. If the MGIT culture needs to be decontaminated, the procedure in the FIND MGIT Manual (equal volume of 4% sodium hydroxide (NaOH)) should be followed (24).

Monitoring of culture contamination rates is discussed in section 5.1.5.3.

9.1.7 Early Positive Signals Versus False-Positive Results

An "early positive signal" on a MGIT culture occurs when the instrument detects growth but the ZN smear and BAP are negative after 72 hours. In these cultures, there is not a sufficient quantity of organisms to be detected by the ZN smear or BAP subculture. Many laboratories report that early positive signal cultures eventually yield either MTB or contaminants after reincubation. Rarely are these truly false-positive MGIT results. A true false-positive MGIT result is defined as a culture that is instrument-positive, but found to be smear-negative and subculture-negative for mycobacteria or other bacteria after 42 days of incubation (end of MGIT protocol). False-positive rates of 0.5 to 3.5% have been reported for the automated MGIT systems (52, 53).

9.1.8 Culture-positive on or after 42 Days

A MGIT tube may flag positive at 42 days or show visual evidence of growth when removed on day 42, and in either case be determined to be MTB. Another rare event is when the MGIT tube flags positive after 42 days in the MGIT instrument; if for example, it was not removed promptly after the end of the 42-day protocol. In these cases, the study team should be contacted to discuss how to report the result.

9.1.9 Using MGIT Cultures for Isolate Storage

Some laboratories prefer to use positive MGIT cultures for isolate storage instead of making bacterial suspensions in Middlebrook 7H9 broth with OADC and glycerol, as this saves time and the need for additional media. See section 16.4.5 for details.

9.1.10 Reporting Study Data

9.1.10.1 Reporting Culture Results

Interpretation of results according to the algorithms provides a definitive result (i.e., MTBC). However, the study sponsor will specify the extent of speciation required for the study. The laboratory is encouraged to identify to species level, in the case of NTM, and record it even if not required for the study.

In the Flow Charts, the term "Report" refers to the final laboratory report for the study data. Preliminary reports are not included in the flow charts. The results for each step on the Flow Charts must be recorded on the MGIT culture worksheet, an important laboratory source document for clinical trials. Only the final culture result will be recorded on the laboratory report form. Furthermore, some laboratories may not be able to use the same wording for reporting due to their Laboratory Information System or other constraints. It is not a problem as long as the steps in the Flow Charts are followed and study data are reported according to the terminology specified by the network/study sponsor.

Note that a "Negative signal" will be reported as "Negative for MTBC" and not as simply "Negative." Results are reported in the context of a clinical trial and not as a routine diagnostic test, which means the culture is used to rule in the presence of MTB and the fact that other organisms are not detected is irrelevant. If the instrument result is negative and there is no visual evidence of growth in tube, then the MGIT culture is negative for MTB. Laboratories can report to the clinicians the results per their standard terminology, but for entry into the study database, it must be indicated as "Negative for MTBC."

Note that an "NTM" identified by the Hain CM test will be reported as either "Positive for NTM," "Positive for MTBC and NTM," or "Contaminated and positive for NTM," depending on the results. The name of the mycobacterial species identified should be recorded on the source document and can be reported to the clinicians if this is routine practice in the laboratory. If the Hain CM test is negative but all three controls (Conjugate Control, Universal Control, and Genus Control) are positive, report as "Positive for NTM." Proceed to do the Hain AS test if requested by the clinicians or routine laboratory practice. Since NTM speciation is not required for TB drug trials, performing the Hain AS test is usually not necessary. If the Hain CM test result is "high GC content positive", this means the organism is related to the Mycobacterium genus (AFB-positive and high GC content) but is not MTBC or NTM. This is considered a contaminant and thus reported as contaminated.

If a technical problem is encountered (e.g., MGIT tube dropped or lost, ZN-positive but identification (ID) tests failed), there should be an option on the laboratory report form to indicate "No result".

9.1.10.2 Reporting Time to Detection

Although the Unloaded Positive Tubes Report includes TTD for all positive tubes, only the TTD values for pure cultures positive for MTBC are recorded on the worksheet. No TTD values should be reported for contaminated cultures and for cultures with NTM. If the first round of confirmatory tests of a positive MGIT culture is negative ("early positive signal") and upon reincubation repeat testing indicates MTB, the first TTD value is reported. TTD values should be reported in days and hours.

9.1.10.3 Culture Reporting Terminology

The following terminology is suggested for the study or laboratory case report form (CRF). Use of standardized terminology will allow harmonization of MGIT culture results among the network laboratories in a study, across studies, and across trial networks. All possible culture results are included

in the options and are straightforward, conclusive, and compatible with the Clinical Data Interchange Standards Consortium (CDISC) terminology.

MGIT culture result options:

1. Negative for MTBC
2. Positive for MTBC
3. Positive for MTBC and contaminated
4. Positive for MTBC and NTM
5. Positive for NTM
6. Contaminated
7. Positive for NTM and contaminated
8. No result (tube broken, lost, etc.)

9.2 Key Elements of MGIT Culture

The volume of the resuspended pellet used to inoculate the MGIT tube is an element of standardization of the culture procedure. Using the same inoculum volume enables results from all the participating laboratories to be compared. The manufacturer specifies this volume.

Following the algorithms depicted in the flow charts (section 9.5) is critical to detecting and identifying MTB or ruling out the presence of MTB. Failure to detect MTB could potentially impact the microbiology endpoints of the trial. See Table 9-2 for a summary.

Table 9-2. Key Elements of the MGIT Culture

Key Element	Effect	Impact
Inoculate each MGIT tube with 0.5 mL of the resuspended sputum pellet	Standardization of quantitative culture results	Comparability of results
Work up all MGIT cultures (positive and negative) according to the MGIT culture algorithms in the flow charts	Isolation/detection of MTB	Microbiology endpoints

9.3 MGIT Culture Internal Quality Controls

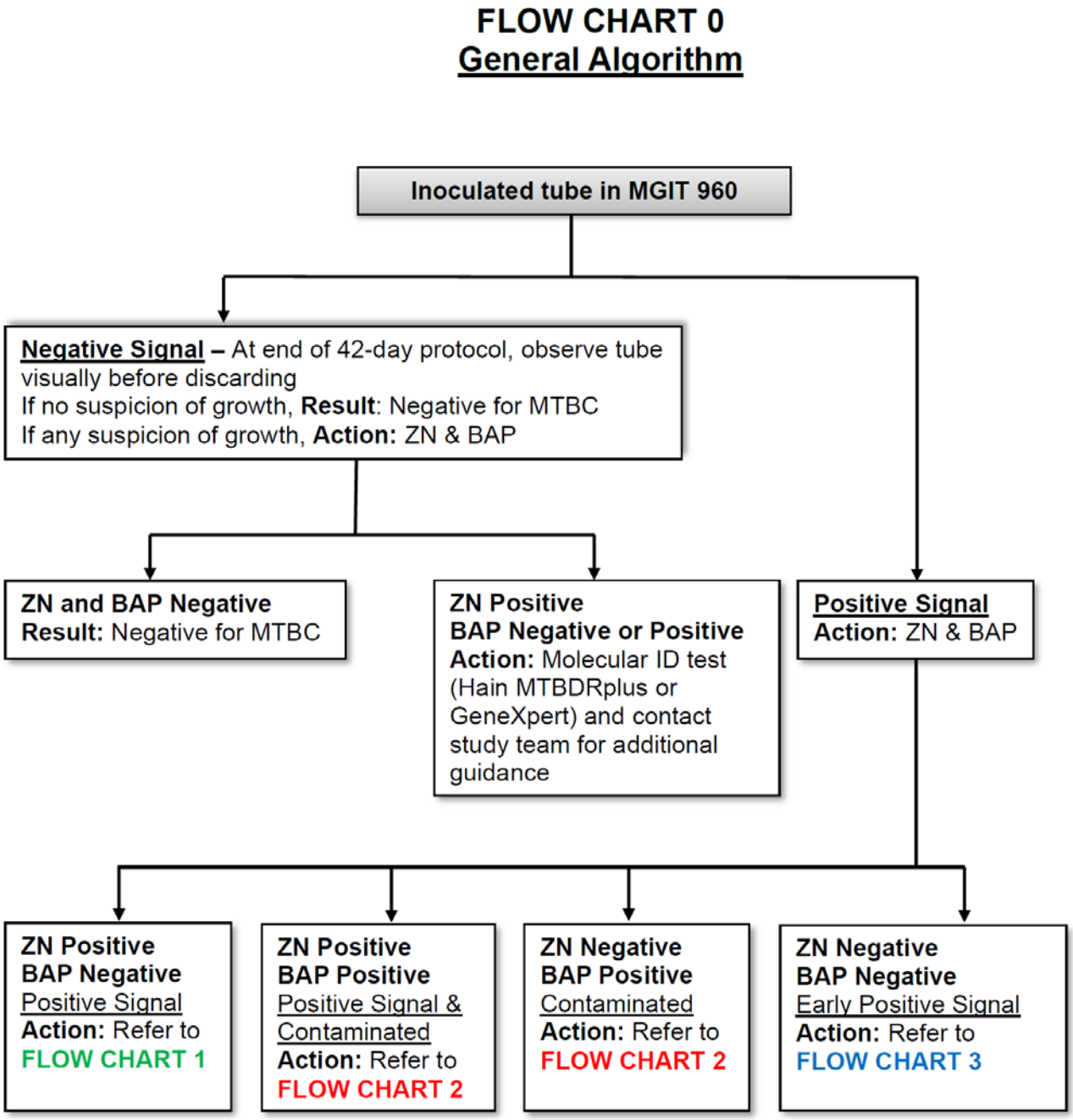
Assessing the sterility and growth performance of the MGIT media before putting the media into use is different than monitoring the quality of the culture procedure with internal quality controls (QCs). There are no internal controls for the MGIT culture SOP; however, the internal controls in the respiratory specimens processing SOP are inoculated into MGIT media; hence, there are culture results (qualitative and TTD) associated with these controls. These results are quality indicators (QI) of both the specimen processing procedure and the MGIT culture. Refer to section 5.1.3 for details on quality testing MGIT medium, Growth Supplement, and PANTA.

9.4 MGIT Culture Checklist

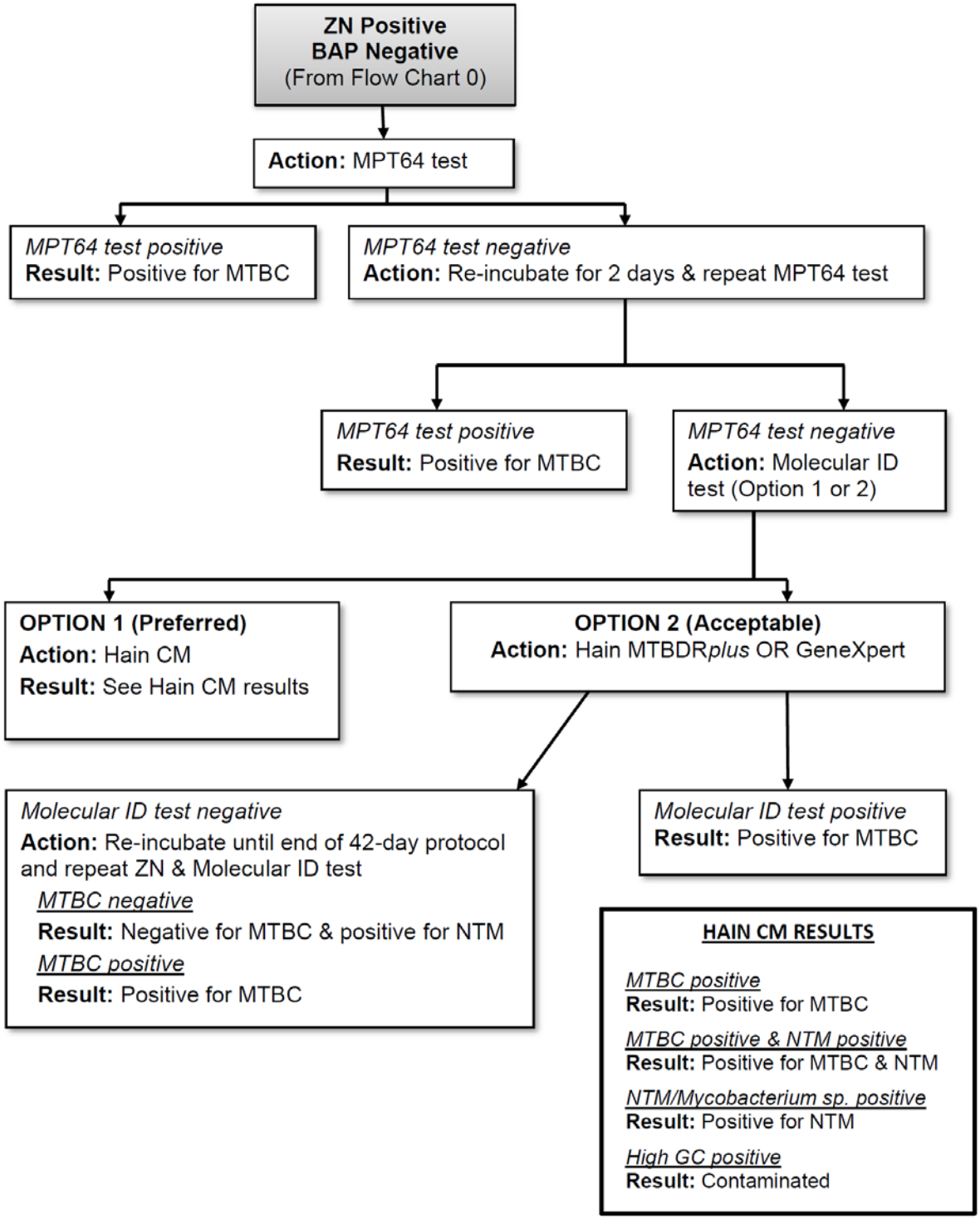
See section 17.5.

9.5 MGIT Culture Algorithms

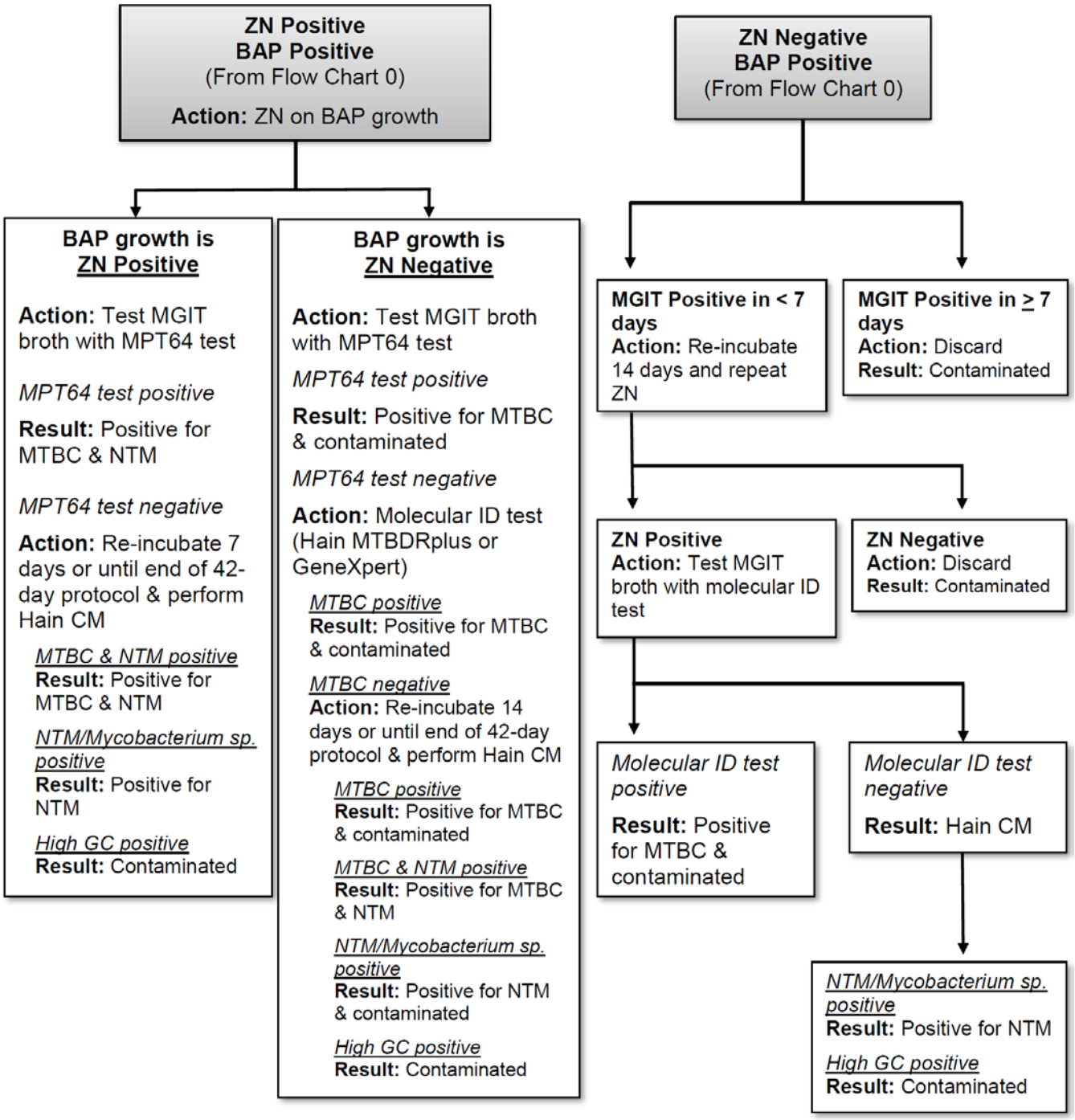
The algorithms have been adapted from the GLI Mycobacteriology Laboratory Manual (3).



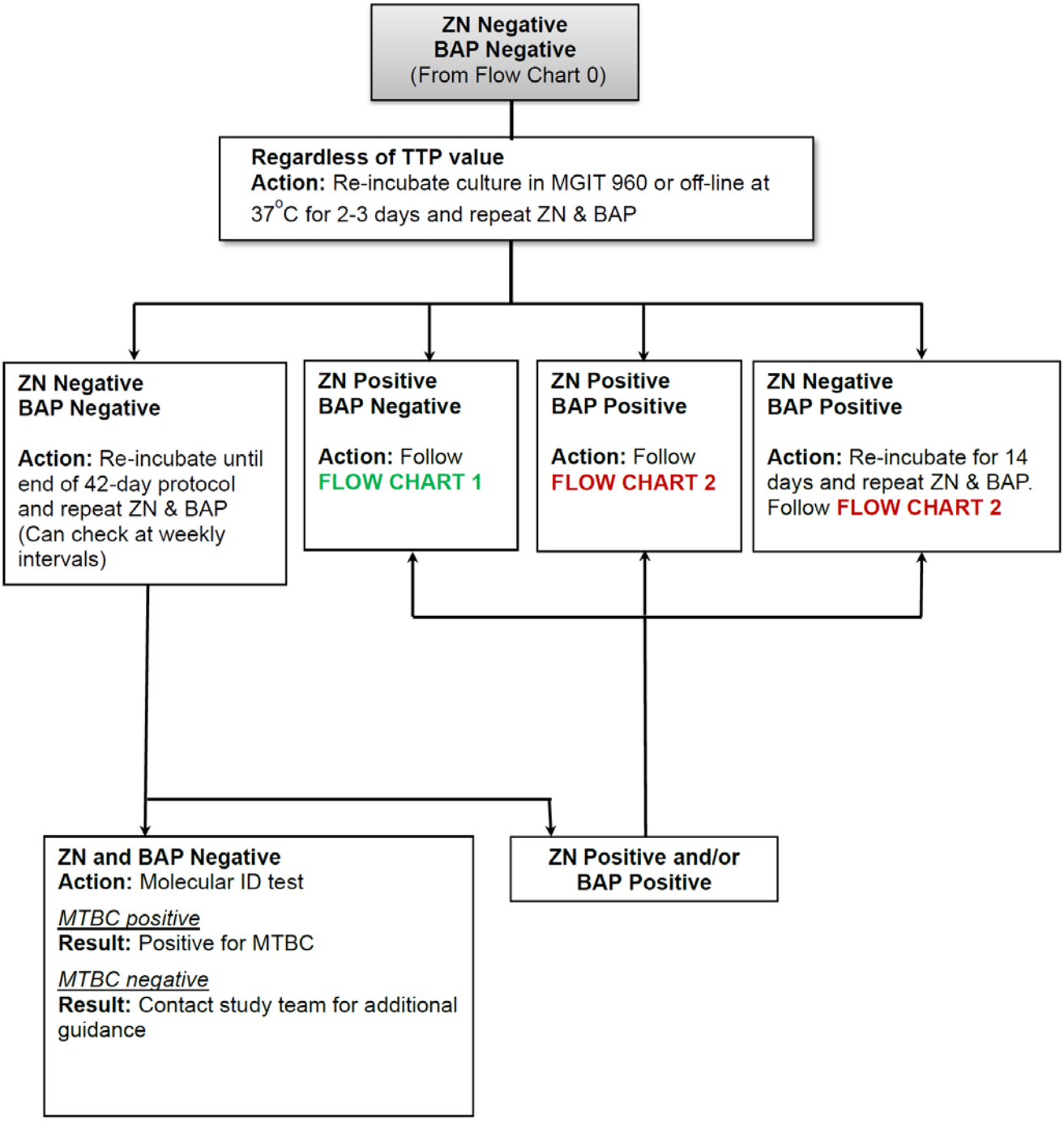
FLOW CHART 1
Positive Signal



FLOW CHART 2
Contaminated MGIT Cultures



FLOW CHART 3
Early Positive Signal



10 Solid Media Culture

10.1 Background Information

10.1.1 Types of Solid Media

This section encompasses culture of sputum specimens on solid media, both Lowenstein-Jensen (LJ) and Middlebrook agar, as well as assessment of *Mycobacterium tuberculosis* (MTB) growth on solid medium in a semi-quantitative manner. Although LJ and Middlebrook 7H11 are the most commonly used, the Key Elements and important technical points described are applicable to other types of solid media, such as Ogawa and Middlebrook 7H10.

Although there are several different solid media available for cultivating mycobacteria, most are variations of egg-potato base or albumin-agar base media (3, 5, 54, 55). There is no general consensus on which medium is best for routine isolation. The choice is usually based on personal experience, laboratory tradition, or both. The unpublished experience in some laboratories has been that some strains grow on one type of solid medium and not another. Before liquid culture was adopted for MTB isolation, some laboratories used both an egg-based and an agar-based medium to optimize recovery. Overall, egg-based media have been more popular because carbon dioxide (CO_2) incubation is required for agar-based media and many laboratories do not have a CO_2 incubator. Furthermore, the OADC supplement adds to the cost of Middlebrook agar media.

Historically, most clinical trials of anti-tuberculosis (TB) drugs have been conducted with the widely used and readily available LJ medium. Drug regimens were developed using 2-month culture conversion on LJ media as a surrogate for response to anti-TB therapy. Detection of MTB in sputum after 1 and 2 months of treatment is a widely used endpoint in clinical trials. Thus, the solid medium providing the highest sensitivity and specificity when the tubercle bacilli are under drug pressure should be the medium of choice for determining response to therapy and specifically, the 2-month sputum conversion rates. When used in the diagnosis of TB and for monitoring the response to anti-TB therapy, egg-based and agar-based media have been assumed to be equivalent for isolating MTB, in the absence of published reports to the contrary. There has been only one study to date that has prospectively compared non-selective media (LJ, Middlebrook 7H10, and Middlebrook 7H11) and media with selective antibiotics (Middlebrook 7H10S, Middlebrook 7H11S) to a constructed reference standard and mycobacteria growth indicator tube (MGIT) 960 culture. These results showed that Middlebrook 7H11S had the highest MTB positivity rate and the lowest contamination rate after 2 months of anti-TB therapy (56). To expand on these findings, another study is ongoing in different geographic locations where the isolation and contamination rates are likely to vary [Centers for Disease Control (CDC) TB Trials Network (TBTC) Study 36, Platform Project 1].

It is important to note that Middlebrook agar medium with selective antibiotics has always been the medium used for the quantitative sputum culture, which is the basis of early bactericidal activity (EBA) studies. The selective media, Middlebrook 7H10S and Middlebrook 7H11S, were developed by Mitchison et al. (57) to recover MTB from sputum without the need to decontaminate the digested sputum. Reliable

colony counts can only be obtained from sputum that has not been decontaminated. The quantitative culture procedure has recently been implemented in Phase II studies to measure change in bacillary load from baseline to 2 months of therapy (15, 17). Furthermore, Middlebrook 7H11S, instead of LJ, is being used in a Phase II TB trial to determine the experimental drug area under the curve(AUC)/minimum inhibitory concentration (MIC) that provides the shortest time to sputum culture conversion on solid medium (58).

Either in-house prepared or commercially prepared solid media can be used for culture. The source is the laboratory's preference or ability to make or purchase the media. The important factor is conducting proper QC testing (growth performance and sterility) before using (see section 5.1.3 for details).

10.1.2 Need for Solid Culture

With the high sensitivity of MGIT culture and similar contamination rate to Middlebrook 7H11S, it is debatable as to whether or not there is a need for solid culture media in clinical trials. Presently, there are two reasons to use both. Culture conversion on solid media at 2 months remains a critical marker of response to therapy. A similar marker has yet to be defined for MGIT culture. Combining solid and MGIT media improves recovery of MTB in the event the MGIT culture is contaminated and the solid media culture is not.

The type of solid medium to use in a clinical trial will either be specified by the study sponsor, or the laboratory will be allowed to use its medium of choice. The medium type must not change during the course of the study. Also, the source, either commercial vendor or prepared in house, must be consistent throughout the study.

10.1.3 Identification of Positive Cultures

If the MGIT culture performed on the same specimen is positive for MTB, usually it is not necessary to proceed to a full identification (ID) of the positive culture as per the MGIT algorithms (section 9.5). In this case, do a Ziehl Neelsen (ZN) stain of the colonies resembling MTB and if acid-fast bacilli (AFB) positive, a presumptive ID can be reported, e.g., "Positive for AFB". The extent of ID of growth on replicate cultures of the same specimen should be specified in the study-specific guidance documentation.

If the MGIT culture is either negative for MTB, contaminated, or positive for non-tuberculosis mycobacteria (NTM), and growth on the solid medium resembles MTB, perform a ZN stain of colonies. If AFB-positive, proceed to identify with an MPT64 antigen test as per the MGIT algorithms (section 9.5). In these cases, a definitive ID of MTB should be reported. If contaminant growth is also present, report "Positive for MTB complex (MTBC) and contaminated." Determining the amount of MTB growth may not be possible in the presence of contaminants.

10.1.4 Reading and Reporting

In some laboratories, the amount of growth on solid media may not be determined or reported (i.e., only qualitative results are reported for the diagnostic specimens). In clinical trials, the plates with MTB growth are read in a semi-quantitative manner (i.e., colonies are counted according to the reporting scheme shown in Table 10-1). The amount of growth is reported only when the culture is positive for MTB and is recorded in another/separate field on the laboratory report form. If the culture has MTB and contaminant growth, an attempt should be made to quantify the amount of MTB growth. If not possible, report "No result" in the Growth Quantity field, and indicate that no counting was performed on the solid culture worksheet.

Table 10-1. Standardized Reporting Scheme for Solid Culture [adapted from (3)]

Growth	What to Record	What to Report
None	No growth	Negative for MTBC
1-9 colonies	Actual number, Identification (ID) result if done	Actual number Positive for AFB/MTBC
10-100 colonies	1+, ID result if done	1+ Positive for AFB/MTBC
101-200 colonies	2+, ID result if done	2+ Positive for AFB/MTBC
> 200 colonies (too numerous to count or confluent)	3+, ID result if done	3+ Positive for AFB/MTBC
MTB growth in the presence of contamination; count the colonies or grade as above if possible	Colony count or grade, Positive for AFB, ID result if done, and contaminated	Colony count or grade Positive for AFB/ MTB and contaminated
Other mycobacterial growth	AFB-positive, NTM	AFB-positive/NTM
Contaminated	Contaminated	Contaminated

10.1.4.1 Solid Culture Reporting Terminology

The following terminology is suggested for the study or laboratory case report form (CRF). Use of standardized terminology will allow harmonization of solid media culture results among the network laboratories in a study, across studies, and across trial networks. All possible culture results are included with the terminology being straightforward, conclusive, and compatible with the CDISC terminology.

Solid culture result options:

1. Negative for MTBC

2. Positive for MTBC
3. Positive for MTBC and contaminated
4. Positive for MTBC and NTM
5. Positive for NTM
6. Contaminated
7. Positive for NTM and contaminated
8. Positive for AFB (definitive identification performed on liquid culture performed at same time)
9. No result

10.2 Key Elements of Solid Media Culture

The amount of inoculum on the solid media must be the same to allow for culture results from the various laboratories to be combined for analysis. Whole plates should be used instead of biplates to accommodate 0.2 mL. If the slope is too small, a commercial source of slopes with a larger surface area must be sought. It is not possible to use two slopes or both sides of a biplate, each inoculated with 0.1 mL, because there is no provision for combining results from two slopes/sides, or reporting two sets of results on the laboratory report form.

Incubation in CO_2 is a requirement for cultures on Middlebrook agar (7H10/7H11, non-selective and selective) (23). CO_2 enhances the growth of MTB on the media, thus allowing colonies to be evident sooner, and maximizing isolation when there is a low amount of MTB in the sputum specimen, such as when the sputum is approaching culture negativity.

The incubation time is specified to ensure recovery of MTB, if present. The optimal time differs depending on whether the MTB strains are drug susceptible or drug resistant (3). See Table 10-2 for a summary.

Table 10-2. Key Elements of Solid Media Culture

Key Element	Effect	Impact
Inoculate solid media (slope or plate) with 0.2 mL of resuspended sputum pellet	Standardization of the amount of sputum pellet cultured	Comparability of results
Incubate Middlebrook agar plates in 5-10% CO_2	Isolation of MTB	Microbiology endpoints
Incubate solid media for at least 6 weeks before reporting a negative result; at least 8 weeks for trials with MDR-TB participants	Isolation of MTB	Microbiology endpoints

10.3 Solid Culture Internal Quality Controls

Assessing sterility and growth performance of the solid culture media before putting the media into use is different than monitoring the quality of the culture procedure with internal quality controls (QCs). There are no internal controls for the solid media culture standard operating procedure (SOP); however, the internal controls in the respiratory specimens processing SOP are inoculated onto solid media; hence, there are culture results (qualitative and semi-quantitative) associated with these controls. These results

are quality indicators (QI) of the specimen processing procedure and solid media culture. Refer to section 5.1.3 for details on quality testing solid media.

10.4 Solid Media Culture Checklist

See section 17.6.

11 MPT64 Antigen Identification

11.1 Background Information

11.1.1 Introduction

Commercial kits for the rapid identification (ID) of *Mycobacterium tuberculosis* complex (MTBC) are based on the detection of the MPT64 antigen. MPT64 antigen is a predominant protein that is transported to the cell surface and secreted when *Mycobacterium tuberculosis* (MTB) is viable and actively replicating. MPT64 antigen tests are widely available; including the mycobacteria growth indicator tube (MGIT) TBcID (Becton Dickinson), SD BIOLINE TB Ag MPT64 (Standard Diagnostics), Capilia TB-Neo (Tauns Laboratories), and TB Ag MPT64 Rapid (KAT Medical South Africa). These kits are reported to be highly sensitive and specific, thus are considered to be equivalent in terms of performance, although few comparative studies have been conducted (59). MPT64 antigen tests are lateral flow, immunochromatographic assays that can be used to test liquid cultures or suspensions of colonies from solid media cultures. These tests are simple to use and provide results in only 15 minutes, which is a significant improvement over the several hours or days required by other available tests. The MPT64 Antigen Kit is the preferred culture ID method.

MPT64 antigen tests have been widely adopted for the work-up of Ziehl-Neelsen (ZN) positive MGIT cultures in clinical trials for several reasons – they are fast, easy to perform, highly accurate, and inexpensive. Furthermore, no special equipment or technical training is required. Additional advantages for its use in the clinical trial setting are being able to reliably distinguish MTBC from nontuberculous mycobacteria (NTM), and confirm MTBC in ZN-positive MGIT cultures having contaminant growth. The outcome of the trial (i.e., assessment of the experimental treatment regimens) is based on the time of treatment when the sputum becomes culture-negative for MTBC and the number of consecutive MTBC-negative cultures. To accurately assess sputum culture conversion, every ZN-positive culture needs to be tested to confirm MTBC. In some individuals, NTM replaces MTBC following effective drug treatment, so one cannot assume that the acid fast bacilli (AFB) in the MGIT culture are MTBC. In some settings, NTM infections are common and occasionally, concomitant tuberculosis (TB) and NTM infections are observed; thus, distinction between the two is important. Generally contaminated culture rates increase the longer individuals are on treatment, and the MPT64 antigen test is reliable even in the presence of contaminants.

11.1.2 Avoiding False-Negative MPT64 Antigen Test Results

An important consideration of the test is the amount of time the culture has been incubated, as the sensitivity of the assay relies on the amount of MPT64 antigen secreted in the medium. To optimize detection of the antigen, it is recommended to incubate the MGIT culture 2 additional days once the culture signals positive (3). Some laboratories monitor the Growth Units (GU) and test on day 1 of instrument positivity if the GU is $\geq$ 300, and those with GU < 300 are reincubated and tested on day 2 (50). With either approach, if the test result is negative, the MGIT tube should be incubated another 2 days and retested with the MPT64 antigen test or a molecular test (see MGIT culture algorithms, section 9.5), thus

ensuring the ID is reliable and avoiding an erroneous result of NTM. Since false negative results can occur due to mutations in the mpt64 gene (60), repeat testing with a MTB molecular test is a good option.

11.2 Key Elements of MPT64 Antigen Identification

A definitive result is critical at all specimen collection times; thus, it is imperative to test at least one AFB-positive culture at each time point with the MPT64 antigen kit.

Confidence in ID results comes when kit performance is regularly tested with appropriate positive and negative controls.

Failure to confirm the presence of MTB in an AFB-positive culture, or erroneous results due to kit performance potentially has several consequences in a trial, depending on the specimen collection time point. These are: (1) early withdrawal of the participant from the study; (2) inaccurate assessment of treatment arm performance in an adaptive-design clinical trial; and (3) inaccurate assessment of time to culture conversion to negative which is a study endpoint. See Table 11-1 for a summary.

Table 11-1. Key Elements of MPT64 Antigen ID

Key Element	Effect	Impact
Confirm presence of MTB vs. non-MTB at each trial time point when culture is AFB-positive	Isolation of MTB	Microbiology endpoints
Include positive and negative controls at least once per week or with each batch of cultures	Accuracy of MTB identification	Microbiology endpoints

11.3 MPT64 Antigen Identification Internal Quality Controls

In addition to the internal reagent control present in the device, positive and negative controls are tested with each batch of cultures as described in Table 11-2. Controls should be suspensions of relevant bacteria, either positive MGIT cultures (with a GU >300) or dense suspensions prepared with colonies from a solid media culture. Although reference strains are recommended, clinical isolates with documented identification can be used. Quality control (QC) testing of new lots of MPT64 antigen kits is described in section 5.1.3.

Table 11-2. MPT64 Antigen ID Internal Quality Controls

Element	Description
Positive control	Suspension of MTB H37Rv or MTB H37Ra. To facilitate frequent testing, the MGIT cultures of the positive processing control can be used as the positive internal control.

Table 11-2. MPT64 Antigen ID Internal Quality Controls	
Negative control	Suspension of *M. kansasii* or *M. fortuitum*. MGIT cultures of *M. kansasii* or *M. fortuitum* prepared for the performance test of new lots of MGIT medium, Growth Supplement, and PANTA can be used for the negative internal control.
Placement of controls in processing batch	Not applicable.
Frequency of testing	Include positive and negative controls each time the test is performed, or at least once weekly.

11.4 MPT64 Antigen Identification Checklist

See section 17.7.

12 MGIT Drug Susceptibility Testing (DST)

12.1 Background Information

12.1.1 Introduction

Drug susceptibility testing (DST) of *Mycobacterium tuberculosis* (MTB) can be performed phenotypically and genotypically. Phenotypic testing is based on whether or not the organism can grow in the presence of the antibiotic. Genotypic testing is based on detecting the presence of wild-type sequences or mutations in genes known to be associated with antibiotic susceptibility or resistance. Phenotypic susceptibility testing is presently regarded as the gold standard (61); however, it is time consuming since it relies on growth of the organism. In clinical trials, phenotypic DST is performed to confirm results from molecular or genotypic-based DST methods used in screening individuals for study eligibility, and to determine if tuberculosis (TB) participants are susceptible to the drugs in the treatment arm to which they are randomized. Confirmation of drug susceptibility in a timely manner ensures participant safety and is an important ethical consideration in TB drug trials.

The BACTEC Mycobacterial Growth Indicator Tube (MGIT) system is the most commonly used DST method for first-line and second-line anti-TB drugs in clinical trials. This is due to: (1) the instrument, media, and cultures with the appropriate inoculum density are readily available, since the studies require the laboratories to use the MGIT system for culture; (2) all components of the system are standardized; (3) the interpretation of results is performed by the MGIT instrument; and (4) BACTEC MGIT drug concentrations comply with World Health Organization (WHO) guidelines on critical concentrations (62). Other phenotypic DST methods are covered in section 13.

12.1.2 Discrepant Results between Genotypic and Phenotypic Methods

Occasionally discrepancies may be observed, e.g., results from the genotypic test indicate isoniazid (INH) or fluoroquinolone (FLQ) susceptibility and from the phenotypic test INH or FLQ resistance (63, 64). Since resistance to these two drugs can be due to mutations not included in the molecular tests, resistance cannot be detected by these tests. This scenario is relevant in drug susceptible TB studies, where the participant would have been enrolled and later discontinued from the study due to drug resistance. Molecular tests are highly accurate in detecting drug resistance so lack of phenotypic DST confirmation is uncommon. Rarely false rifampin (RIF) resistance is observed with Xpert® MTB/RIF (silent *rpoB* mutation or *rpoB* wildtype sequence); however, these strains would be RIF susceptible by phenotypic testing (65). There are few specific *rpoB* mutations associated with low level clinically relevant RIF resistance (resistance detected by molecular tests) which are found to be RIF susceptible by MGIT DST (66, 67). These strains are resistant by other proportion methods thus using one of these methods to resolve the discrepancy would be a logical approach, providing alternative phenotypic methods are available.

12.1.3 Direct Versus Indirect Methods

There are two basic approaches to DST, the direct and indirect methods. In the direct test, the drug-containing media are directly inoculated with a smear-positive sputum. The indirect test is performed with a bacterial suspension made from a pure culture (MGIT or solid media). It is feasible to rely on the direct DST during the initial diagnosis or screening phase for a clinical trial. The major advantage would be to rapidly obtain DST results for the newly developed TB drugs where the gene target or mutations associated with resistance have not been defined. This approach has not been adopted by the clinical trials community despite results being available in considerably less time than with indirect DST.

12.1.4 Proportion Method – the Preferred Phenotypic Method

The MGIT DST (direct and indirect) is based on the proportion method. In this method, the isolate is susceptible if the critical concentration of the drug inhibits 99% of the growth. The isolate is resistant if the proportion of growth in the presence of the critical concentration is greater than 1% in relation to the amount of growth in the drug-free medium. With the MGIT system, the instrument determines this relationship during the defined protocol time and interprets the results automatically. For phenotypic testing in clinical trials, the proportion method is preferred.

Drugs available from Becton Dickinson for testing in the MGIT system are INH, RIF, ethambutol (EMB), pyrazinamide (PZA), and streptomycin. For second-line drugs, the following are available: amikacin, kanamycin, capreomycin, ofloxacin, and moxifloxacin.

12.1.5 Qualitative Versus Quantitative Minimum Inhibitory Concentration Tests

Whether the isolate is susceptible or resistant to the critical concentrations has no relationship to the concentrations attainable in humans. Empiric concentrations are used for qualitative testing, and it is assumed that the individual will likely not respond to the treatment with a particular drug if the isolate is resistant to the critical concentration (62). Minimum inhibitory concentration (MIC) determination is an indirect quantitative test. MIC results can be correlated with the drug concentrations attainable in humans. In clinical trials, MIC testing is often performed on isolates obtained from enrolment/randomization specimens and from culture-positive specimens occurring after 2 months of treatment. In this situation, only the study/experimental drugs are tested and presently the testing is performed by a reference laboratory.

12.1.6 MGIT DST Turn-Around-Time

DST should be performed as soon as possible once the MGIT culture is confirmed as being pure and having MTB. This enables the study team to have the confirmatory DST results in a timely manner in the event the participant must be withdrawn from the study due to a discrepant DST result. Furthermore, problems arise when MGIT cultures are held for DST at a later date. Often cultures are contaminated once the PANTA activity diminishes or the MTB are no longer viable. Furthermore, the preparation of the inoculum

at the appropriate density is challenging, e.g., if the inoculum is too dense, false resistance results are observed, especially with PZA (68).

12.1.6 Testing Non-BACTEC MGIT Drugs

Some of the second-line drugs that may need to be tested are not available from Becton Dickinson. The technical points related to testing these drugs are the following:

- Obtain drugs from a reputable manufacturer (e.g., Sigma).
- Prepare stock solutions based on the potency of the lot number of the drug.
- Dissolve drugs in the solvent recommended by the manufacturer.
- Make stock solutions at 1000 µg/mL or 10-fold higher than the drug's working concentration.
- Filter stock solutions of drugs to sterilize.
- Stock solutions must be stored in small volumes in cryovials for up to 12 months at -70°C/-80°C, or up to the date of drug powder expiry, whichever is sooner.
- Once thawed, stock solution vials must be used immediately and not refrozen.
- The working concentration is calculated based on the final/critical concentration in the MGIT tube and 100 µL being added to the MGIT tube. The stock solution is diluted with sterile distilled water to achieve the working solution concentration.
- A 5 or 8-tube Antimicrobial Susceptibility Test (AST) carrier is used to program the instrument for the 13-day protocol. If using Epicenter, you can program the protocol time for 13 days.
- The drugs should always be placed in the same order in the carrier.
- If there are open spaces in the carrier (following the last drug-containing tube), blank MGIT tubes are placed in these spaces.
- If not using Epicenter, interpret the results accordingly: when growth control reaches 400 GU (growth units) within 4 to 13 days, susceptible = GU result of the drug tube is < 100 GU, and resistant = GU result of the drug tube is $\geq$ 100 GU. If using Epicenter, interpretation will be made by the TBExist software.
- If not using Epicenter, record the names of the drugs on the Unloaded AST Set Report. If using Epicenter, define the names of drugs tested in the TBExist software.

12.1.7 Working Up X200 and X400 MGIT Errors

Invalid MGIT DST results are reported on the Unloaded AST Set Report when certain conditions occur that affect the interpretation of test results. These are indicated as X200 and X400 errors.

X200 errors are reported when the Growth Control tube has not reached a GU result of 400 in the specified protocol time. Insufficient amount of MTB in inoculum or a slow growing drug-resistant isolate are the most common reasons for X200 errors. Refer to Flow Chart 4 (section 12.5) for management of these errors.

X400 errors are reported when the Growth Control tube has a GU result of $\geq$ 400 before day 4, which suggests the tube is contaminated or over-inoculated (bacterial density of inoculum is too high). Refer to

Flow Chart 5 (section 12.5) for management of these errors. Both flow charts have been adapted from the Global Laboratory Initiative (GLI) Mycobacteriology Laboratory Manual (3).

12.1.8 Interpretation of Results and Reporting

Results for the BACTEC MGIT drugs are interpreted by the instrument and appear on the Unloaded AST Set Report. The interpretation of the non-BACTEC MGIT drugs is as described above in 12.1.6.

DST results for all drugs must be reported as either:

1. Susceptible
2. Resistant
3. Indeterminate (Note: indeterminate can be due to control tube failure, X200 error, X400 error, or quality control [QC] failure)
4. No result (Note: no result would be due to a nonviable culture)

Most study and laboratory case report forms (CRFs) require reporting the critical concentration tested for each of the drugs. This ensures the WHO recommended concentration (62) was tested, and when high and low concentrations are tested, these are distinguished (e.g., INH, 0.2 µg/mL and 1.0 µg/mL; moxifloxacin, 0.5 µg/mL and 2.0 µg/mL).

12.2 Key Element of MGIT DST

The Key Element is including an internal QC when testing isolates. QC strain MTB H37Rv is the preferred reference strain; however, MTB H37Ra or another well-characterized strain that is susceptible to all standard anti-TB agents can be used. Timely and quality results are required to ensure the safety of study participants (i.e., the participants are susceptible to the drugs being administered in the trial and compliant with the study's eligibility requirements related to drug susceptibility or resistance). See Table 12-1 for a summary.

Table 12-1. Key Element of the MGIT DST

Key Element	Effect	Impact
Include drug-susceptible QC strain MTB at least once per week or with each batch of isolates tested	Quality of DST results	Participant enrollment and safety

12.3 MGIT DST Internal Quality Controls

MGIT DST internal QC testing is outlined in Table 12-2. The frequency of QC testing depends on how often DST is performed. Typically, DST is carried out once a week and the QC strain is included in the batch. If performed less frequently, QC testing is performed with each batch of isolates tested. QC testing new lots of media, growth supplement, and drugs is described in section 5.1.3.

Table 12-2. MGIT DST Internal Quality Controls	
Element	**Description**
Positive control	MTB H37Rv Alternatively, MTB H37Ra or another well-characterized strain susceptible to all standard anti-TB agents
Negative control	Not applicable
Placement of controls in processing batch	Not applicable
Frequency of testing	At least weekly or with each batch of isolates tested

12.4 MGIT DST Checklist

See section 17.8.

12.5 MGIT DST Flow Charts for x200 and x400 Errors

Flow Chart 4: DST Work-up for x200 Errors from MGIT 960

NOTE: All work must be done in the biological safety cabinet

Prepare new MGIT culture from *primary* culture tube:

- Vortex tube well; let sit 5-10 minutes.
- Make a 1:100 dilution of the culture tube using saline or 7H9 broth.
- Supplement a new MGIT tube with 0.8 ml MGIT Growth Supplement ***without*** PANTA.
- Inoculate 0.5 ml of the 1:100 diluted sample into the new MGIT tube.
- Cap tube tightly; mix well by gently inverting 3-4 times.
- Enter into instrument and monitor until tube turns positive.
- When tube turns positive, screen for purity with ZN and BAP.
- If purity verified, re-incubate tube until it is 3 - 5 days old; record GU (Growth Unit) value on lab worksheet.

↓

Prepare new control and drug-containing media:

- Remove drugs from freezer; let thaw in refrigerator while labeling tubes and adding supplement.
- Aseptically add **0.8** ml SIRE or PZA supplement to each control and drug tube as appropriate.
- Aseptically add **0.1** ml (100 µl) of each drug to its appropriately labeled tube.

↓

Set-up new DST:

- Vortex 3-5 day old culture tube well.
- Let tube sit 5-10 minutes to settle big clumps. Use the supernatant broth without disturbing the sediment.
- Using transfer pipette, aseptically add **0.5 ml** of MGIT culture broth to each *drug* tube; **DO NOT DILUTE** culture.
- Recap tubes tightly.
- Mix by inverting gently 3-4 times.
- Inoculate control tube(s) with 0.5 ml of diluted MGIT culture broth as per routine procedure; i.e., 1:10 dilution for PZA; 1:100 dilution for SIRE.
- Enter tubes into the instrument.
- Monitor until instrument signals as complete.

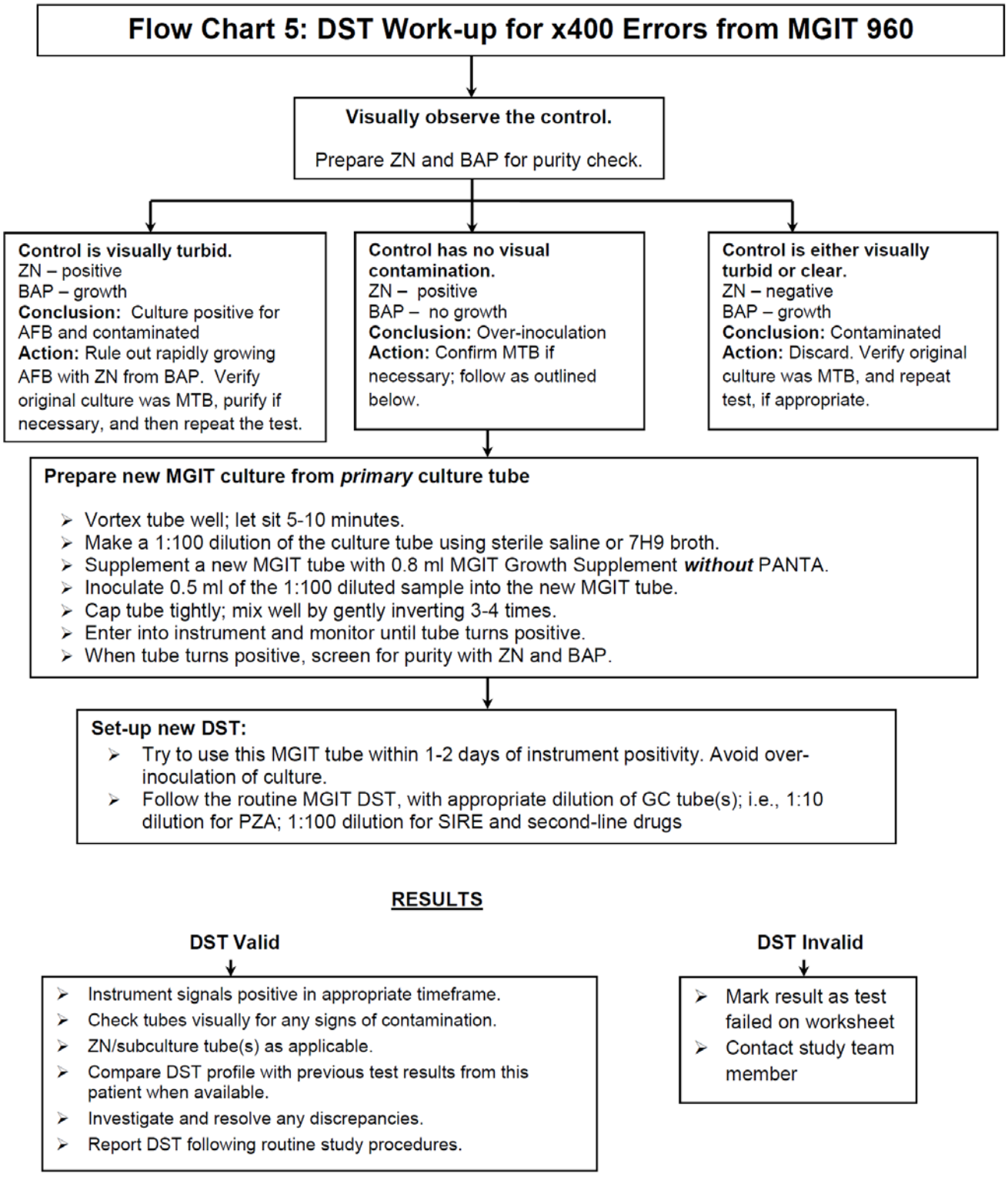
RESULTS

DST Valid ↓

- Instrument signals positive in appropriate timeframe.
- Check tubes visually for any signs of contamination.
- ZN/subculture tube(s) as applicable.
- Compare DST profile with previous test results from this patient when available.
- Investigate and resolve any discrepancies.
- Report DST following routine study procedures.
- If successful, note in internal records that a modified procedure was used to obtain DST results, and monitor frequency of X200 errors

DST Invalid ↓

- Mark result as test failed
- Contact study team member


Flow Chart 5: DST Work-up for x400 Errors from MGIT 960
Visually observe the control.
Prepare ZN and BAP for purity check.
Control is visually turbid.
ZN – positive
BAP – growth
Conclusion: Culture positive for AFB and contaminated
Action: Rule out rapidly growing AFB with ZN from BAP. Verify original culture was MTB, purify if necessary, and then repeat the test.
Control has no visual contamination.
ZN – positive
BAP – no growth
Conclusion: Over-inoculation
Action: Confirm MTB if necessary; follow as outlined below.
Control is either visually turbid or clear.
ZN – negative
BAP – growth
Conclusion: Contaminated
Action: Discard. Verify original culture was MTB, and repeat test, if appropriate.
Prepare new MGIT culture from primary culture tube
Vortex tube well; let sit 5-10 minutes.
Make a 1:100 dilution of the culture tube using sterile saline or 7H9 broth.
Supplement a new MGIT tube with 0.8 ml MGIT Growth Supplement without PANTA.
Inoculate 0.5 ml of the 1:100 diluted sample into the new MGIT tube.
Cap tube tightly; mix well by gently inverting 3-4 times.
Enter into instrument and monitor until tube turns positive.
When tube turns positive, screen for purity with ZN and BAP.
Set-up new DST:
Try to use this MGIT tube within 1-2 days of instrument positivity. Avoid over-inoculation of culture.
Follow the routine MGIT DST, with appropriate dilution of GC tube(s); i.e., 1:10 dilution for PZA; 1:100 dilution for SIRE and second-line drugs
RESULTS
DST Valid
Instrument signals positive in appropriate timeframe.
Check tubes visually for any signs of contamination.
ZN/subculture tube(s) as applicable.
Compare DST profile with previous test results from this patient when available.
Investigate and resolve any discrepancies.
Report DST following routine study procedures.
DST Invalid
Mark result as test failed on worksheet
Contact study team member

13 Solid Media Drug Susceptibility Testing (DST)

13.1 Background Information

13.1.1 Introduction

Standardization of non-commercial DST methods is difficult. Thus the BACTEC Mycobacterial Growth Indicator Tube (MGIT) DST method, a commercial, automated method, is the preferred method for clinical trials. A section on the solid media proportion method is included in the Sourcebook because it is the reference method for phenotypic DST and often used in testing second-line drugs. Its use can be:

- The standard against which new methods are evaluated
- To resolve problematic BACTEC MGIT 960 results
- To test additional drugs that cannot be, or have not been, evaluated for testing by the MGIT 960 system
- To test different drug concentrations that cannot be, or have not been, evaluated for testing by the MGIT 960 system

13.1.2 Proportion Method -- the Preferred Method

The three methods used for determining drug susceptibility are: the absolute concentration method, resistance ratio method, and proportion method. The proportion method is most widely used and the preferred method when used as the reference method. The proportion method is based on critical concentration of the drug and critical proportion of *Mycobacterium tuberculosis* (MTB) growing on drug-containing media. When using solid media resistance is expressed as the percentage of colonies on drug-containing media compared with colonies on drug-free media. Resistance is based on the fact that there is a certain proportion of drug-resistant mutants above which therapeutic success is less likely to occur. This percentage is 1% (5).

13.1.3 Solid Media Types

Either Middlebrook agar or Lowenstein-Jensen (LJ) can be used. LJ media with recommended drug concentrations are available commercially in some settings. Typically, laboratories make the media themselves so they can select which drugs they want to test. For agar media, quadrant plates are prepared (four drugs per plate) which saves incubator space when testing a large number of drugs. With Middlebrook, the option is 7H10 or 7H11 agar. The Middlebrook 7H11 agar is preferred because it promotes better growth, especially of the drug-resistant strains (69).

Critical concentrations often differ, depending on the type of solid media. Refer to the critical concentrations published by the World Health Organization (WHO) when performing this DST method (62).

13.1.4 Other Phenotypic DST Methods

WHO has conditionally recommended certain non-commercial tests for detecting rifampin (RIF) resistance as an interim solution, pending the laboratory's development of genotypic capacity for RIF resistance detection (29). These methods include microscopic observation of drug susceptibility, nitrate reductase assay, and colorimetric redox indicator. Some can be used as a direct test for detecting *Mycobacterium tuberculosis* (MTB) and drug resistance. However, their use is not intended to replace conventional culture and DST. Since laboratories participating in TB drug trials have the capacity to perform MGIT DST, MTBDR*plus*, and Xpert® MTB/RIF assays, these non-commercial methods are not used. Results from these methods may be available from diagnostic testing prior to screening and enrollment in the study.

13.1.5 Reading and Reporting Results

Solid media are examined macroscopically. After 1 week of incubation, plates/slopes are examined for contamination and again after 3 weeks of incubation for presence of growth. Strains showing resistance at this time can be reported as drug resistant. If no growth, plates/slopes are incubated for a total of 4 weeks (28 days).

DST results for all drugs must be reported as one of the following:

1. Susceptible
2. Resistant
3. Indeterminate *(Note: indeterminate can be due to a drug-free control plate/slope failure or quality control [QC] failure)*
4. No result (Note: no result can be due to a nonviable culture)

Most study and laboratory case report forms (CRFs) require reporting the critical concentration tested for each of the drugs. This ensures that the WHO recommended concentration (62) was tested, and, when high and low concentrations are tested, these are distinguished (e.g., isoniazid [INH], 0.2 µg/mL and 1.0 µg/mL; moxifloxacin, 0.5 µg/mL and 2.0 µg/mL).

13.2 Key Elements of Solid Media Proportion DST

There are four Key Elements that relate to the accuracy or overall quality of DST results. If the solid media proportion DST is used in context of a clinical trial (e.g., phenotypic confirmation of molecular test results for fluoroquinolones (FLQ)); the potential impact would be on the participant's safety or inclusion in the study.

When using agar media, access to oxygen and carbon dioxide (CO_2) is critical for optimal growth of the MTB and optimal activity of the drugs; thus, plates must be placed in a CO_2 incubator and sealed with gas-permeable tape or bags to prevent drying and allow exposure to oxygen and CO_2.

Plates and slopes must be incubated long enough to allow slow-growing strains time to yield adequate growth, both on drug-free and drug-containing media. However, the plates/slopes cannot be incubated

longer than 28 days as some of the drugs lose their activity, especially if they were made 1-2 weeks before being used. Drug-free control plates/slopes must have at least 50 distinct colonies, demonstrating that the density of the inoculum was sufficient.

As a reference method, the solid media proportion DST is performed less frequently; thus, QC testing is performed with each batch of isolates tested.

Timely and quality results are required to ensure the safety of study participants (i.e., the participants are susceptible to the drugs being administered in the trial, and compliant with the study's eligibility requirements related to drug susceptibility or resistance). See Table 13-1 for a summary.

Table 13-1. Key Elements of Solid Media Proportion DST

Key Element	**Effect**	Impact
Incubate agar plates in the presence of 5-10% CO_2	Accuracy of DST results	Participant enrollment and safety
Incubate plates/slopes up to 28 days before reporting a susceptible result	Accuracy of DST results	Participant enrollment and safety
Drug-free control plates/slopes must have at least 50 distinct colonies for a valid test	Accuracy of DST results	Participant enrollment and safety
Include a drug-susceptible QC strain with each batch of isolates tested	Quality of DST results	Participant enrollment and safety

13.3 Solid Media DST Internal Controls

Solid media DST internal controls are described in Table 13-2. QC testing new lots of media and drugs is described in section 5.1.3.

Table 13-2. Solid Media DST Internal Controls

Element	**Description**
Positive control	Suspension of MTB H37Rv Alternatively, MTB H37Ra or another well-characterized strain that is susceptible to all standard anti-tuberculosis (TB) agents
Negative control	Not applicable
Placement of controls in processing batch	Not applicable
Frequency of testing	With each batch of isolates tested

13.4 Solid Media Proportion DST Checklist

See section 17.9.

14 Hain Line Probe Assays (LPAs)

14.1 Background Information

14.1.1 Introduction

Molecular assays have become important adjuncts to traditional culture-based procedures, as they provide more rapid identification of mycobacterial species, including distinguishing species of the *Mycobacterium tuberculosis* complex (MTBC), as well as the rapid detection of drug resistance-associated mutations in *M. tuberculosis* (MTB). The Hain GenoType® line probe assays (LPAs) (MTBDR*plus*, MTBDR*sl*, Common Mycobacteria (CM)/Additional Species (AS), and MTBC) are based on line probe technology involving multiplex polymerase chain reaction (PCR) amplification and binding of amplicons to specific oligonucleotide probes immobilized on a nitrocellulose membrane strip during the reverse hybridization process. Presently, the LPAs are the most appealing molecular tests in full-service tuberculosis (TB) laboratories because of their versatility and comprehensive testing menu.

Molecular identification and drug susceptibility testing (DST) may be performed on clinical specimens as well as DNA isolated from cultures. This Sourcebook focuses on the use of MTBDR*plus* and MTBDR*sl* assays as direct tests (i.e., DNA isolated from resuspended sediment of a processed sputum specimen). However, the Key Elements and critical steps described herein are also applicable to the MTBDR*plus* and MTBDR*sl* assays when used as indirect tests (cultures/isolates), and to the CM, AS, and MTBC LPAs for species identification. A description of these applications can be found in the MGIT culture section (section 9), including the algorithms (section 9.5). The various Hain LPAs available are listed in Table 9-1.

14.1.2 MTBDR*plus* Version 2 Kits – the Preferred LPAs

The MTBDR*plus* version 2 kit has replaced version 1 because it is more sensitive. The MTBDR*sl* assay, version 2, is now available and reportedly has superior sensitivity than version 1 in detecting fluoroquinolone (FLQ) and kanamycin resistance (70). In some countries, MTBDR*sl* version 2 kits are not yet available (i.e., awaiting local approval before they can be imported and used as a TB diagnostic test). In these situations, the "RUO – research use only" kits can be purchased if needed for a clinical trial.

14.1.3 LPAs as Screening Tests

In the context of clinical trials, the MTBDR*plus* and MTBDR*sl* assays are used as screening tests to determine if the individual has drug-susceptible or drug-resistant tuberculosis (TB). These results indicate whether or not the individual fulfills specific study eligibility criteria. The MTBDR*plus* assay is used to distinguish drug-susceptible from multi-drug-resistant (MDR)-TB. In drug-susceptible TB studies, the MTBDR*plus* assay is preferred over the Xpert MTB/RIF assay when the frequency of isoniazid (INH) mono-resistance is high in the population, since the Xpert® MTB/RIF assay does not test INH. In MDR-TB trials, the MTBDR*sl* assay is critical in assessing FLQ resistance, as drugs in this class are often included in the experimental treatment regimens. Although individuals are evaluated with these molecular tests and may

be randomized to a treatment arm of a study, the presence of MTB and DST results must be confirmed by phenotypic tests (i.e., culture, identification, and DST) (61).

14.1.4 Procedure Precautions

As with any commercial product, all steps in the procedure must be performed in accordance with the manufacturer's package insert.

Since sputum sediments (resuspended pellets) and culture suspensions tested with the LPAs will contain viable MTB, the DNA extraction procedure must be carried out in a biosafety cabinet (BSC) using biosafety practices appropriate for MTB. Preferably, this is done in a biosafety level (BSL)-3 or BSL-2 laboratory that is a part of the TB laboratory and by separate staff from those performing the molecular assay.

When performing molecular assays, the main steps of the procedure are physically separated into these areas: PCR master mix preparation, assay setup (addition of DNA to the reaction mixture) and amplification, and detection. Staff carrying out more than one step in the procedure in 1 day must move from one area to another in only one direction (master mix preparation → assay setup → amplification → detection).

14.1.5 LPA Turnaround Time

Turnaround time (TAT) is a critical component of screening tests; ensuring results are obtained in a timely manner for determining enrolment eligibility. MTBDR*plus* and MTBDR*sl* results should be available to the study team within 48 hours of receipt of the sputum specimen in the laboratory. TATs of TB tests are quality indicators (QI), and in a diagnostic setting, a TAT of 72 hours is acceptable for LPAs.

14.1.6 Interpretation of Results and Reporting

Results are interpreted by visually examining the hybridized DNA bands on the LPA strips, or by scanning the LPA strips with the automated GenoScan®. The following terminology is suggested for reporting on the study or laboratory case report form (CRF). Note that drug susceptibility results are reported only when MTBC is detected. Also, reported results should be final (i.e., if an indeterminate result is obtained, the test should be repeated before reporting this result as final).

- Final result of the HAIN MTBDR*plus*/MTBDR*sl* assay:

  1. MTBC detected
  2. MTBC not detected
  3. Indeterminate (Note: indeterminate can be due to a gene locus failure or mixed infection)

- DST result if MTBC is detected:

1. Susceptible
2. Resistant
3. Indeterminate (Note: indeterminate can be due to a gene locus failure or heteroresistance)

Study-specific requirements may include reporting the mutation observed when a result is resistant or only one drug from the MTBDR*s*/ assay (e.g., FLQ).

14.2 Key Elements of Hain LPAs

Performing the test in accordance with the manufacturer's package insert ensures the accuracy of the test results. In a clinical trial where the LPA is used during the screening phase to determine drug susceptibility or resistance, inaccurate results could potentially: (1) impact the participant's safety, if treated with a drug(s) to which he/she is resistant; and (2) be a late exclusion criterion and require the participant to be withdrawn from the study to comply with the protocol.

Confidence in the accuracy of test results comes when kit performance is regularly tested with appropriate positive and negative controls. The key element pertaining to running internal controls addresses the need for accurate results, to avoid issues that could potentially risk participant safety or protocol compliance. See Table 14-1 for a summary.

Table 14-1. Key Elements of the Hain Line Probe Assays

Key Element	Effect	Impact
Perform procedures according to the manufacturer's instructions in the package insert	Accuracy of MTB and drug-resistance detection	Participant safety Protocol compliance
Include positive and negative controls with each batch of specimens or one batch per week	Accuracy of MTB and drug-resistance detection	Participant safety Protocol compliance

14.3 Hain LPA Quality Controls

14.3.1 LPA Internal Device Controls

To validate the proper functioning of the kit components, each strip includes control zones. These controls are different from internal quality controls (QC) (described below) that assess the overall performance of the assay. The Conjugate Control (CC) zone checks the binding of the conjugate on the strip and correct chromogenic reaction. The Amplification Control (AC) zone checks for successful amplification reaction. The Locus Control (LC) zones (labeled per gene target) check the optimal sensitivity of the reaction for each of the tested gene loci. All control zones must give the expected results for the test results to be valid.

14.3.2 LPA Internal Quality Controls

Hain LPA controls are described in Table 14-2. QC testing of new lots of Hain kits is described in section 5.1.3.

Table 14-2. Hain LPA Internal Quality Controls

Element	Description
Positive control	An in-house prepared control (e.g., resuspended pellet of the specimen processing positive control) (see section 7.3). Alternatively, a relevant bacterial suspension. For MTB LPAs, use MTB; for CM/AS LPAs, use a nontuberculous mycobacterium (NTM). Note: DNA samples are not to be used as positive controls, since this circumvents the ability to assess proficiency of the DNA extraction step.
Negative control	Molecular grade or sterile water.
Placement of controls in processing batch	The positive control(s) is always placed after the last test sample in the run and is followed by the negative control(s).
Frequency of testing	Controls are included with each test batch or tested at least once weekly.

14.4 Hain LPAs Checklist

See section 17.10.

15 Cepheid Xpert® MTB/RIF

15.1 Background Information

Molecular assays have become important adjuncts to traditional culture-based procedures, as they provide more rapid identification of mycobacterial species, including distinguishing species of the *Mycobacterium tuberculosis* complex (MTBC), as well as the rapid detection of drug resistance-associated mutations in *Mycobacterium tuberculosis* (MTB). The Xpert® MTB/RIF (GeneXpert; Cepheid, Sunnyvale, CA) is a closed, self-contained platform (cartridge) test for the extraction, amplification, and detection of both MTB and rifampicin (RIF) resistance mutations, which are markers for multi-drug-resistant tuberculosis (MDR-TB). It is a nested, real-time polymerase chain reaction (PCR) with five molecular beacons for mutations within the RIF-resistance determining region (*rpoB* gene) that have specificity for MTBC.

The Xpert® MTB/RIF assay is self-contained, easy-to-use, and automated, advantages that explain its wide implementation in clinic settings where sputum is collected from individuals suspected of having tuberculosis (TB). The test can be performed with raw (unprocessed) sputum, or concentrated sediments prepared from sputum, the latter requiring a traditional laboratory environment. If using raw sputum, test results can be available within 2 hours of sputum collection. Although the assay simultaneously detects MTB and RIF resistance, in some settings it is used solely for detecting MTB, as a primary diagnostic test (along with smear microscopy). In other patient populations, such as treatment failures and retreatment cases, it is used to rule out MDR-TB.

15.1.1 Xpert® MTB/RIF as a Screening Test

In the context of clinical trials, the Xpert® MTB/RIF assay is used as an alternative to the Hain MTBDR*plus* assay in screening potential study participants for MDR-TB. These results indicate whether or not the individual fulfills specific study eligibility criteria.

Although the MTBDR*plus* assay has the advantage of being able to detect isoniazid (INH) resistance in addition to RIF, the Xpert® MTB/RIF assay is desirable with its shorter turnaround time (TAT) and ability to test raw sputum. Xpert MTB/RIF; thus, it can easily be performed at the time of the direct sputum smear in the clinic. Also, the PCR cycle threshold results (Ct values) are indicative of the amount of MTB in the sputum specimen and can be used to predict smear-positivity. That is, Ct values $\leq$ 22 are indicative of medium to high MTB and highly likely to be smear-positive (71). Thus, in the absence of sputum smear results, the Ct value is examined along with the Xpert® MTB/RIF assay results in screening individuals for enrollment eligibility. Although individuals can be evaluated and may be randomized to a treatment arm of a study with either of these molecular tests, the presence of MTB and drug susceptibility result must be confirmed by phenotypic tests (i.e., culture, identification, and DST).

Although the most common use of the Xpert® MTB/RIF assay in a clinical trial will be in screening individuals, the Xpert® MTB/RIF assay may be used to confirm the identification of acid-fast bacilli (AFB)

positive mycobacteria growth indicator tube (MGIT) culture as described in the MGIT culture algorithms, section 9.5.

15.1.2 Procedure Precautions

As with any commercial product, all steps in the procedure must be performed in accordance with the manufacturer's package insert.

Since raw sputum specimens, sputum sediments (resuspended pellet), and culture suspensions tested in the Xpert® MTB/RIF assay contain viable MTB, the transfer of samples to the sample tubes should be handled inside a biosafety cabinet (BSC) using biosafety practices appropriate for MTB. Preferably, this is done in a BSL-3 or BSL-2 containment laboratory, which is a part of the TB laboratory. Although the sample is considered non-infectious after the 15-minute incubation step (72), it is preferable to load the cartridge with the liquefied sample inside the BSC. When performing the Xpert® MTB/RIF assay in a clinic setting it is desirable to work in a well-ventilated room and to minimize aerosolization of raw sputum when transferring to the sample tubes.

15.1.3 Xpert® MTB/RIF Assay Turnaround Time

The Xpert® MTB/RIF TAT is covered in the Sourcebook because it is important to report these results in a timely manner to facilitate enrolment of participants in the study. Although the testing time is about 2 hours, the test may not be performed immediately upon receipt. Xpert® MTB/RIF results should be available to the study team within 48 hours of receipt of the sputum specimen in the laboratory, which is in-line with the TAT for molecular tests (22, 29).

15.1.4 Interpretation of Results and Reporting

The GeneXpert instrument generates the results from measured fluorescent signals and calculation algorithms. The following terminology is suggested for reporting on the study and laboratory case report forms (CRFs). Results reported should be final (i.e., if invalid, or an error, or no result is obtained, the test must be repeated before reporting these results as final).

Final Xpert® MTB/RIF result for MTB detection:

1. Detected
2. Not detected
3. Invalid (Note: invalid can be due to Sample Processing Control [SPC] failure, specimen not properly processed, or PCR inhibition)
4. Error (Note: error can be due to a number of system/assay errors)
5. No result (Note: no result is due to insufficient data when the ongoing test is stopped)

Final Xpert® MTB/RIF result for RIF resistance, if MTB is detected:

1. Detected
2. Not detected
3. Indeterminate (Note: indeterminate is due to an insufficient signal for the *rpoB* gene)

Reporting of Ct values and semi-quantitative results (high, medium, low, and very low positive results) are not recommended by manufacturers, but may be required by the study.

15.1.5 Future Xpert® MTB/RIF Assays

15.1.5.1 Xpert® MTB/RIF Ultra

Xpert® MTB/RIF Ultra is now available (73-77). The multiple developments of the Xpert® MTB/RIF Ultra increased the analytical sensitivity by lowering the limit of detection to an equivalent to 5 CFU/mL. It has a new sample processing cartridge that doubled the amount of purified DNA delivered to the PCR reaction. Four newly designed probes that detected mutations in the *rpoB* gene replaced the five Xpert® MTB/RIF real-time probes. Real-time MTB-detecting probes targeting IS6110 and IS1081 were added. Cartridge fluidics and PCR cycling were optimized. Ultra uses the same semi-quantitative categories used in the Xpert® MTB/RIF assay (high, medium, low and very low) as well as the addition of a new semi-quantitative category "trace" that corresponds to the lowest bacillary burden for MTB detection (74).

Sensitivity of MTB detection is reported to be equivalent to liquid culture. Also, higher sensitivity in MTB detection than Xpert® MTB/RIF in smear-negative and HIV-infected individuals, and improved accuracy of RIF resistance detection have been reported. This is an important advance of the assay as a diagnostic test, but may not have impact on its use as a screening test for clinical trials where sensitivity is not required. Xpert® MTB/RIF Ultra, with its increased sensitivity, detects TB DNA in some individuals with prior TB disease; thus, Xpert® MTB/RIF Ultra results must be considered along with clinical history during screening. It is expected that the Xpert® MTB/RIF will be replaced by Xpert® MTB/RIF Ultra when available in countries where clinical studies are performed.

15.1.5.2 Xpert® MTB/RIF Extensively Drug-Resistant

An Xpert® MTB/RIF assay for extensively drug-resistant TB (XDR-TB) detecting MTB and resistance to fluoroquinolones (FLQ) and AG/CP is currently being evaluated (78). Such an assay, or any other molecular assay for detection of MTB and drug resistance, could be used for screening individuals. The Key Elements and important technical points are likely to be the same or very similar to those described in the Hain line probe assays (LPAs) and Cepheid Xpert® MTB/RIF sections (sections 14.2 and 15.2).

15.2 Key Element of Xpert® MTB/RIF

Performing the test in accordance with the manufacturer's package insert ensures the accuracy of the test results. In the context of a clinical trial where the Xpert® MTB/RIF assay is used during the screening phase to determine drug susceptibility or resistance, inaccurate results could potentially: (1) impact the participant's safety, if treated with a drug(s) to which he/she is resistant; and (2) be a late exclusion

criterion and require the participant to be withdrawn from the study to comply with the protocol. See Table 15-1 for a summary.

Table 15-1. Key Element of Xpert® MTB/RIF

Key Element	Effect	Impact
Perform Xpert® MTB/RIF assay according to the manufacturer's package insert	Accuracy of MTB and RIF resistance detection	Participant safety Protocol compliance

15.3 Quality Controls

15.3.3 Xpert® MTB/RIF Internal Assay Controls

To validate the proper functioning of the assay, each test includes a Sample Processing Control (SPC) and Probe Check Control (PCC). The SPC ensures the sample was correctly processed. The SPC verifies that conditions for lysis of MTB have occurred if the organisms are present and verifies that specimen processing is adequate. Additionally, this control detects specimen-associated inhibition of the real-time PCR reactions and acts as an internal positive control.

The Probe Check Controls (PCC), QC1 and QC2, check several reactions and functions of the assay. Before the start of the first and second reactions of the nested PCR assay, the GeneXpert® Instrument System measures the fluorescence signal from the QC1 and QC2 probes (reaction 1) and the *rpo*B and SPC probes (reaction 2) to monitor bead rehydration, reaction-tube filling, probe integrity and dye stability.

The SPC and PCC must give the expected results for the test result to be valid.

15.3.4 Xpert® MTB/RIF External Controls

External controls (Maine Molecular Quality Controls, Inc., Saco, Maine) are provided by Cepheid when certifying the assay prior to implementation (section 5.1.3). The INTROL™ TB controls are non-infectious, synthetic MTB DNA encapsulated in chemically fixed and killed bacterial cells, thus allowing for assessment of DNA extraction. These are recommended for testing new lots of cartridges and when performing annual calibration. They are not used as QC samples in routine testing of patient specimens. No additional QC testing is necessary if the new lots give acceptable results with the INTROL™ External Run Controls. Instrument and assay are calibrated annually using INTROL ™ External Run Controls.

15.4 Cepheid Xpert® MTB/RIF Checklist

See section 17.11.

16 Storage

16.1 Storage of Sputum

A separate standard operating procedure (SOP) has been developed for sputum processing for storage and tuberculosis (TB) biomarker research (40). It describes the procedure to process and store sputum specimens until they are shipped to a reference laboratory, or a formal repository for future TB research investigations.

16.2 Storage of the Sputum Pellet

Some references mention to keep the leftover pellet for up to one week in the refrigerator and use it in case of a contaminated culture. It is preferable to decontaminate the mycobacteria growth indicator tube (MGIT) bottle instead (section 9.1.6). Little has been published on the value and optimal storage conditions of leftover sediment/pellet for recovering *Mycobacterium tuberculosis* (MTB). Storage of sputum pellets is not required for TB clinical trials. However, if stored after processing and kept at -70°C to -80°C, they could be used for the development and validation of new TB diagnostic/biomarker tests.

16.3 Storage of Leftover Extracted DNA

The GenoLyse® extracted DNA used for the Hain line probe assays (LPAs) (section 14) may need to be stored for future testing if specified by the study. Once the GenoLyse® extraction is complete, it is critical that the top layer of the extracted DNA be immediately removed from the pellet containing the debris, and aliquoted into a sterile 1.5 mL cryotube. The tubes can be stored at -20°C or -80°C.

16.4 Storage of TB Isolates

16.4.3 Short-Term Storage on LJ

Short-term storage of positive cultures ensures that growth is available if repeat/further testing is needed. MTB recovered from all positive MGIT cultures (or solid media if MGIT has no growth or is contaminated) should be subcultured to Lowenstein-Jensen (LJ) slopes at the same time that identification tests are done (instead of batching for later subculture). When sufficient pure growth is observed, dense suspensions of this growth should be prepared in broth for long-term storage (see below). The short-term LJ slopes can be stored at room temperature for 6 months, or refrigerated for one year before being discarded, provided that all necessary testing has been completed and all frozen stock aliquots (long-term storage) have been appropriately stored. Tubes should be tightly sealed and wrapped with parafilm. More details can be found in the Global Laboratory Initiative (GLI) Mycobacteriology Laboratory Manual (3).

16.4.4 Long-Term Storage from LJ

Generally, not all MTB isolates from each MTB-positive culture will be stored long term. The sputum collection time intervals with MTB-positive cultures will be specified by the study; for example, MTB

isolates from two positive cultures before the participant is randomized to a study drug regimen (screening/baseline), and MTB isolates from any positive culture occurring after 2 months of receiving the study drug regimen. The short-term LJ subcultures are used to prepare the frozen isolates for long-term storage. Growth on LJ slopes should be luxuriant, pure (without contaminants), and less than 8 weeks old. Dense bacterial suspension is prepared in the broth in which it will be frozen. Preferably, the isolates are stored in Middlebrook 7H9 broth with OADC and 15% glycerol at -70°C to -80°C (3). It is recommended that at least two aliquots be frozen for each MTB isolate. These aliquots should not be discarded without prior verification from the study team.

Alternative to Middlebrook 7H9 broth, the Microbank™ system (Pro-Lab Diagnostics; Round Rock, TX) can be used. Each Microbank™ vial contains approximately 25 sterile beads and a cryopreservative. The specially treated beads are of a porous nature, allowing microorganisms to readily adhere onto the bead surface. A dense suspension of MTB is prepared in the cryopreservative using fresh MTB growth from a solid medium, or a dense suspension of MTB is prepared in sterile phosphate buffered saline (PBS) and placed into the Microbank™ vial. The Microbank™ vial sits for 2 minutes to allow the isolate to bind to the beads and then the PBS and/or cryopreservative is removed. Once the beads are free of liquid, the vial is ready to be frozen. Current studies with the Microbank™ system are being performed to evaluate the longevity of MTB isolates stored in this manner.

16.4.5 Long-Term Storage from MGIT

Some laboratories prefer to use positive MGIT cultures for isolate storage instead of making bacterial suspensions in Middlebrook 7H9 broth with OADC and glycerol, as this saves time and the need for additional media. The following precautions must be taken if this method is used for preserving MTB. MGIT cultures must contain a high density of viable MTB and be without contaminants or non-tuberculosis mycobacteria (NTM). Thus, cultures must: (1) have been incubated until the Growth Units (GU) are close to 900; (2) have been subcultured to demonstrate purity; and (3) not be older than 42 days regardless of whether they were incubated offline, stored at room temperature, or stored at 4-8°C since becoming positive. Samples can be removed from a well-mixed broth or from the bottom of a settled broth. If stored at -70°C, isolates should be viable up to 3 years (79).

17 Mycobacteriology Laboratory Checklists

Word versions of the checklists can be found on the HIV AIDS Network Coordination (HANC) website: https://www.hanc.info/labs/labresources/procedures/Pages/actgImpaactLabManual.aspx.

17.1 Quality Assurance Checklist

A. Laboratory Information	
Laboratory Name:	
Laboratory Address:	
Completed By:	
Date Completed:	

B. Relevant Standard Operating Procedures (SOPs)		
SOP No.	**SOP Title**	**Version No.**

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*				
Key Elements	**Y**	**N**	**NA**	**Comments**
1. Include positive and negative controls at least once each day that specimen processing is performed.				
2. Include positive and negative smears with each batch of patient slides when performing smear microscopy.				
3. Include positive and negative controls at least once per week or with each batch of cultures when performing MPT64 antigen test.				
4. Include a drug-susceptible QC strain of MTB at least once per week or with each batch of isolates when performing DST.				
5. Include positive and negative controls with each batch of specimens/isolates or one batch per week when performing Hain LPAs.				
Important Technical Points	**Y**	**N**	**NA**	**Comments**
1. Check specimen collection time and time of receipt in the laboratory to ensure transport was within allowable time limits.				

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*				
2. Check specimen transport box to ensure specimens were kept cool during transport.				
3. QC testing of new lots of stains with positive and negative smears before using.				
4. Examination of sputum smears is monitored for consistency between technologists				
5. QC testing of new lots of MGIT medium, Growth Supplement, and PANTA according to the instructions in the FIN.				
6. QC testing new lots of in-house prepared media for sterility, growth performance, and selectivity of antibiotic containing media.				
7. QC testing commercially prepared media for sterility, growth performance, and selectivity of antibiotic containing media if stored $\geq$ 6 weeks.				
8. QC testing of new lots of identification kits (MPT64 antigen test) with positive and negative controls before using.				
9. QC testing of new lots of MGIT media, Growth Supplement, and drugs with control strains before using for routine DST.				
10. QC testing of new lots of molecular test kit/reagents with positive and negative controls before using.				
11. Participation in external quality assessment programs which include panels for all TB tests performed by the laboratory.				
12. Testing EQA panels in the same manner as clinical specimens, cultures, and isolates.				
13. Daily check of equipment temperature and CO_2 concentration, if applicable.				
14. Routine maintenance and cleaning of the incubators.				
15. Routine maintenance of the MGIT and GeneXpert instruments per manufacturer's recommendations.				
16. Annual certification of the BSC.				
17. Annual service contract for the MGIT instruments.				
18. Cleaning and maintenance schedule for all equipment.				
19. Comparing of results to the corresponding results on worksheets/logs/registers (laboratory source documents) to ensure accuracy before reporting.				

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*				
20. Review of all final test results by a second person to minimize reporting errors.				
21. Routine collection of QI data.				
22. Monthly review of QC activities by the laboratory supervisor or designee, including a review of QC, EQA, QM, and QI results.				
23. Documentation of corrective and preventative actions when QC, EQA, QM, and QI results show unsatisfactory performance or deviation from baseline/normal values.				
24. Reporting of smear microscopy results within 24-48 hours of specimen receipt in the laboratory.				
25. Reporting of molecular test results within 48-72 hours of specimen receipt in the laboratory.				

D. Additional Notes/Comments

17.2 Specimen Collection, Transport, and Laboratory Receipt Checklist

A. Laboratory Information	
Laboratory Name:	
Laboratory Address:	
Completed By:	
Date Completed:	

B. Relevant Standard Operating Procedures (SOPs)		
SOP No.	**SOP Title**	**Version No.**

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.* ***Note that specimen refers to respiratory specimen and gastric aspirate/lavage.***

Key Elements	Y	N	NA	Comments
1. Participants must rinse their mouths with boiled/sterile/bottled or distilled water prior to collection.				
2. Collecting at least 3-5 mL of sputum. Larger volumes are preferred. A minimum of 1 mL is acceptable but not optimal to recover TB.				
3. Collecting at least 5-10 mL of gastric aspirate. Larger volumes are preferred. A minimum of 1 mL is acceptable but not optimal to recover TB.				
4. Collecting gastric aspirate after a minimum fasting period of at least 4 hours. Early morning collection is preferred.				
5. Gastric aspirate must be pH neutralized as soon as possible after aspiration, unless the laboratory can neutralize or process the specimen within 4 hours of collection.				
6. Storing specimens in a refrigerator or cool box (2-8^{o}C) if not transported to the laboratory within 1 hour of collection.				
7. Transporting specimens to the laboratory in a cool box (2-8^{o}C) as soon as possible after collection. Respiratory specimens must be delivered to the laboratory as soon as possible and/or within 24 hours of collection. Delays of up to 3 days in transport from the clinic to the laboratory are allowable if the transport distance is long, and if agreed upon by the study/protocol team.				

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.* ***Note that specimen refers to respiratory specimen and gastric aspirate/lavage.***				
8. Storing specimens in a refrigerator or cool box (2-8°C) if not processed within 1 hour of receipt at the laboratory.				
9. Neutralizing the gastric aspirate specimen within 4 hours of collection (if the specimen was not pH neutralized at the site of collection), if not immediately processed.				
Important Technical Points	**Y**	**N**	**NA**	**Comments**
1. Procedures for the collection, transport, and receipt of all mycobacteriology specimens.				
Infection control measures during specimen collection. Refer to network SOP (31)				
2. Collecting the gastric content by aspiration first, as lavage introduces dilution. If adequate volumes are not obtained, lavage can be performed using sterile water or saline.				

D. Additional Notes/Comments

17.3 Specimen Processing Checklist

A. Laboratory Information	
Laboratory Name:	
Laboratory Address:	
Completed By:	
Date Completed:	

B. Relevant Standard Operating Procedures (SOPs)		
SOP No.	**SOP Title**	**Version No.**

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*				
Key Elements	**Y**	**N**	**NA**	**Comments**
1. Using the entire respiratory specimen in processing. If more than 10 mL, a procedure is used to reduce the starting volume.				
2. Decontaminating specimens with a final sodium hydroxide (NaOH) concentration of 1.0-1.5%.				
Decontaminating specimens in NALC-NaOH for 15-20 minutes at room temperature prior to adding phosphate buffered saline (PBS) pH 6. 8.				
3. Centrifuging the decontaminated specimen at 3000 x *g* for 15-20 minutes. A refrigerated centrifuge is preferred.				
4. Resuspending the sediment in PBS (pH 6.8) to a final volume of 1.5-2.0 mL.				
5. Setting up cultures immediately following the suspension of the decontaminated, concentrated specimen.				
6. Including positive and negative controls at least once each day that specimen processing is performed.				
Important Technical Points	**Y**	**N**	**NA**	**Comments**
1. Performing all procedural steps of in a Class II BSC.				
2. Not working with more specimens than can be placed in the centrifuge at one time.				

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*				
3. Labeling each specimen collection container/tube/plate/slide with the participant identification, specimen number, and date of processing/inoculation.				
4. Bringing all specimens and reagents to room temperature prior to processing.				
5. Opening only one tube at a time (tube caps can be loosened to ensure ease of processing) to avoid cross-contamination and potential mix-ups.				
6. Vortexing the specimen for 5-10 seconds (every 5 minutes) or placing on a shaker at approximately 60 RPM during the decontamination step of the procedure.				
7. Carrying out centrifugation in sealed biosafety buckets.				
8. Processing or decontaminating specimens only once.				

D. Additional Notes/Comments

17.4 Smear Microscopy Checklist

A. Laboratory Information	
Laboratory Name:	
Laboratory Address:	
Completed By:	
Date Completed:	

B. Relevant Standard Operating Procedures (SOPs)		
SOP No.	**SOP Title**	**Version No.**

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*				
Key Elements	**Y**	**N**	**NA**	**Comments**
1. Including positive and negative smears with each batch of patient slides.				
2. Reporting results according to the WHO/UNION grading scale as per the Global Laboratory Initiative Sputum Microscopy Handbook.				
Important Technical Points	**Y**	**N**	**NA**	**Comments**
1. Preparing smears and drying slides are performed in a Class II BSC.				
2. Recording lot numbers and expiry dates of staining reagents used.				
3. Testing each new lot/batch of in-house and commercially prepared staining reagents before putting into use.				
4. Labeling each slide with patient identification, specimen number, and date of smear.				
5. Using the purulent and/or blood-tinged portion of the sputum specimen for making the direct smear.				
6. Using a well-mixed pellet suspension for making the concentrated smear.				
7. Using a well-mixed MGIT broth for making the smear. Do not vortex the tube excessively and do not sample the broth from the bottom of the MGIT tube.				
8. Leaving the slides in the BSC until they have air-dried.				
9. Fixing the slides before staining so that the MTB are nonviable. This should be done before removing the slides from the BSC when feasible.				

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*				
10. If using a hot plate or slide warmer, keeping the slides for at least 2 hours at 65-75°C. If using a drying oven, keep for the slides at least 20-30 minutes at 85-90°C. *If a heat fixation method is not used, indicate "NA" and report the fixation method in the "Comments" section*.				
11. Placing the slides on a staining rack so they are at least 1 cm apart to prevent transfer of material from one slide to another.				
12. Not overheating or drying out the slides while heating carbol fuchsin-flooded smears.				
13. Protecting fluorescent smears from light and examining them immediately. If the smears are to be read later, place the slides in a covered box.				
14. Avoiding touching the slide with the tip of the dropper when dispensing oil. Always wipe oil from the oil immersion lens after each slide is read.				
15. Reporting results to the clinic/study team within 48 hours of sputum receipt in the laboratory.				
16. Confirming all scanty results.				
17. Having all positive and 10% of negative slides reviewed by a second person before reporting.				
18. Periodically comparing microscopic observations between staff to ensure accuracy and reproducibility (internal QA).				
19. Staff participation in an external quality assessment (EQA) program.				
20. Maintaining equipment (e.g., slide warmer, microscope).				
21. Following safety precautions while preparing and using stains and staining solutions.				

D. Additional Notes/Comments

17.5 Mycobacteria Growth Indicator Tube (MGIT) Culture Checklist

A. Laboratory Information	
Laboratory Name:	
Laboratory Address:	
Completed By:	
Date Completed:	

B. Relevant Standard Operating Procedures (SOPs)		
SOP No.	**SOP Title**	**Version No.**

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*

Key Elements	Y	N	NA	Comments
1. Inoculating each MGIT tube with 0.5 mL of the resuspended pellet.				
2. Working up all MGIT cultures (positive and negative) according to the MGIT culture algorithms in the flow charts.				
Important Technical Points	**Y**	**N**	**NA**	**Comments**
1. Performing all procedures in a Class II BSC.				
2. Performing procedures in accordance with the manufacturer's package insert and the FIND MGIT Manual (24).				
3. Keeping MGIT tubes closed until ready for the specimen or PANTA/Growth Supplement addition.				
4. Checking the storage conditions and expiration date of the lyophilized PANTA (maintain at 2-8°C).				
5. Reconstituting PANTA in 15 mL of MGIT Growth Supplement (OADC). Use an aseptic technique to avoid introducing contaminants. The Growth Supplement must be measured even if provided in a 15-mL volume, as the actual volume may vary. Note: a smaller amount (e.g., 10 mL) of OADC may be used to increase the PANTA concentration to help control high contamination rates.				
6. Ensuring PANTA is completely dissolved in OADC solution when reconstituted.				

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*				
7. Storing reconstituted PANTA at 2-8°C and using it within 5 days. Do not freeze PANTA.				
8. Adding 0.8 mL of MGIT Growth Supplement/PANTA to each MGIT tube just prior to inoculating the specimen.				
9. Adding MGIT Growth Supplement/PANTA to the MGIT tube using a sterile transfer device. Using a repeat pipettor with a sterile cotton plugged tip helps in reducing contamination.				
10. Labeling the MGIT tube with patient identification, specimen number, and date of inoculation.				
11. Opening only one MGIT tube at a time and minimizing the time each cap is removed from the tube.				
12. Keeping the cap tightly closed and not shaking the tube during the incubation; this helps in maintaining the oxygen gradient in the medium.				
13. Recording the instrument-generated time to detection (TTD) in days and hours.				
14. Using a well-mixed MGIT broth for making the smear. Do not vortex the tube excessively and do not sample the broth from the bottom of the MGIT tube.				
15. Preparing a blood agar plate (BAP) for all positive cultures to rule out contamination.				
16. Inspecting all negative cultures for growth prior to discarding.				
17. Performing daily/monthly MGIT instrument maintenance. *If referenced in a separate SOP, indicate "YES" and report in the "Comments" section*.				
18. Including a procedure for using MGIT broth if Xpert® MTB/RIF is used in the work-up of positive MGIT cultures. *If referenced in a separate SOP (Xpert SOP), indicate "YES" and report in the "Comments" section*.				
19. Using an equal volume of 4% NaOH for decontaminating the MGIT culture per the FIND MGIT Manual.				
20. Inoculating a positive sputum processing control (resuspended pellet) in an MGIT tube to monitor NaOH killing of MTB.				

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*				
21. Inoculating a negative sputum processing control (resuspended pellet) in an MGIT tube to check for sterility of the processing reagents and monitor for cross-contamination.				
22. Prior to putting new lot numbers of media, Growth Supplement, and PANTA into use, checking the performance by culturing suspensions of MTB, *M. kansasii*, and *M. fortuitum* prepared as described in the FIND MGIT Manual.				

D. Additional Notes/Comments

17.6 Solid Media Culture Checklist

A. Laboratory Information	
Laboratory Name:	
Laboratory Address:	
Completed By:	
Date Completed:	

B. Relevant Standard Operating Procedures (SOPs)		
SOP No.	**SOP Title**	**Version No.**

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*				
Key Elements	**Y**	**N**	**NA**	**Comments**
1. Inoculating solid media (tube or plate) with 0.2 mL of resuspended pellet. *If slopes/plates cannot accommodate 0.2 mL, discuss alternatives with the study sponsor*.				
2. Incubating Middlebrook agar plates in 5-10% CO_2.				
3. Incubating solid media for at least 6 weeks before reporting a negative result; at least 8 weeks for trials with MDR-TB participants.				
Important Technical Points	**Y**	**N**	**NA**	**Comments**
1. Performing all procedural steps in a Class II BSC. Slopes/plates can be read outside the BSC, if kept closed/sealed.				
2. Using one tube or one plate per inoculation unless otherwise specified by the study sponsor.				
3. Streaking inoculum on the slope/plate for isolation of individual colonies.				
4. Sealing the plates with gas-permeable tape.				
5. Incubating the LJ slopes in a slanted position with the caps loosened in 5-10% CO_2 for a minimum of 2 weeks, if a CO_2 incubator is available. After 2 weeks, tighten the caps.				
6. Visualizing colony morphology using a strong direct light from an anglepoise lamp or a dissecting microscope.				
7. Working up growth resembling MTB using presumptive and definitive identification methods.				

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*				
8. Recording growth results weekly on the laboratory worksheet.				
9. Recording growth at the final reading interval according to the WHO/UNION standardized reporting scheme.				
10. Inoculating a positive sputum processing control (resuspended pellet) on a solid medium to monitor NaOH killing of MTB.				
11. Inoculating a negative sputum processing control (resuspended pellet) on a solid medium to check for sterility of the processing reagents and monitor for cross-contamination.				
12. Quality control (QC) testing each new lot/batch of in-house prepared media for sterility, growth performance, and selectivity of antibiotic containing media.				
13. QC testing commercial media after prolonged storage ($\geq$ 6 weeks) in the same manner as for a new lot/batch of in-house prepared media.				
14. Not using expired media.				

D. Additional Notes/Comments

17.7 MPT64 Antigen Identification Checklist

A. Laboratory Information	
Laboratory Name:	
Laboratory Address:	
Completed By:	
Date Completed:	

B. Relevant Standard Operating Procedures (SOPs)		
SOP No.	**SOP Title**	**Version No.**

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*

Key Elements	Y	N	NA	Comments
1. Confirming the presence of MTB vs. non-MTB at each trial time point when the culture is AFB positive.				
2. Including positive and negative controls at least once per week or with each batch of cultures.				
Important Technical Points	**Y**	**N**	**NA**	**Comments**
1. Performing all procedural steps in a Class II BSC.				
2. Performing procedures in accordance with the manufacturer's package insert.				
3. Performing an MPT64 antigen test only on cultures confirmed as being AFB positive.				
4. Testing the MGIT culture within 2-5 days of the instrument signaling positive.				
5. Testing colonies from the solid media within 2-4 weeks of visible growth.				
6. Inverting or vortexing the MGIT tube before sampling the culture for testing.				
7. Preparing a density of colony suspension equivalent to the 0.5 McFarland standard.				
8. The internal reagent control present in the device must give an expected result for a valid test result.				
9. Describing causes of possible false-negative results.				
10. Re-incubating and retesting all negative MGIT cultures in accordance with the MGIT culture algorithms.				

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*				
11. Testing new lot numbers with negative and positive controls before putting the MPT64 antigen kits into use.				

D. Additional Notes/Comments

17.8 MGIT Drug Susceptibility Testing (DST) Checklist

A. Laboratory Information	
Laboratory Name:	
Laboratory Address:	
Completed By:	
Date Completed:	

B. Relevant Standard Operating Procedures (SOPs)		
SOP No.	**SOP Title**	**Version No.**

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*				
Key Elements	**Y**	**N**	**NA**	**Comments**
1. Including a drug-susceptible QC strain of MTB at least once per week or with each batch of isolates tested. *Report the reference strain used in the "Comments" section*.				
Important Technical Points	**Y**	**N**	**NA**	**Comments**
1. Setting up DST in a Class II BSC.				
2. Reconstituting BACTEC drugs as specified in the manufacturer's package inserts.				
3. Storing reconstituted BACTEC drugs at -20°C or lower for up to 6 months, or up to the date of original expiry (whichever is sooner).				
4. For non-BACTEC drugs, use recommended WHO critical concentrations.				
5. For non-BACTEC drugs, preparing stock solution based on the potency of the lot number of the powdered/lyophilized drug.				
6. For non-BACTEC drugs, using the manufacturer's recommended solvent. If the solvent is other than water, use only a sufficient amount of solvent to solubilize the drug powder, then dilute to a final stock concentration with sterile distilled water.				
7. For non-BACTEC drugs, preparing stock solutions at 1000 µg/mL or 10-fold higher than the drug's working concentration, and sterilizing by membrane filtration.				

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*				
8. For non-BACTEC drugs, storing small volumes of sterile stock solutions in cryovials, carefully sealed, for up to 12 months at -70°C or -80°C, or up to the date of drug powder expiry (whichever is sooner).				
9. For non-BACTEC drugs, the final concentration in the MGIT tube is equivalent to the critical concentration for the respective drugs as per WHO.				
10. For non-BACTEC drugs, calculating working concentrations based on the final/critical concentration in the MGIT tube and 100 µL volume being added to the MGIT tube. Dilute the stock solution with sterile distilled water to achieve the working solution.				
11. Once thawed, using the drugs (reconstituted BACTEC drugs or stock solutions of non-BACTEC drugs) immediately.				
12. NOT refreezing or storing thawed reconstituted drugs/drug stock solutions (once thawed, discard unused drugs).				
13. Using a pure MGIT culture or pure isolate from solid medium for preparing DST inoculum.				
14. When preparing inoculum from solid media culture, using fresh growth harvested within 15 days of the appearance of growth on the solid medium.				
15. When using a seed culture from either MGIT or solid medium, time to detection (TTD) must be ≥ 4 days for use as AST inoculum. If the tube becomes positive in < 4 days, prepare a new seed culture using an inoculum containing a lower number of MTB (less dense).				
16. The day the MGIT tube signals positive by the MGIT instrument is Day 0.				
17. Incubating the MGIT culture for at least 1 day and no more than 5 days after the MGIT instrument signals positive prior to use in DST.				
18. An MGIT tube which has been positive for more than 5 days must be subcultured into a fresh MGIT tube with Growth Supplement (designated seed culture) and incubated in the MGIT instrument until positive prior to use in DST.				

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*				
19. If using a Day 1 or Day 2 positive MGIT tube, leaving the tube to stand for 5-10 minutes to allow large clumps to settle to the bottom of the tube, and using the undiluted supernatant for inoculation.				
20. If using a Day 3-5 positive MGIT tube, vortexing and allowing the tube to stand for 5-10 minutes to remove large clumps, then diluting 1 mL of the supernatant in 4 mL sterile saline (1:5 dilution) prior to inoculation.				
21. Placing all MGIT tubes in the correct sequence in the appropriate AST carrier set (i.e., for a SIRE, 5-tube carrier: Growth Control, streptomycin, INH, RIF, ethambutol; for a PZA, 2-tube carrier: Growth Control, PZA).				
22. For second-line drugs, using the AST carrier that will hold the number drugs being tested plus the control (e.g., 8-tube carrier: Growth Control, ofloxacin, moxifloxacin, amikacin, kanamycin, capreomycin, p-aminosalicylic acid, ethionamide). If the carrier set is not full, place blank MGIT tubes in the open positions at the end of the carrier. If needed, two carriers can be used, but each carrier must have a Growth Control in position 1. Refer to the FIND MGIT Manual for all possible AST set configurations.				
23. For second-line drug testing, entering the carrier as either "undefined" drugs and manually interpreting using Growth Units (GU) values from the unloaded AST set report, OR entering the carrier as a SIRE drug protocol where each second-line drug is coded as a first-line drug, and the instrument will interpret the results when the test is complete. Always load the tubes in the carrier set in the same order and maintain a record of drug designations (e.g., undesignated Drug #1 = ofloxacin or streptomycin = ofloxacin).				
24. If MGIT DST results are invalid, referring to the flow charts for work-up of X200 and X400 errors.				
25. Checking all "resistant" tubes visually for signs of contamination immediately after removing the tubes from the instrument.				
26. If contamination is suspected, performing a ZN smear and subculture to BAP.				

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*				
27. If contamination is confirmed, referring to the X400 error flow chart.				
28. When drug resistance is observed in a patient for the first time, performing a ZN smear and subculture to BAP to ensure the growth is not due to contaminants or NTM.				
29. When PZA resistance is observed in a patient for the first time, repeating the PZA test using a lower density inoculum to ensure this is not a false resistant result.				
30. If X200 errors are frequently observed in a specific patient population (e.g., MDR/XDR-TB patients), a Day 3-5 culture can be used undiluted as a first step, as described in the X200 error flow chart.				
31. When testing new lots of MGIT media, Growth Supplement, and drugs, including a clinical isolate known to be MDR-TB, if drug-resistant isolates are routinely tested.				
32. When testing new lots of MGIT media, Growth Supplement, and drugs, including clinical isolates known to be mono-resistant to FLQ and AG/CP, or pre-XDR-TB isolates (resistant to INH, RIF, and either FLQ or AG/CP) if second-line DST is to be performed.				

D. Additional Notes/Comments

17.9 Solid Media Proportion DST Checklist

A. Laboratory Information	
Laboratory Name:	
Laboratory Address:	
Completed By:	
Date Completed:	

B. Relevant Standard Operating Procedures (SOPs)		
SOP No.	**SOP Title**	**Version No.**

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*				
Key Elements	**Y**	**N**	**NA**	**Comments**
1. Incubating plates/slopes in the presence of 5-10% CO_2.				
2. Incubating plates/slopes up to 28 days before reporting a susceptible result.				
3. Drug-free control plates/slopes must have at least 50 distinct colonies for a valid test.				
4. Including a drug-susceptible QC strain of MTB at least once per week or with each batch of isolates tested. *Report the reference strain used in the "Comments" section*.				
Important Technical Points	**Y**	**N**	**NA**	**Comments**
1. Setting up DST in a Class II BSC.				
2. Performing DST when MGIT culture or solid media culture becomes positive and is confirmed as MTB, if DST is required.				
3. Preparing drug stock solution based on the potency of the lot number of the powdered/lyophilized drug.				
4. Using the manufacturer's recommended solvent. If the solvent is other than water, use only a sufficient amount of solvent to solubilize the drug powder, then dilute to the final stock concentration with sterile distilled water.				

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*				
5. Preparing stock solutions at 1000 µg/mL or 10-fold higher than the drug's working concentration, and sterilizing by membrane filtration.				
6. Storing small volumes of sterile stock solutions in cryovials, carefully sealed, for up to 12 months at -70°C or -80°C, or up to the date of drug powder expiry (whichever is sooner).				
7. Calculating working concentrations based on the final/critical concentration in solid medium and volume of the medium being prepared. Dilute the stock solution with sterile distilled water to achieve the working solution.				
8. Once thawed, using the drugs immediately.				
9. NOT refreezing or storing the thawed reconstituted drugs/drug stock solutions (once thawed, discard unused drugs).				
10. Instructions on preparing drug-containing media. *If referenced in a separate SOP, indicate "Y" and report in the "Comments" section*.				
11. When preparing inoculum from solid media culture, using fresh growth within 15 days of appearance on the solid medium.				
12. When preparing inoculum from a positive MGIT culture, incubating the tube until the density of the culture is at least the density of the McFarland Standard #1.				
13. Preparing the initial suspension at a density of the McFarland Standard #1 using a pure culture of MTB.				
14. Preparing thee final working suspensions by diluting McFarland Standard #1 suspension 10^{-2} and 10^{-4} (1:100 & 1:10000 dilutions).				
15. Inoculating drug-containing and drug-free media with volume specified in procedure.				
16. Sealing the plates with gas-permeable tape.				
17. Incubating the LJ slopes in a slanted position with the caps loosened in 5-10% CO_2 for a minimum of 2 weeks, if a CO_2 incubator is available. After 2 weeks, tighten the caps.				
18. Visualizing colony morphology using a strong direct light from an anglepoise lamp or a dissecting microscope.				

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*				
19. Recording weekly growth results on a worksheet/log/register.				
20. Procedure for interpreting and reporting results, including a formula for determining resistance (> 1%) or susceptibility (≥ 99%).				
21. If second-line drug testing is performed and/or MDR-TB isolates are tested frequently, testing appropriate resistant strains periodically (e.g., monthly) or with new lots of medium and drugs.				

D. Additional Notes/Comments

17.10 Hain Line Probe Assays Checklist

A. Laboratory Information	
Laboratory Name:	
Laboratory Address:	
Completed By:	
Date Completed:	

B. Relevant Standard Operating Procedures (SOPs)		
SOP No.	**SOP Title**	**Version No.**

C. Do your laboratory SOP(s) include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*

Key Elements	**Y**	**N**	**NA**	**Comments**
1. Performing procedures in accordance with the manufacturer's package inserts.				
2. Including positive and negative controls with each batch of specimens/cultures or one batch per week.				
Important Technical Points	**Y**	**N**	**NA**	**Comments**
1. Areas for master-mix preparation, DNA template addition/PCR, and detection are physically separated. *Describe how the laboratory is physically arranged to separate these activities in the "Comments" section*.				
2. Staff carrying out more than one step in the procedure in one day must move in one direction (master mix prep → assay setup → amplification → detection).				
3. Procedures for isolating DNA from resuspended pellets and cultures are according to the manufacturer's instructions in the package insert.				
4. MTB in resuspended pellets and cultures must be nonviable if DNA extraction (GenoLyse® procedure) is conducted outside the BSC.				
5. For the MTBDR*plus* assay, MTB must be detected for INH and RIF results to be valid.				
6. For the MTBDR*sl* assay, MTB must be detected for fluoroquinolone and aminoglycoside/cyclic peptide results to be valid.				

C. Do your laboratory SOP(s) include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*				
7. For the MTBDR*plus* and MTBDR*sl* assays, if indeterminate results are obtained, repeating with newly extracted DNA from the same specimen. If still indeterminate, request another specimen.				
8. Results should be available to clinical staff within 48 hours of sputum receipt in the laboratory.				
9. QC testing new lots of LPA kits with appropriate controls before using.				

D. Additional Notes/Comments

17.11 Cepheid Xpert® MTB/RIF Checklist

A. Laboratory Information	
Laboratory Name:	
Laboratory Address:	
Completed By:	
Date Completed:	

B. Relevant Standard Operating Procedures (SOPs)		
SOP No.	**SOP Title**	**Version No.**

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*				
Key Elements	**Y**	**N**	**NA**	**Comments**
1. Performing the procedure in accordance with the manufacturer's package insert.				
Important Technical Points	**Y**	**N**	**NA**	**Comments**
1. Results of internal controls, Probe Check Control, and Specimen Processing Control must be as expected for the test results to be valid.				
2. If invalid, no result, or error is obtained, repeating the test with the same specimen. If still invalid, no result, or error, request another specimen for retesting.				
3. MTB must be detected for RIF results to be valid.				
4. Description on the reporting of semi-quantitative results (high, medium, low, very low) and Ct (PCR cycle threshold) values, if required by the study sponsor.				
5. Results must be available to clinical staff within 48 hours of sputum receipt in the laboratory.				
6. Routine maintenance of the GeneXpert instrument. *If referenced in a separate SOP, indicate "Y" and report in the "Comments" section*.				
7. Mean Ct values of the Specimen Processing Control and an internal control in the assay are monitored.				
8. New lots of cartridges are tested with INTROL™ External Run Controls.				

C. Do your laboratory SOPs include instructions for the following? *Indicate "Yes (Y)," "No (N)," or "Not Applicable (NA)" for each response below. Justify or explain all "N" or "NA" responses in the "Comments" column.*				
9. Instrument and assay are calibrated annually with INTROL™ External Run Controls.				

D. Additional Notes/Comments

18 Appendices

18.1 Study Protocol Review Form

Refer to section 3 for more details. The objective of this form is to help TB laboratory specialists who may be called upon to serve on a study protocol team or create TB laboratory-associated study documents.

Section I: Study Information
Network/Study Number/Version:
Title:
Principal Investigator(PI) or Chair:
Participating Sites:
Population:
Intervention (drug regimen):

Section II: Study Review Questions	
Instructions: Review the study protocol and answer the following questions as they relate to **TB microbiology ONLY** (i.e., TB mycobacteriology as well as TB biomarkers). Please refer to the section and page in the study protocol. Pages may have changed in the study protocol by the time feedback is given to the study team. Use box to respond to the questions and indicate comments or questions for the study team.	
1. Is drug efficacy an objective of the study? If yes, identify the objective(s) that relates to drug efficacy.	☐ YES ☐ NO
Comments/Questions:	
2. What are the study outcomes? Identify the microbiology endpoints according to their ranking (primary, secondary, exploratory and tertiary).	
Comments/Questions:	
3. Are the inclusion criteria related to TB microbiology? If yes, identify these.	☐ YES ☐ NO
Comments/Questions:	
4. Are there conditions for premature discontinuation or late exclusion after enrollment related to TB microbiology? If yes, identify these.	☐ YES ☐ NO
Comments/Questions:	
5. Is the participant allowed to be on anti-TB drugs at the time of enrollment or randomization? If yes, identify these. If not, what length of time is required for the participant to be off anti-TB drugs?	☐ YES ☐ NO
Comments/Questions:	

6. Identify the specimens collected and TB tests done at screening (e.g., for mycobacteriology testing, biomarkers, or transcriptional analysis)	
Comments/Questions:	
7. Identify how far in advance of enrollment can the screening tests be performed	
Comments/Questions:	
8. Determine if a laboratory outside of the study network can perform TB screening tests	☐ YES ☐ NO
Comments/Questions:	
9. Identify the specimens collected and TB tests done at enrollment/randomization	
Comments/Questions:	
10. Identify the specimens collected and TB tests done after enrollment	
Comments/Questions:	
11. Are specific TB test methods/assays specified? If yes, identify these	☐ YES ☐ NO
Comments/Questions:	
12. Are any TB drugs to be tested by a phenotypic or molecular method? If yes, identify the drugs and method, if specified.	☐ YES ☐ NO
Comments/Questions:	
13. Determine if some TB tests are to be performed in a central reference laboratory and not at the local/site laboratory	☐ YES ☐ NO
Comments/Questions:	
14. Identify any sub-studies related to TB microbiology	☐ YES ☐ NO
Comments/Questions:	

Section III: Feedback to Study Protocol Team
Instructions: Indicate other aspects that need to be clarified, modified, or added. Please refer to the section and page in the study protocol. Pages may have changed in the study protocol by the time feedback is given to the study team.

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20 References

1. Bark CM, Furin J, Johnson J. Approaches to clinical trials of new anti-TB drugs. Clin Invest 2012;2(4):359–70

2. Global Laboratory Initiative Stop TB Partnership. Laboratory Diagnosis of Tuberculosis by Sputum Microscopy – The Handbook 2013 [Available from: http://www.stoptb.org/wg/gli/assets/documents/TBLabDiagnosisSputum%20Microscopy_Handbook.pdf.

3. Global Laboratory Initiative Stop TB Partnership. Mycobacteriology Laboratory Manual. First Edition. 2014 [Available from: http://www.stoptb.org/wg/gli/assets/documents/gli_mycobacteriology_lab_manual_web.pdf.

4. American Society for Microbiology. Clinical Microbiology Procedures Handbook. 3rd Edition ed. Washington, D.C: American Society of Microbiology; 2010.

5. Kent PT, Kubica GP. Public Health Mycobacteriology: a Guide for Level III Laboratory. Atlanta: US Department of Health and Human Services, Centers for Disease Control; 1985.

6. World Health Organization. Laboratory services in tuberculosis control. Part II: Microscopy. WHO/TB/98.258 Geneva: WHO; 1998 [Available from: http://whqlibdoc.who.int/hq/1998/WHO_TB_98.258_(part2).pdf.

7. Clinical and Laboratory Standards Institute. Laboratory Detection and Identification of Mycobacteria; Approved Guideline, M48-A 2008 [

8. Global Laboratory Initiative Stop TB Partnership. GLI Training Package on Xpert MTB/RIF 2016 [Available from: http://www.stoptb.org/wg/gli/trainingpackages.asp.

9. Global Laboratory Initiative Stop TB Partnership. GLI Training Package on Culture in Solid and Liquid Media 2016 [Available from: http://www.stoptb.org/wg/gli/trainingpackages.asp.

10. Global Laboratory Initiative Stop TB Partnership. GLI Training Package on DST by Phenotypic and Molecular Methods 2016 [Available from: http://www.stoptb.org/wg/gli/trainingpackages.asp.

11. Global Laboratory Initiative Stop TB Partnership. GLI Training Package on LPA 2016 [Available from: http://www.stoptb.org/wg/gli/trainingpackages.asp.

12. DAIDS. Guidelines for Good Clinical Laboratory Practice Standards. Final Version 3.0 2013 [Available from: http://www.niaid.nih.gov/LabsAndResources/resources/DAIDSClinRsrch/Documents/gclp.pdf.

13. ACTG-IMPAACT. Key Elements of Mycobacteriology Laboratory Procedures. Version: 2 July 2015. 2015 [Available from: https://www.hanc.info/labs/labresources/procedures/ACTGIMPAACT%20Lab%20Manual/Harmonized%20Key%20Elements%20v1.9_070215.pdf.

14. Tuberculosis Trials Consortium (TBTC). TBTC Study 31/ACTG 5349 Mycobacteriology Laboratory Reference Manual 2016 [Available from: https://member.actgnetwork.org/cms/dl/27739.

15. TB Alliance. A Phase 2 Open Label Partially Randomized Trial to Evaluate the Efficacy, Safety and Tolerability of Combinations of Bedaquiline, Moxifloxacin, PA-824 and Pyrazinamide in Adult Subjects With Drug-Sensitive or Multi Drug-Resistant Pulmonary Tuberculosis. (NC-005) 2014 [Available from: https://clinicaltrials.gov/ct2/show/NCT02193776.

16. TB Alliance. Protocol NC-005-(J-M-Pa-Z) Microbiology Manual 2014 [Available from: https://www.tballiance.org/downloads/publications/NC005_Myco%20Lab%20Manual_19Dec14_Final.pdf.

17. TB Alliance. Shortening Treatment by Advancing Novel Drugs (STAND)(NC-006) 2014 [Available from: https://clinicaltrials.gov/ct2/show/study/NCT02342886.

18. Jensen PA, Lambert LA, Iademarco MF, Ridzon R. Guidelines for preventing the transmission of Mycobacterium tuberculosis in health-care settings: MMWR Recomm Rep. Vol. 54 RR-17; pages 1-141; 2005 [Available from: http://www.cdc.gov/tb/publications/guidelines/infectioncontrol.htm.

19. World Health Organization. Tuberculosis laboratory biosafety manual, WHO/HTM/TB/2012.11 2012 [Available from: http://www.who.int/tb/publications/2012/tb_biosafety/en/.

20. World Health Organization. WHO policy on TB infection control in health-care facilities, congregate settings and households, WHO/HTM/TB/2009.419: World Health Organization; 2009 [Available from: http://whqlibdoc.who.int/publications/2009/9789241598323_eng.pdf.

21. pSMILE. Laboratory Biosafety Inspection Checklist [Available from: http://resources.psmile.org/resources/process-control/section-specific-information/microbiology/mycobacteriology/Pro6.7-C-19%20TB%20Lab%20Self%20Inspection%20Checklist.xls/view.

22. Association of Public Health Laboratories. Mycobacteriology tuberculosis: Assessing Your Laboratory 2013 [Available from:

https://www.aphl.org/AboutAPHL/publications/Documents/ID_2013Aug_Mycobacterium-Tuberculosis-Assessing-Your-Laboratory.pdf.

23. Pfyffer GE, Palicova F. Mycobacterium: General Characteristics, Laboratory Detection, and Staining Procedures. In: Murray PR, Baron EJ, American Society for M, editors. Manual of Clinical Microbiology. 10th. Washington, DC.: ASM Press; 2011. p. 472-502.

24. Siddiqi S, Rusch-Gerdes S. MGIT Procedure Manual For BACTEC™ MGIT 960™ TB System (Also applicable for Manual MGIT) Mycobacteria Growth Indicator Tube (MGIT) Culture and Drug Susceptibility Demonstration Projects 2006 [Available from: http://www.finddiagnostics.org/export/sites/default/resource-centre/find_documentation/pdfs/mgit_manual_nov_2007.pdf.

25. Parsons LM, Somoskovi A, Gutierrez C, Lee E, Paramasivan CN, Abimiku Al, et al. Laboratory Diagnosis of Tuberculosis in Resource-Poor Countries: Challenges and Opportunities. Clinical microbiology reviews. 2011;24(2):314-50.

26. European Centre for Disease Prevention and Control. Handbook on TB laboratory diagnostic methods for the European Union 2016 [Available from: http://ecdc.europa.eu/en/publications/_layouts/forms/Publication_DispForm.aspx?List=4f55ad51-4aed-4d32-b960-af70113dbb90&ID=1449.

27. The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). Guideline for Good Clinical Practice. ICH Harmonised Tripartite Guideline. E6(R1). Current Step 4 version, dated 10 June 1996. (including the Post Step 4 corrections). 1996 [Available from: https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Efficacy/E6/E6_R1_Guideline.pdf.

28. McCarthy KD, Metchock B, Kanphukiew A, Monkongdee P, Sinthuwattanawibool C, Tasaneeyapan T, et al. Monitoring the performance of mycobacteriology laboratories: a proposal for standardized indicators. The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease. 2008;12(9):1015-20.

29. Global Laboratory Initiative Stop TB Partnership. GLI Practical Guide to TB Laboratory Strengthening 2017 [Available from: http://www.stoptb.org/wg/gli/assets/documents/GLI_practical_guide.pdf.

30. pSMILE. SMILE Validation Guidelines and Templates / Mycobacteriology [Available from: http://resources.psmile.org/resources/equipment/smile-validation-guidelines/mycobacteriology.

31. ACTG-IMPAACT. Site Tuberculosis Infection Control Guidelines 2017 [Available from: https://www.hanc.info/labs/labresources/procedures/Pages/actgImpaactLabManual.aspx.

32. ACTG-IMPAACT. Collection Storage Transport Expectorated Sputum SOP, LTC SOP 070 v1.0. ACTG/IMPAACT Laboratory Manual. 2014 [Available from: https://www.hanc.info/labs/labresources/procedures/ACTGIMPAACT%20Lab%20Manual/70-Collection%20Storage%20Transport%20Expectorated%20Sputum-LTC-SOP-70v1.0-2014-07-01.pdf.

33. World Health Organization. Guidance for national tuberculosis programmes on the management of tuberculosis in children, Second Edition, WHO/HTM/TB/2014.03 2014 [Available from: http://www.who.int/tb/publications/childtb_guidelines/en/.

34. Curry International Tuberculosis Center. Tuberculosis Infection Control: A Practical Manual for Preventing TB 2011 [Available from: http://www.currytbcenter.ucsf.edu/products/view/tuberculosis-infection-control-practical-manual-preventing-tb.

35. Curry International Tuberculosis Center. Pediatric Tuberculosis: A Guide to the Gastric Aspirate (GA) Procedure 2014 [Available from: http://www.currytbcenter.ucsf.edu/products/view/pediatric-tuberculosis-guide-gastric-aspirate-ga-procedure.

36. Global Laboratory Initiative Stop TB Partnership. GLI Guide to TB Specimen Referral Systems and Integrated Networks 2017 [Available from: http://stoptb.org/wg/gli/assets/documents/GLI_Guide_specimens_web_ready.pdf.

37. Diagnostic Standards and Classification of Tuberculosis in Adults and Children. AmJRespirCrit Care Med. 2000;161(4 Pt 1):1376-95.

38. Parashar D, Kabra SK, Lodha R, Singh V, Mukherjee A, Arya T, et al. Does neutralization of gastric aspirates from children with suspected intrathoracic tuberculosis affect mycobacterial yields on MGIT culture? Journal of clinical microbiology. 2013;51(6):1753-6.

39. Strong BE, Kubica GP. Isolation and Identification of Mycobacterium tuberculosis. A Guide for the Level II Laboratory. Atlanta: US Department of Health,Education,and Welfare; 1981. 143 pp p.

40. ACTG-IMPAACT. Sputum processing for storage and tuberculosis biomarker research LTC SOP 074 v1.0. ACTG/IMPAACT Laboratory Manual. 2015 [Available from: https://www.hanc.info/labs/labresources/procedures/ACTGIMPAACT%20Lab%20Manual/74-Sputum%20Processing%20for%20Storage-LTC-SOP-74v1-2015-10-16.pdf.

41. Demers AM, Boulle A, Warren R, Verver S, van Helden P, Behr MA, et al. Use of simulated sputum specimens to estimate the specificity of laboratory-diagnosed tuberculosis. The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease. 2010;14(8):1016-23.

42. Somoskovi A, Hotaling JE, Fitzgerald M, O'Donnell D, Parsons LM, Salfinger M. Lessons from a proficiency testing event for acid-fast microscopy. Chest. 2001;120(1):250-7.

43. Gebre N, Karlsson U, Jonsson G, Macaden R, Wolde A, Assefa A, et al. Improved microscopical diagnosis of pulmonary tuberculosis in developing countries. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1995;89(2):191-3.

44. den Hertog AL, Daher S, Straetemans M, Scholing M, Anthony RM. No added value of performing Ziehl-Neelsen on auramine-positive samples for tuberculosis diagnostics. The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease. 2013;17(8):1094-9.

45. Chedore P, Th'ng C, Nolan DH, Churchwell GM, Sieffert DE, Hale YM, et al. Method for inactivating and fixing unstained smear preparations of mycobacterium tuberculosis for improved laboratory safety. Journal of clinical microbiology. 2002;40(11):4077-80.

46. World Health Organization. Fluorescent light-emitting diode (LED) microscopy for diagnosis of tuberculosis: policy statement. WHO/HTM/TB/2011.8 2011 [Available from: http://apps.who.int/iris/bitstream/10665/44602/1/9789241501613_eng.pdf?ua=1&ua=1.

47. Minion J, Pai M, Ramsay A, Menzies D, Greenaway C. Comparison of LED and conventional fluorescence microscopy for detection of acid fast bacilli in a low-incidence setting. PloS one. 2011;6(7):e22495.

48. Reisner BS, Gatson AM, Woods GL. Use of Gen-Probe AccuProbes to identify Mycobacterium avium complex, Mycobacterium tuberculosis complex, Mycobacterium kansasii, and Mycobacterium gordonae directly from BACTEC TB broth cultures. J Clin Microbiol. 1994;32(12):2995-8.

49. Gomathi NS, Devi SM, Lakshmi R, Ramachandran R, Wares DF, Kumar V, et al. Capilia test for identification of Mycobacterium tuberculosis in MGIT-positive cultures. The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease. 2012;16(6):788-92.

50. Vadwai V, Sadani M, Sable R, Chavan A, Balan K, Naik A, et al. Immunochromatographic assays for detection of Mycobacterium tuberculosis: what is the perfect time to test? Diagn Microbiol Infect Dis. 2012;74(3):282-7.

51. TBCARE I U, Global Laboratory Initiative. TB CARE I Laboratory Tools [Available from: http://www.tbcare1.org/publications/toolbox/lab/.

52. Hanna BA, Ebrahimzadeh A, Elliott LB, Morgan MA, Novak SM, Rusch-Gerdes S, et al. Multicenter evaluation of the BACTEC MGIT 960 system for recovery of mycobacteria. Journal of clinical microbiology. 1999;37(3):748-52.

53. Badak FZ, Kiska DL, Setterquist S, Hartley C, O'Connell MA, Hopfer RL. Comparison of mycobacteria growth indicator tube with BACTEC 460 for detection and recovery of mycobacteria from clinical specimens. Journal of clinical microbiology. 1996;34(9):2236-9.

54. World Health Organization. Laboratory services in tuberculosis control. Part III: Culture. WHO/TB/98.258 Geneva: WHO; 1998 [Available from: http://whqlibdoc.who.int/hq/1998/WHO_TB_98.258_%28part3%29.pdf.

55. Rieder H, Deun AV, Kam KM, Kim SJ, Chonde TM, Trebucq A, et al. Priorities for Tuberculosis Bacteriology Services in Low-Income Countries Paris, France.2007 [Available from: http://www.theunion.org/what-we-do/publications/technical/priorities-for-tuberculosis-bacteriology-services-in-low-income-countries.

56. Joloba ML, Johnson JL, Feng PJ, Bozeman L, Goldberg SV, Morgan K, et al. What is the most reliable solid culture medium for tuberculosis treatment trials? Tuberculosis. 2014;94(3):311-6.

57. Mitchison DA, Allen BW, Carrol L, Dickinson JM, Aber VR. A selective oleic acid albumin agar medium for tubercle bacilli. J Med Microbiol. 1972;5(2):165-75.

58. OPTIQ. Efficacy and Safety of Levofloxacin for the Treatment of MDR-TB (Opti-Q) 2013 [Available from: https://clinicaltrials.gov/ct2/show/NCT01918397.

59. Brent AJ, Mugo D, Musyimi R, Mutiso A, Morpeth S, Levin M, et al. Performance of the MGIT TBc identification test and meta-analysis of MPT64 assays for identification of the Mycobacterium tuberculosis complex in liquid culture. Journal of clinical microbiology. 2011;49(12):4343-6.

60. Basu I, Bower JE, Henderson GK, Lowe O, Newton S, Vaughan R, et al. False-negative BD MGIT TBc Identification Test results in routine tuberculosis diagnosis: a New Zealand perspective. The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease. 2015;19(9):1073-5.

61. Dominguez J, Boettger EC, Cirillo D, Cobelens F, Eisenach KD, Gagneux S, et al. Clinical implications of molecular drug resistance testing for Mycobacterium tuberculosis: a TBNET/RESIST-TB consensus statement. The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease. 2016;20(1):24-42.

62. World Health Organization. Updated critical concentrations for first-line and second-line DST. May 2012 2012 [Available from: http://www.stoptb.org/wg/gli/assets/documents/Updated%20critical%20concentration%20table_1st%20and%202nd%20line%20drugs.pdf.

63. Torres JN, Paul LV, Rodwell TC, Victor TC, Amallraja AM, Elghraoui A, et al. Novel katG mutations causing isoniazid resistance in clinical M. tuberculosis isolates. Emerg Microbes Infect. 2015;4(7):e42.

64. Tomasicchio M, Theron G, Pietersen E, Streicher E, Stanley-Josephs D, van Helden P, et al. The diagnostic accuracy of the MTBDRplus and MTBDRsl assays for drug-resistant TB detection when performed on sputum and culture isolates. Scientific reports. 2016;6:17850.

65. Mathys V, van de Vyvere M, de Droogh E, Soetaert K, Groenen G. False-positive rifampicin resistance on Xpert(R) MTB/RIF caused by a silent mutation in the rpoB gene. The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease. 2014;18(10):1255-7.

66. Ocheretina O, Escuyer VE, Mabou MM, Royal-Mardi G, Collins S, Vilbrun SC, et al. Correlation between genotypic and phenotypic testing for resistance to rifampin in Mycobacterium tuberculosis clinical isolates in Haiti: investigation of cases with discrepant susceptibility results. PloS one. 2014;9(3):e90569.

67. Van Deun A, Aung KJ, Bola V, Lebeke R, Hossain MA, de Rijk WB, et al. Rifampin drug resistance tests for tuberculosis: challenging the gold standard. Journal of clinical microbiology. 2013;51(8):2633-40.

68. Piersimoni C, Mustazzolu A, Giannoni F, Bornigia S, Gherardi G, Fattorini L. Prevention of false resistance results obtained in testing the susceptibility of Mycobacterium tuberculosis to pyrazinamide

with the Bactec MGIT 960 system using a reduced inoculum. Journal of clinical microbiology. 2013;51(1):291-4.

69. Heifets LB. The mycobacteriology laboratory. Past, present, and future. Clin Lab Med. 1996;16(3):513-25.

70. Brossier F, Guindo D, Pham A, Reibel F, Sougakoff W, Veziris N, et al. Performance of the New Version (v2.0) of the GenoType MTBDRsl Test for Detection of Resistance to Second-Line Drugs in Multidrug-Resistant Mycobacterium tuberculosis Complex Strains. Journal of clinical microbiology. 2016;54(6):1573-80.

71. Rachow A, Zumla A, Heinrich N, Rojas-Ponce G, Mtafya B, Reither K, et al. Rapid and accurate detection of Mycobacterium tuberculosis in sputum samples by Cepheid Xpert MTB/RIF assay--a clinical validation study. PloS one. 2011;6(6):e20458.

72. Banada PP, Sivasubramani SK, Blakemore R, Boehme C, Perkins MD, Fennelly K, et al. Containment of bioaerosol infection risk by the Xpert MTB/RIF assay and its applicability to point-of-care settings. Journal of clinical microbiology. 2010;48(10):3551-7.

73. Samuel G. Schumacher PN, Catharina C. Boehme, Jerrold Ellner, David Alland, Susan E. Dorman, Claudia M. Denkinger. A Multicenter Diagnostic Accuracy Study Of The Xpert Ultra For Tuberculosis Diagnosis. Abstract 76LB. CROI February 13–16, 2017 2017 [Available from: http://www.croiconference.org/sessions/multicenter-diagnostic-accuracy-study-xpert-ultra-tuberculosis-diagnosis.

74. World Health Organization. WHO Meeting Report of a Technical Expert Consultation: Non-inferiority analysis of Xpert MTB/RIF Ultra compared to Xpert MTB/RIF. WHO/HTM/TB/2017.04 2017 [Available from: http://apps.who.int/iris/bitstream/10665/254792/1/WHO-HTM-TB-2017.04-eng.pdf.

75. FIND. Report for WHO: A multicentre non-inferiority diagnostic accuracy study of the Ultra assay compared to the Xpert MTB/RIF assay 2017 [Available from: https://www.finddx.org/wp-content/uploads/2017/03/Ultra-WHO-report_24MAR2017_FINAL.pdf.

76. Global Laboratory Initiative Stop TB Partnership. Planning for country transition to Xpert® MTB/RIF Ultra cartridges 2017 [Available from: http://www.stoptb.org/wg/gli/assets/documents/GLI_ultra.pdf.

77. Chakravorty S, Simmons AM, Rowneki M, Parmar H, Cao Y, Ryan J, et al. The New Xpert MTB/RIF Ultra: Improving Detection of Mycobacterium tuberculosis and Resistance to Rifampin in an Assay Suitable for Point-of-Care Testing. mBio. 2017;8(4).

78. Erica Lessem. 2016 PIPELINE REPORT. The Tuberculosis Diagnostics Pipeline 2016 [Available from: http://www.pipelinereport.org/2016/tb-diagnostics.

79. Huang TS, Chen YS, Lee SS, Tu HZ, Liu YC. Preservation of clinical isolates of Mycobacterium tuberculosis complex directly from MGIT culture tubes. Ann Clin Lab Sci. 2005;35(4):455-8.