REPUBLIC OF UGANDA

Tuberculosis Laboratory Diagnostic Network Manual

2nd Edition

October 2023

Foreword

Uganda is one of the 30 high TB & HIV burden countries and one of the 20 countries that contribute to 83% of missing people with TB in the globe. For all the years up to 2010, diagnosis of TB relied on microscopy whose sensitivity is about 50% and can be as low as 25 % among People living with HIV (PLHIV) and diagnosis of drug resistant TB (DR-TB) relied on culture whose result turnaround time (TAT) was on average 3 months. In the last ten years there has been rapid development in the TB diagnostic land scape with WHO recommending a number of diagnostic tools to support early, accurate and rapid diagnosis of TB. In the quest to end TB by 2030 Uganda like other TB & HIV burden countries has adopted a number of TB diagnostic tools and strategies and hence the urgent need for a revised TB Laboratory Network manual (Current version was written in 2014), for the over 1600 laboratories at all levels of TB diagnostic services in the country.

Although the manual is written to meet local needs, it is written in line with international guidelines, specifically those of the World Health Organization (WHO). This 2nd edition of the manual has been improved with more elaborate descriptions and pictures of the different TB test methods and processes from the Peripheral Laboratory level to the National Reference level stipulating the minimum diagnostic expectations and facilitating easy grasp of the key principles and practices in TB diagnosis.

The Ministry of Health is strongly committed to accurate, reliable Quality assured TB diagnostics in Uganda. I congratulate the NHLDS, NTRL and all stakeholders on this achievement and strongly recommend this manual to all involved in TB diagnosis and control services in Uganda and beyond.

Dr. Henry G. Mwebesa

Director General of Health Services, Ministry of health

Acknowledgements

The review and update of the first edition of the National Tuberculosis Laboratory Network manual has only been possible because of the kind and generous support of the UKAID/GIZ backup Project in Uganda

The following individuals were involved directly in the revision of the different sections of the updated manual: Abdunoor Nyombi, Anitah Katuramu, Charles Manyonge, Didas Tugumisirize, Faith Alikoba, Hasfah Ssentamu, Jane Babirye, Kangave Fredrick, Mary Akumu, Mary Akumu, Nakiwala Dorothy, Nyombi Abdunoor and Oola Denis of the NTRL; Christopher Okiira, Ogwok Patrick and Patricia Akello of NHLDS and Didas Atwembere of Mbale Regional Referral Hospital and Robert Wagubi of Mbarara Regional Referral Hospital.

The following individuals are also acknowledged for providing expert review and editing of the final copy of the National Tuberculosis Laboratory Network manual, Patricia Akello (NHLDS), Denis Oola, Charles Manyonge, Abdnoor Nyombi, Beatrice Orena,Okeba Newton, Jane Babirye, Anita Katuramu, Kangave Fredrick, Patrick Ademun all of NTRL and Dr. Arinaitwe Moses-NTLP-MOH.

Special gratitude is extended to Professor Moses Joloba, Head of the NTRL, and to Dr. Susan Nabada, Commissioner of Laboratory Services. Their invaluable wisdom, leadership, and guidance were instrumental throughout the manual's revision process.

We express our heartfelt gratitude to the UKAID/GIZ Backup Project for their unwavering support. Their generous provision of resources has not only facilitated the review process but also enabled the printing and distribution of the revised Network manual to all testing facilities across the country. Your contributions have been instrumental in enhancing our capabilities and ensuring the widespread availability of crucial information. Thank you once again for your invaluable assistance.

List of abbreviations

AFB Acid fast bacilli

AHPC Allied Health Professionals council

BSC Biosafety Cabinet

CPHL Central Public Health Laboratories

CSF Cerebral Spinal Fluid

Cts Cycle threshold

CXR Chest X-ray

DHO District Health Officer

DLFP District Laboratory Focal person

DNA Deoxyribonucleic acid

DOT Directly Observed Therapy

DR TB Drug Resistance TB

DST Drug Susceptivity Testing

DTLS District TB/Leprosy Supervisor

e-CBSS Electronic Case Based Surveillance System

ELISA Enzyme Linked Immunosorbent Assay

EPTB Extra Pulmonary TB

EQA External Quality assessment

FM Fluorescent Microscopy

HC Health Center

HMIS Health Management Information System

IGRA Interferon-gamma release assay

IPC Infection Prevention and Control

LF LAM Lateral flow urine lipoarabinomannan assay

LJ Lowenstein Jensen

LPA Line Probe Assay

LQMS Laboratory Quality Management System

MDR Multi Drug Resistance

MGIT Mycobacterium Growth Indicator Tubes

MKIT- Mycobacterium KIT

MOH Ministry of Health

MSDS Material Safety Data sheet

MTB Mycobacterium Tuberculosis

NHLDS National Health Laboratory and Diagnostic services

NTLP National TB and Leprosy program

NTRL National TB Reference Laboratory

PCR Polymerase Chain Reaction

pDST Phenotypic Drug Susceptibity Testing

PLHIV People Living with HIV

RRH Regional Referral Hospital

RR Rifampicin Resistant

SOP Standard Operating Procedure

TAT Turnaround Time

TB LAMP- Loop-mediated isothermal amplification

VHTs Village health Teams

WHO World Health Organization

XDR-TB Extensively drug-resistant TB

ZN Ziel Nielsen

Contents

Definition

Accreditation

A procedure by which an authoritative body gives formal recognition that an organization is competent to carry out specific tasks.

Quality Assurance (QA)

Is the total process that guarantees that the final results reported by the laboratory are as accurate as possible. QA involves inspecting samples, reviewing transcriptional measures, using the most reliable assays, and verifying reports.

Quality Control (QC) - Also called Internal Quality Control.

Includes all means by which the laboratory personnel performing testing control the process, including checking of the instruments, new lots of reagents, samples preparation, results reporting, etc. It is a systematic internal monitoring of working practices, technical procedures, equipment, and materials.

CHAPTER 1

1.0 Introduction

Uganda is one of the 30 high TB & HIV burden countries and one of the 20 countries that contribute to 83% of missing people with TB. The epidemiology of TB in Uganda is characterized by gender (male: female prevalence ratio of 4:1), residence (urban: rural prevalence 2:1), geographical disparities and existence of hot spots, as well as similar socio-economic-cultural drivers of the epidemic in all districts (TB prevalence survey, 2014/2015). According to the Global TB Report 2022, Uganda is one of the 5 Countries where the number of newly diagnosed with TB in 2021 recovered to or beyond the 2019 levels. Of the 30 high-TB-burden countries, Uganda was among those with the highest levels of treatment coverage in 2021. In the same year, Uganda was also among the six high-TB-burden Countries that reached or passed the first milestone of a 35% reduction in TB deaths compared with 2015. According to the WHO 2022 Report, the incidence of TB stood at 199/100,000, the treatment coverage was 82% and the TB treatment Success Rate for Drug-sensitive TB stood at 85%. For all the years up to 2010, diagnosis of TB relied on microscopy, whose sensitivity is about 50% and can be as low as 25% among People living with HIV (PLHIV), and diagnosis of drug-resistant TB (DR-TB) relied on culture, whose result turnaround time (TAT) was on average 3 months. In the last ten years, there has been rapid development in the TB diagnostic landscape, with WHO recommending several diagnostic tools to support early, accurate and rapid diagnosis of TB. In line with the WHO END TB Strategy, Uganda has adopted numerous TB diagnostic tools and approaches to strengthen the capacity of the diagnostic network to respond to the TB Epidemic in the country.

1.2 Background

The National TB Reference Laboratory is the diagnostic arm of the National TB and Leprosy control Programme (NTLP) and one of the reference Laboratories under the department of National Health Laboratory and Diagnostic Services (NHLDS) of the Ministry of Health

The NTLP administratively falls under the Department of National Disease Control (NDC) within the Ministry of Health.

The primary role of the National TB Reference Laboratory (NTRL) is to provide technical services for the detection, identification, drug sensitivity testing and surveillance of TB disease. NTRL is also mandated to lead and oversee the National TB laboratory network and ensure that TB diagnosis and monitoring is performed timely, accurately and reliably.

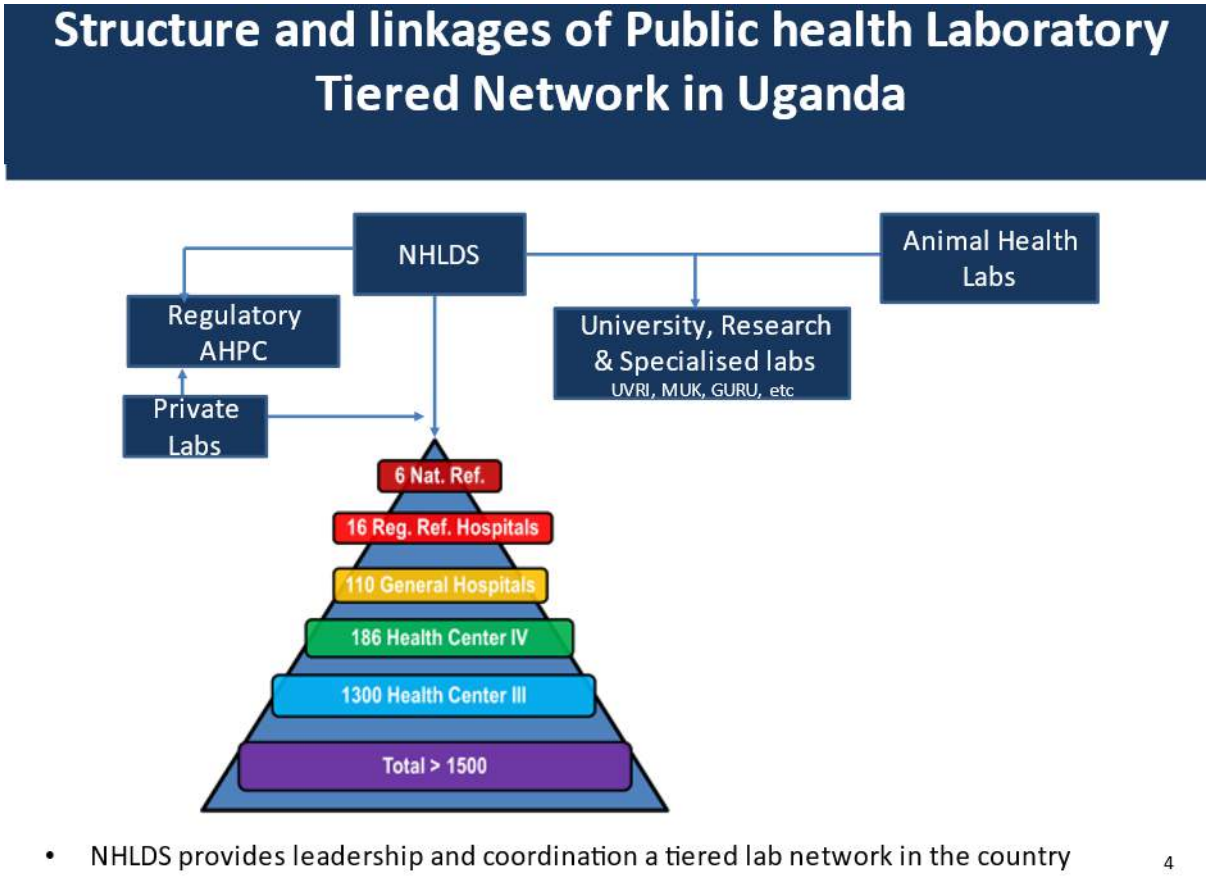
The National Tuberculosis Reference Laboratory (NTRL) is one of the specialized laboratories serving as a national reference centre providing specialized Tuberculosis (TB) testing services. The other reference laboratories include; the National Microbiology Reference Laboratory, (NMRL), and Central Public Health Laboratories (CPHL).

The Private Not for Profit (PNFP) and Private for Profit (PFP) Health Facilities are part of the diagnostic network providing TB services either by referral or onsite testing. In addition, University research laboratories and other training institutions complement the national TB diagnostic network.

A comprehensive, high-quality TB diagnostic network is essential to diagnose TB accurately and rapidly, link diagnosed TB patients to appropriate and timely treatment, and monitor patients on therapy.

TB Laboratory services exist in all public health facilities starting from HC III. The range and complexity of testing services increase with the level of health facility as defined in the national Test menu with each level serving as a reference laboratory for the levels lower than it, except for laboratory hubs which serve as referrals for the same level and below due to enhanced facilities at hubs. TB Diagnostic services are also provided at the community level, using mobile laboratories and community actors.

Figure 1: Structure of National Diagnostic Network



1.3 Context and Rational

This TB Diagnostic manual is intended to elaborate structure and functions that will contribute to the achievement of the strategic objectives of the NTLP and NHLDS. This manual describes the roles and responsibilities of all key players/stake holders involved in the TB diagnostic activities laid out to improve coordination. Efficient coordination will facilitate the uptake of TB prevention services, and improve case finding, treatment outcomes, and multisectoral collaboration.

1.3 Scope of the TB Lab Network Manual

The TB Lab Network Manual provides guidelines for TB testing including, the available platform, Quality control measures and the layout of the diagnostic network. It targets both the public and private sectors within the laboratory diagnostic arena.

This manual specifies the minimum acceptable requirements for laboratories performing TB testing at all levels and defines the roles and responsibilities of various stakeholders in the TB network.

CHAPTER 2

2.0 The National Tuberculosis Laboratory Network

The Uganda TB Laboratory Diagnostic network consists of over 1600 TB microscopy sites; over 290 GeneXpert sites, 40 Truenat sites and 16 TB LAMP sites; TB LF-Urine LAM is performed in over 800 facilities and the IGRA blood test for latent tuberculosis infection (LTBI) in 6 Sites. Culture and Drug susceptibility testing is currently performed in two public laboratories and three research laboratories.

The Tuberculosis Laboratory network consists of closely linked laboratory facilities organized at three tier levels as demonstrated in the structure below:

a) National/central Level National Tuberculosis Reference Laboratory (NTRL)

b) Intermediate level – The Regional Referral Hospital Laboratories (RRHL)

c) Peripheral level – This includes laboratories at district general hospitals, Health Centre IVs, Health Centre IIIs and Private Not for profit and Private For-Profit facilities.

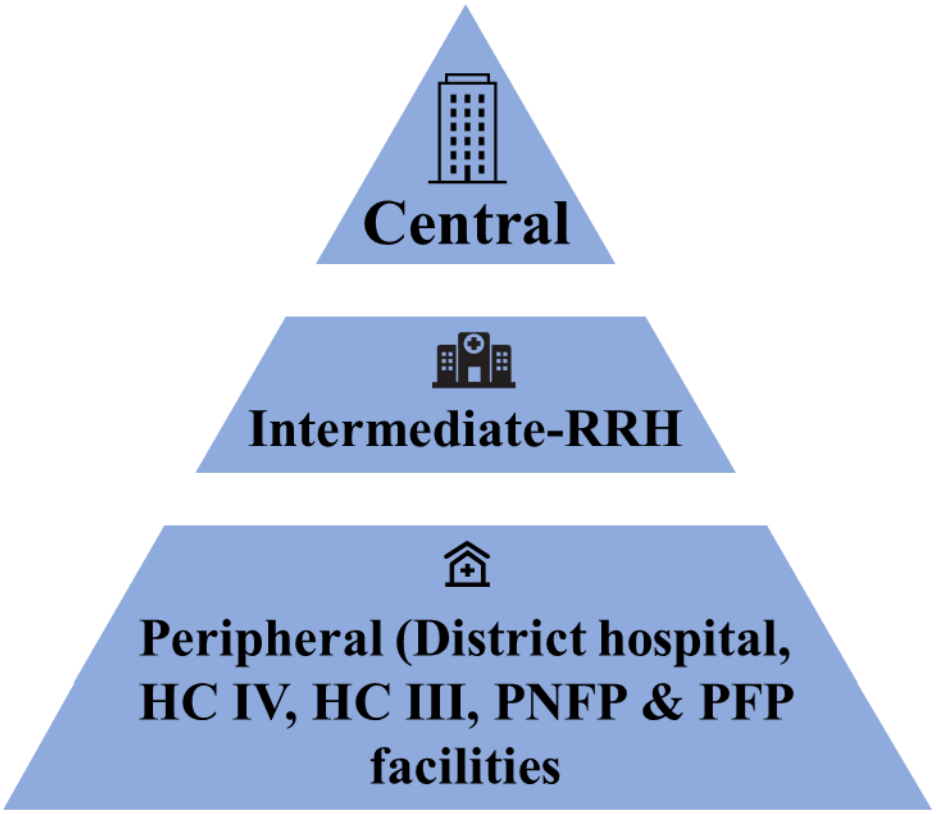


Figure 2: Hierarchy and key actors in the TB Laboratory Network in Uganda.

2.1 Functions at each level

2.1.1 National Level; National TB Reference Laboratory (NTRL)

Core functions of NTRL include the following:

a) Support the TB program at the Ministry of Health

- Policy formulation, resource mobilization, strategic planning and setting diagnostic network standards
- Provide technical services for detection, identification, and drug sensitivity testing.
- Conduct routine and periodic TB disease surveillance.
- Oversee the national TB laboratory network ensuring that TB diagnosis and monitoring is performed accurately and reliably.

b) Train medical and technical staff to handle Tuberculosis and Leprosy

- Improve scientific and technical skills of public health laboratory technical staff.
- Provide short-term refresher training for public health and laboratory practice.
- Ensure continued education in management and leadership.

c) Carry out research with the NTLP in control of Tuberculosis and Leprosy

- Conduct TB surveillance to monitor prevalence and drug resistance patterns to inform policy.
- Evaluate and implement new technologies and analytical methodologies to ensure that laboratories provide state-of-the-art, cost-effective and timely analytical and diagnostic services and support to the public health and health care of communities in Uganda with evidence from validation studies.
- Develop, test and put to use tools to facilitate the adoption of new methods and technologies regarding requisitioning, accessioning and reporting.
- Conduct research to improve laboratory testing.

d) Laboratory improvement through support supervision

- Provide guidance and oversight through training of personnel
- Coordinate and promote quality assurance programs for clinical laboratories through training, consultation, certification, and proficiency testing.
- Serve as the standard of excellence for public and private lab performance

e) Integrated Data Management

- Participate as a key link in national database systems to collect, monitor, and analyze TB laboratory data.
- Inform monitoring and analysis of national TB program indicators to inform decision-making and policy.

- Serve the data needs of epidemiologists, other laboratories, and practitioners, to identify trends and 'sentinel events' of TB disease.

f) Policy development

- Provide scientific and managerial leadership in developing health policy, and in developing, promoting, and integrating public health laboratory science into practice.
- Participate in the development of standards for all TB laboratories, including clinical and research standards.

2.1.2 Intermediate-Level Laboratories

Intermediate laboratories are the regional referral hospital laboratories and have the following functions;

a) Support TB diagnosis

b) Training of staff from peripheral laboratories in the region

c) Supervision and monitoring of peripheral laboratories in the region

d) Conduct quality assurance activities in the respective regions through support supervision and mentorship

e) Support Blinded rechecking EQA activities in the respective regions through second control of slides from their designated districts

f) Monitoring proficiency testing performance of the sites in their respective regions

g) Support in the diagnosis and treatment of TB patients and act as public health laboratories for epidemiological surveillance and control of diseases in the community.

2.1.3 Peripheral Level

Peripheral laboratories are district general hospitals, Health Centre IVs, and Health Centre IIIs, including Private Not for profit and Private Profit facilities.

They perform the following functions;

a) Perform TB testing including smear microscopy, GeneXpert, Truenat, TB LAM, and TB LAMP as designated.

b) Participate in EQA; blinded rechecking and Proficiency Testing (PT).

c) Participate in TB sample referral as per the National sample referral guidelines and TB diagnostic algorithms.

Functions of key actors at each level

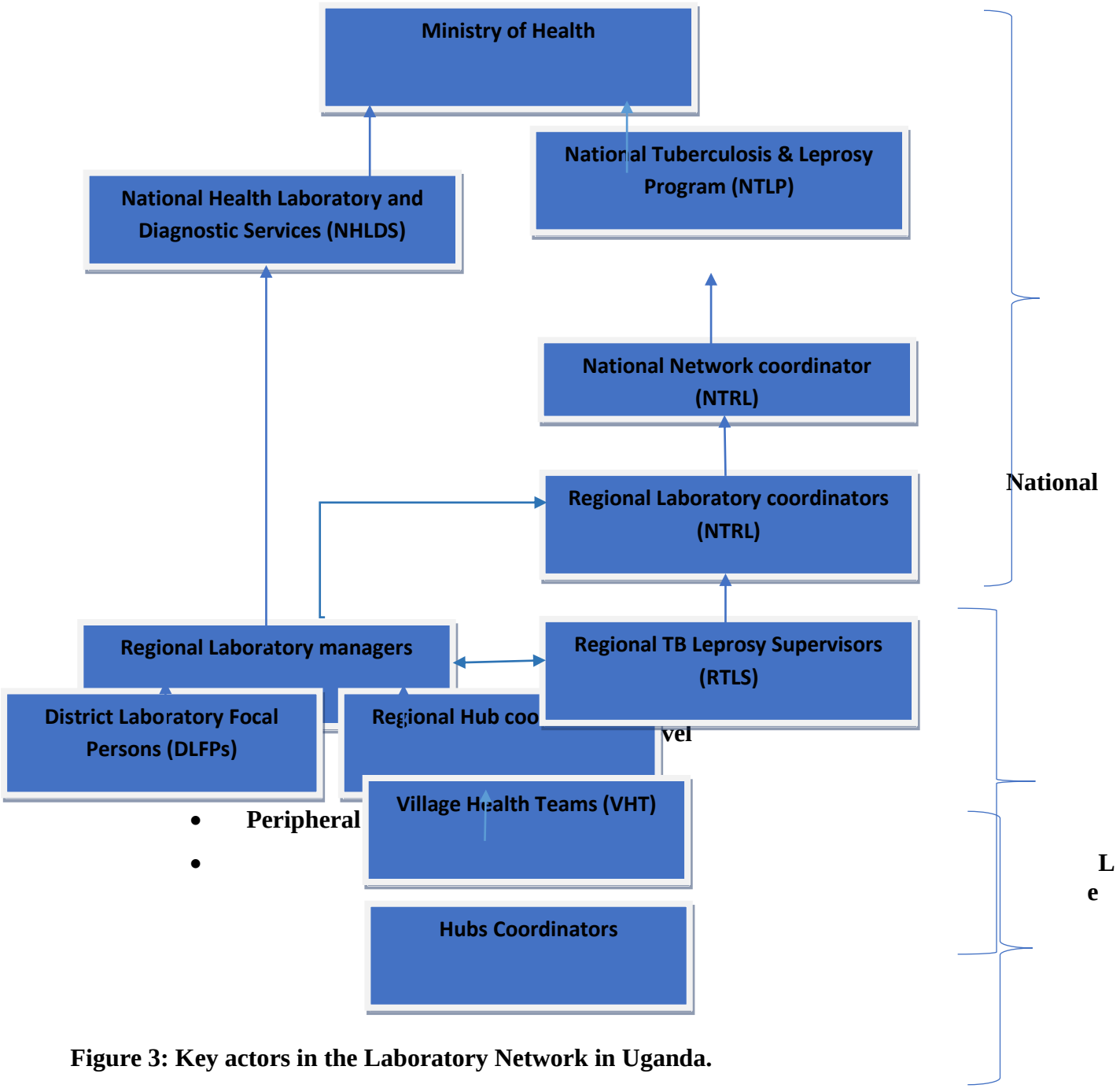


Figure 3: Key actors in the Laboratory Network in Uganda.

1. **Ministry of Health**
2. Ministry of Health through the department of National Health Laboratory and Diagnostic Services (NHLDS)

provides overall policy guidance, coordination, resource mobilization and stewardship.

3. **National Health Laboratory and Diagnostic Services (NHLDS)**

Perform the following roles;

- Provide overall policy guidance to all stakeholders
- Engage with other MOH departments, programs, line Ministries and stakeholders interested in leveraging the operations of the National Sample Referral Transport Network (NSRTN)/Hub system.
- Oversee and supervise activities of the NSRTN.
- Conduct stakeholders' meetings to review of the Network performance
- Ensure availability of commodities necessary for the hub operation activities to Gazette, launch new Hubs, transfer and close others in consultation with other key stakeholders
- Undertake strategic planning for laboratory service
- Mobilize resources for implementation of the laboratory network activities
- Coordinate technical and administrative support supervision to the hubs
- Provide guidance to implementing partners when planning and budgeting for the laboratory Network
- Harmonize operations of Implementing Partners and manage the transition period from one implement partner/project to another

4. **National TB and Leprosy Program (NTLP)**

The overall coordination of TB and Leprosy programming is provided by the National TB and Leprosy Control Program (NTLP), under the department of National Disease Control of the Ministry of Health (MoH).

The NTLP is charged with the following core functions:

(i) Establishment of country-wide facilities for quality diagnosis and treatment of TB and leprosy

(ii) Coordination and supervision of the implementation of TB and leprosy prevention and care

(iii) Prevention and management of leprosy-related disabilities.

5. **Hubs**

Perform the following roles;

- Ensure the testing laboratories within their catchment areas have enough testing materials to execute minimum tests
- Ensure functional diagnostic equipment (CD4, Hematology, Clinical Chemistry, GeneXpert), Stocked with reagents and consumables for all diagnostic equipment and laboratory registers for all laboratory tests at testing facilities

- Order and redistribute all necessary lab supplies
- Ensure the laboratory staff conduct testing of all referred samples and hub facility samples promptly
- Ensure the quality of results produced by testing sites
- Ensure that all the required documentation and record keeping is done in the relevant registers
- Ensure that results of all tests run at the hub are ready for dispatch by the sample transporter less than one week after sample collection
- Ensure proper sample identification, reception, packaging and storage of referral samples for transportation and corrective action
- Keep track of referred samples and return of results to and from reference lab following the TAT
- Have a contingency plan to ensure continuity of testing of referral samples in cases of equipment break down.
- Mentorship of the peripheral health personnel in coordination with the DLFP
- Ensure implementation of the LQMS in the spokes
- Perform quality checks on completeness of information regarding samples for outbreak investigations e.g. name and location of the patient (village, Sub County, county and district)

6. National TB Laboratory Network coordinator

i. Supervise all EQA activities of the regional supervisors and all PT scheme coordinators.

ii. Provide quarterly Network work plans and reports for all activities

iii. Coordinates activities and meetings between NTRL and NTLP and NHLDS and all other partners

iv. Oversees all the activities of the NTRL Proficiency Testing (PT) schemes including Preparation, dispatch, results analysis and issue of feedback to participants.

v. Participate in developing of policies, procedures, guidelines and protocols for both NTLP and NTRL.

vi. Carry out training activities with respect to TB management for all levels

vii. Carry out External Quality Assessment support supervision visits in the regions

viii. Chairs meetings with the EQA regional coordinators as per the Lab schedule

7. Regional laboratory coordinators at NTRL

i. Ensure proper linkage of the TB program, the TB Reference Laboratory to the Regional, District supervisors and the Implementing partners, through providing accurate and timely communications and feedback over all the Network activities.

ii. Oversee all activities of EQA, proficiency testing and Blinded rechecking in the Region.

iii. Participate in developing of policies, procedures, guidelines and protocols for both NTLP and NTRL.

iv. Participate in TB training activities with respect to TB management for all levels

v. Conduct support supervision visits to the regions

vi. Ensure continuity of TB diagnostic activities in the regions

vii. Participate in the TB Performance review meetings in the Regions

viii. Provide quarterly Network work plans and reports for all activities to the Network coordinator

8. Regional Referral Hospital laboratory managers

Perform the following roles;

i. Support adoption and implementation of national policies and guidelines

ii. Coordinate all quality assurance roles within the designated regions, monitor participation and performance of all TB testing laboratories in EQA

iii. Conduct integrated support supervision and mentorship

iv. Conduct training of laboratory staff in the region

v. Support Laboratory Quality Management Systems (LQMS) implementation within the laboratories in the region

vi. Work with IPs in the region to ensure TB related activities are implemented

vii. Ensure timely quarterly regional reports are prepared and disseminated

viii. Conduct/ participate in TB operational research.

9. Hub coordinators

Perform the following roles;

i. Track and monitor TB samples that are referred from the spokes

ii. Ensure timely testing of all TB samples received at the hubs

iii. Ensure timely delivery of results to referring sites

iv. Work with IPs in the region to ensure TB related activities are implemented

v. Ensure delivery of EQA material to the designated sites within the time frame

10. District laboratory focal persons

Custodian of the laboratory services in their designated districts including;

i. Ensuring availability of reagents and supplies at testing sites for timely diagnosis
ii. Ensuring existence and functionality of equipment at testing sites
iii. Ensure all testing sites participate in EQA
iv. Responsible for first control of TB slides for all microscopy testing sites
v. Ensure follow-up of laboratories identified with errors in all TB EQA schemes for corrective action
vi. Conduct support supervisions in their districts and ensure reports are submitted to the regional Lab managers

11. Implementing partners

Perform the following roles;

i. Support the day-to-day operations of the sample transport network which includes but not limited to; provision of fuel for daily routes, service, maintenance and repair of the motor bikes, and other day to day operational needs at the hub
ii. In collaboration with the hub administration and the DHO of the district, the partner recruits and seconds Sample Transporter to the district/hub
iii. Where needed, provide infrastructural, equipment, human resource support to the hubs and funding for EQA activities (e.g. slide sampling and targeted support supervision)
iv. Support the hub administration to build capacity within the hub lab in order to carry out tests for all samples
v. Support and ensure functional diagnostic equipment (CD4, Hematology, Clinical Chemistry, etc) in the facilities
vi. Ensure adequate stock of reagents and consumables for all diagnostic equipment
vii. Ensure availability of laboratory registers for all laboratory tests at testing facilities
viii. Provide support for technical assistance and quarterly TB performance review meetings
ix. Provide comprehensive insurance to the sample transporters
x. Ensure laboratories participate in all EQA schemes
xi. Capacity building of the hub sample transporters
xii. Mobilize resources for hubs

12. Village Health Teams (VHTs)

The VHTs work closely with the health facilities within their placement to ensure the following;

i. Promotion of the uptake of TB services in the community
ii. Referral of presumptive TB cases for TB diagnosis after screening
iii. Guidance in proper TB sample collection and packaging
iv. Contact tracing and linkage of TB patients for treatment
v. Directly observed therapy (DOT) for patients in the community

Refer to the Village Health Teams Handbook, March, 2019 Edition.

CHAPTER 3

3.0 Laboratory biosafety and biosecurity

Laboratory biosafety and biosecurity activities are fundamental to protecting the laboratory workforce and the wider community against unintentional exposures or releases of pathogenic biological agents.

These activities are implemented using a risk assessment framework and through the development of a safety culture which is needed to ensure a safe workplace where adequate measures are applied to minimize the likelihood and severity of any potential exposure to biological agents.

3.1 Risks and transmission of TB

TB is an infectious disease. Transmission occurs when small aerosols containing acid-fast bacilli (AFB) become airborne and are inhaled. When a person coughs, sneezes, sings or vigorously exhales they produce aerosols that could be infectious if the person has pulmonary TB.

Properly trained lab personnel, when working correctly, have a very low risk of infection in a TB laboratory. However, some activities such as talking to infected patients and collecting samples can carry a greater risk.

3.2 Risk assessment

Risk assessment involves stepwise process in which the risk(s) arising from working with a hazard(s) are evaluated and the resulting information is used to determine whether risk control measures can be applied to reduce those risks to acceptable risk level.

Risk is the combination of the probability that a hazard will cause harm and the severity of harm that may arise from contact with that hazard.

TB laboratory activities are assessed according to the risk of generating aerosols, and the bacillary load. For further information on conducting risk assessments, refer to the *'WHO TB Laboratory Biosafety Manual'*

Risk Level	Laboratory Activities	Assessment of Risk
Low Risk	Direct Sputum microscopy; Preparation of specimens for the Xpert MTB RIF/ULTRA assay	Low risk of generating infectious aerosols from specimens, low concentration of infectious particles
Moderate Risk	Processing and concentration of specimens for inoculation on primary culture media; direct molecular testing on processed sputum by a line probe Assay	Moderate risk of generating infectious aerosols from specimen; low concentration of infectious particles

High Risk	Culture manipulation for identification, phenotypic DST, or a line probe assay on cultures	High Risk of generating infectious aerosols from cultures; high concentration of infectious particles

Note

All samples are infectious. Do not shake or stir samples, aerosols may be generated!

Samples may contain pathogens other than TB.

3.4 Infection Prevention and Control (IPC)

IPC is a practical and evidence-based approach intended to prevent exposure and reduce the risk of transmission of infectious agents to patients and health workers. IPC is implemented through the following measures:

a) **Administrative Controls:** These are measures put in place to reduce the amount of TB germs released into room air by a TB patient when he or she coughs, sneezes, laughs or spits, and these include;

- Prompt identification of presumptive TB cases at all service delivery points (SDPs)
- Separate presumed and diagnosed TB patients from others
- Educate patients on cough etiquette and respiratory hygiene
- Reduce the time taken to diagnose TB and initiate treatment

b) **Environmental Controls:** These measures are intended to reduce the concentration of the generated TB pathogens in room air. These include;

- Maximizing natural ventilation in patient waiting areas, outpatient departments, general medical wards and TB wards reduces the risk of TB transmission.
- Mechanical ventilation
- High Efficiency Particulate Air (HEPA) filtration
- Ultraviolet germicidal irradiation (UVGI)
- Open-air shelters with a roof to protect patients from sun and rain are one way of maximizing natural ventilation. Where resources allow, a certified well maintained Biosafety cabinet may be used for testing.

c) **Personal Protective Measures:** These are intended to protect the personnel that interact with the patients and/or patients' samples;

- Personal protective measures are the third and least priority in TB Infection Control.

- Personal protective measures compliment administrative and environmental control measures.
- Health workers should use respirators, while coughing patients should be given face masks.
- A respirator protects the wearer from inhalation of air contaminated with TB germs while masks protect others from the wearer.
- Respirators should meet or exceed the N95 standard
- Health workers in high-risk settings should wear respirators
- Respirators should be tight fitting for effectiveness and the wearers should be fit tested for appropriate size and model of the respirators.

Note: It's not a requirement to use N95 respirators for performing sputum smear microscopy, however, where available, personnel should use N95 when manipulating samples. Surgical masks are not recommended for laboratory personnel when working in the laboratory because they are not designed to protect the wearer; they are designed to stop the wearer from spreading aerosols.

3.5 Laboratory Layout

The Ministry of health's national health laboratory infrastructure guidelines details the designs of laboratories at various levels. The figure xx illustrates the basic layout/requirement for an adequate TB laboratory.

The minimum requirements for a TB laboratory layout include the following:

- Adequate ventilation in the room to protect personnel from airborne infectious droplet nuclei.
- Unilateral flow of air
- Sufficient lighting.
- Walls, ceilings, floors and bench tops are made of smooth, non-absorbent, easy-to-clean and disinfect material that is resistant to corrosion by chemicals.

The laboratory should have distinct sections for the following:

- A bench space or a table (A) for incoming samples (ideally near a window or latch)
- One well-lit area for preparing and staining smears (C)
- One well-lit area for microscopy reading and molecular techniques (B)
- One area for recording and reporting (F)

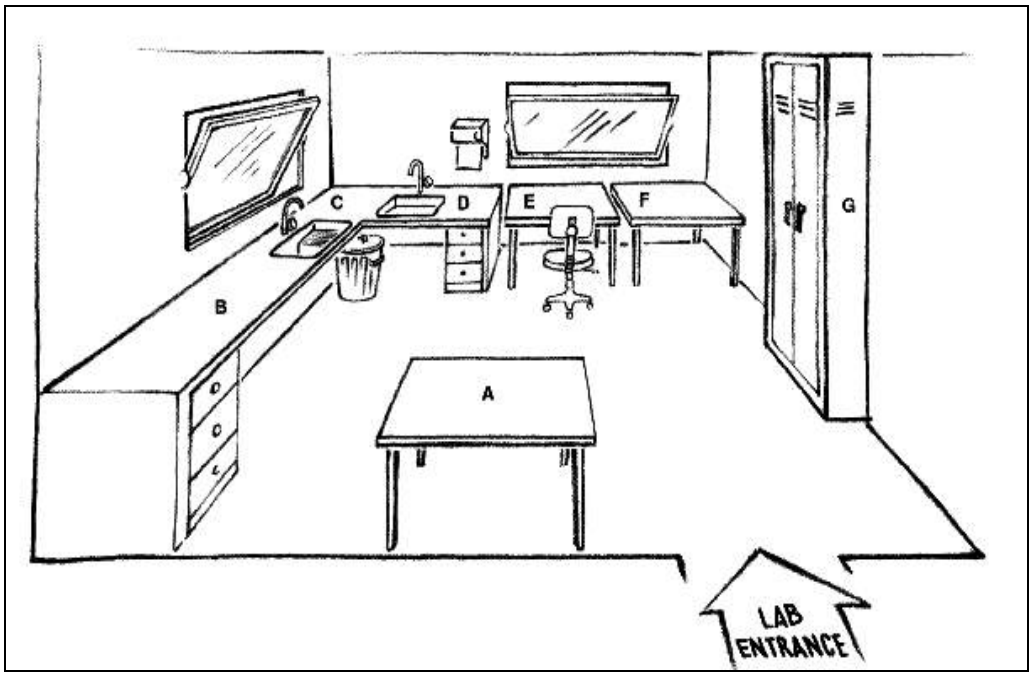


Figure 5: Guide on the flow of work for a TB Laboratory

3.8 Laboratory equipment

3.8.1 Biological Safety Cabinets

A biological safety cabinet (BSC) is not necessary for sputum smear microscopy and molecular techniques. Only laboratories performing culture and drug susceptibility testing need a functioning certified BSC. Never use a lamina flow hood to manipulate TB samples; it can blow TB organisms into the laboratory.

3.8.2 Pipettes

To prevent the generation of aerosols, care should be taken not to blow air forcefully expel liquids/solutions that contain biological agents when using pipettes. It is recommended to use serological pipettes and/or pipette tips that have cotton plugs to reduce contamination of pipetting devices.

3.8.3 Centrifuges

All centrifuges must be operated and maintained according to manufacturers' instructions by authorized personnel. Where safety buckets are available for a centrifuge, these must be used. Sealing rings for buckets must be checked regularly for integrity and replaced if cracks appear. Bucket covers should always be used when spinning biological samples.

3.8.4 Autoclaves

Use of high-pressure, saturated steam is an efficient way of killing TB organisms. Use operator's manual to select optimum temperatures and pressure for effective autoclave cycle.

Use autoclave tapes to verify the completion of an autoclave cycle.

3.9 Aerosols and cross-contamination

The major risk of TB infection in the laboratory is associated with the inhalation of aerosols generated by laboratory processes. Minimizing production of aerosols is the most effective means of staying safe. All aerosols should be considered as potentially infectious. Aerosols once settled onto a surface, may contaminate specimens or cultures, consumables, reagents, equipment, or PPE creating a cross-contamination risk.

Good laboratory practice to minimize aerosol formation and contamination of work surfaces and equipment may include;

a) Separate 'clean' activities (administration, microscopy) from 'dirty' activities (sample reception, smear preparation, staining).

b) Never shake a sputum sample.

c) Carefully open sample containers, the sample may have collected around the thread of the container.

d) Spread the sample onto the slide gently in a regular motion.

Stand away from (perpendicular to) the direction of wind, and prepare smears at an arm's length.

e) Always air-dry smears before heat fixing to avoid aerosol creation.

f) Use disposable wooden applicator sticks or transfer loops for making smears.

g) Always manage laboratory waste according to the national waste management guidelines.

3.9.2 Spill management

Treat all spills as potentially infectious. If a biological spill occurs the following protocol should be followed.

a) Put on a laboratory coat and gloves.

b) Place paper towel or cloth over the spill area and apply the disinfectant solution.

c) Leave covered – minimum 15 minutes to allow time for the disinfectant to act on the biological agent.

d) Clean up the contaminated material and put into the waste container.

e) Clean the surface using 70% v/v alcohol.

f) Wash your hands after the cleanup is complete.

Spill kits, including disinfectant, must be easily accessible to personnel.

Written procedures for cleaning and decontaminating spills should be developed for the laboratory.

3.10 Decontamination and waste management

3.10.1 Waste Management Process

Waste management refers to all activities involved in proper handling of wastes in medical/laboratory settings. These include Waste segregation, waste minimization, storage (handling of waste), treatment, transportation and disposal.

Refer to the *National Biosafety and Biosecurity Manual, 3rd Edition, 2021*.

3.10.2 Disinfectants and their use

Disinfectants are chemicals able to kill most of the micro-organisms on a surface or in a solution. For *Mycobacterium* species including MTB, their high fatty acid cell wall makes them less affected by some disinfectants.

The choice of disinfectant depends upon the material to be disinfected, exposure time, and the relative advantages/disadvantages of a disinfectant.

The disinfectants recommended for use in TB laboratories should contain phenol, chlorine or alcohol at the appropriate concentrations.

Recommended disinfectants and when they should be used

Disinfection Methods	**Surfaces**	**Spills**	**Prepare**
Phenol 5%	Yes	Yes	Daily
Alcohol 70%	Yes	No	Weekly
Hypochlorite 0.5%	No	Yes	Daily

Laboratories should maintain Material Safety Data Sheet (MSDS) for all the disinfectants that are used for their proper management.

All laboratory waste should be treated as potentially infectious. Laboratory staff are responsible for waste management and ensuring that anyone handling waste is properly trained.

3.10.2 Decontamination

Decontamination is a process or treatment that renders a medical device, instrument or environment surfaces safe to handle.

Sterilization, disinfection and antisepsis are all forms of decontamination:

Disinfection is a process of destroying microorganisms but not necessarily spores.

Sterilization is a process of destruction of all organisms, including spores.

Aseptic technique is a set of procedures by medical staff in which added precautions, such as use of sterile gloves and instruments, are used to prevent contamination of sterile equipment, medicine, or environment by microorganisms.

Consideration should be given to the microorganism being targeted, the characteristics of a specific disinfectant, methods to be used, and environmental issues.

Categories of laboratory waste

CATEGORY OF LABORATORY WASTE MATERIAL	**TREATMENT**
Contaminated sharps (hypodermic needles,	Must be collected in puncture-proof containers

scalpels, knives and broken slides).	fitted with covers and treated as infectious
Contaminated material for incineration	Must be decontaminated onsite OR stored safely before transportation to another site for decontamination and disposal.
Liquid waste (including potentially contaminated liquids) for disposal in the sanitary sewer system	Must be incinerated onsite. Should be decontaminated before disposal in the sanitary sewer

Chapter 4

4.0 Sample collection, transport and management at different levels

4.1 Sample types

Different TB laboratory tests may require different sample types for the diagnosis of TB among presumptive TB cases as outlined below;

TB ASSAY	**SAMPLE TYPE**
Microscopy	Sputum
GeneXpert	Sputum, stool, lymph node aspirate or tissue, Pleural fluid, CSF and Gastric Aspirates or any other body fluid suspected to have TB bacilli.
TB Loop Mediated Isothermal Amplification assay (TB LAMP)	Sputum
Truenat assay	Sputum
Line Probe Assay (LPA)	Processed sputum and TB isolates
TB Lateral Flow Lipoarabinomannan assay (TB LAM)	Urine
TB Culture and phenotypic DST (Lowenstein Jensen (LJ), Multiplexing Kit (Mkit), Mycobacteria Growth Indication Tube, (MGIT) and Broth Microdilution (BMD))	Sputum, lymph node aspirate or tissue, Pleural fluid, CSF and Gastric Aspirates or any other body fluid suspected to have TB bacilli.
Interferon Gamma Release Assay- (IGRA Test) for Latent TB infection	Blood
C-Reactive Protein	Blood

4.2 Sputum collection criteria.

Collect two sputum samples from a new patient for sputum microscopy examination:

a) Supervised **spot** sample at first visit.

b) **Early morning** sample on the next day.

*Alternatively, **Spot-spot** sample collection strategy can be followed i.e. two consecutive spot sputum samples, collected on the same day, may be performed. This is especially useful when there is a risk of the patient not being able to return to the clinic the following morning.*

For Nucleic Acid Amplification Tests such as GeneXpert, Truenat, TB LAMP, one sample is sufficient for TB testing.

Treatment follow up; Collect one -sputum sample for each follow-up during treatment.

4.2.2 Sputum sample Collection.

When a patient coughs the risk of infection is very high. Transmission of TB occurs because infectious droplets are released into the air when an infected patient coughs.

Samples should be collected outside the laboratories so that infectious droplets are diluted in an open, well-ventilated area, as far away as possible from people. Facilities should designate a 'coughers' corner' or sputum booth for sputum collection.

Safety precautions taken to minimize infections among health workers includes the following;

- Instruct the patient to cover their mouth when coughing
- Collect sputum outside the laboratory, preferably outside the building and well away from other people (coughers' corner).
- Never collect sputum samples from undesignated areas including:
  Laboratories, toilets, waiting rooms, reception rooms and any enclosed space.

Safety should be observed when collecting and handling non sputum specimens such as urine, biopsies, aspirates, CSF, Blood, stool due to the risk of contracting other infectious pathogens (other than *M.Tuberculosis*) that may be present in such specimens.

4.1.4 Sputum Containers

A good sputum container should be:

a) Wide mouthed
b) Leak proof
c) Provided with a tight-fitting lid (preferably screw-capped)
d) Easily disposable
e) Sterile
f) Transparent
g) Unbreakable
h) Have a frosted section for labelling

Sputum containers should be clearly labelled (on the sides and not the lid) with the patient's name, gender and date.

Ideal Sample Container

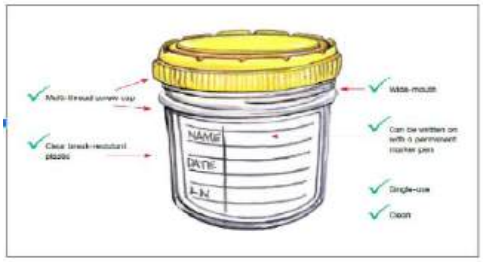


Figure 6: Sputum container

4.2 Procedure for sputum collection.

a) Explain to the patient, the reason for sputum examination.

b) Explain how many samples are needed and why.

c) Give the patient the labelled sputum container and demonstrate to the patient how to open and close the container. Explain the difference between sputum and saliva and the importance of bringing out sputum deep from the lungs

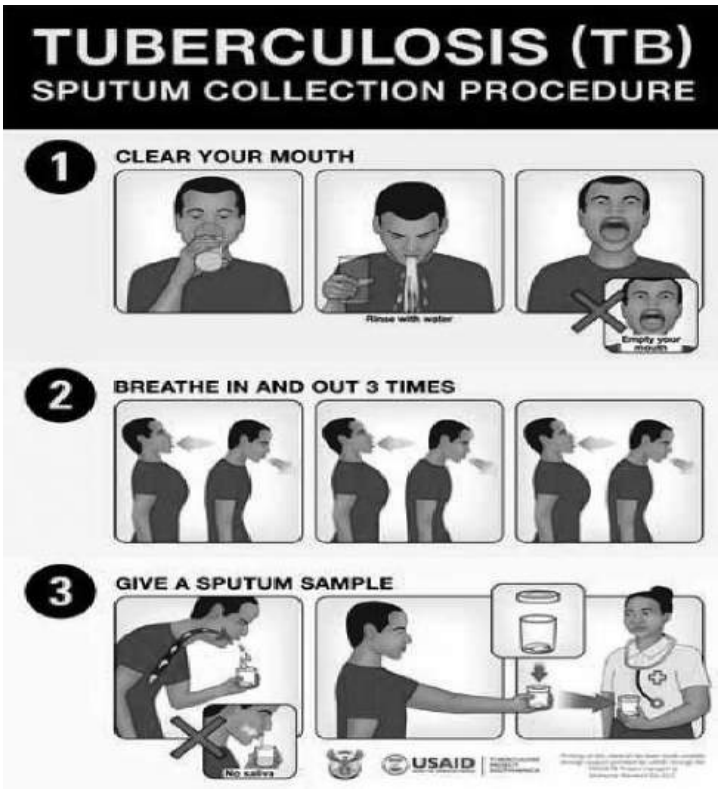


Figure 7(*Left*): Correct procedure of collecting sputum.

Spot Sample.

Give the patient the container and bring them to the nearby sputum collecting area/coughers corner and then instruct him by demonstrating with actual actions:

a) Ask the patient to rinse his or her mouth with plain water.
b) Inhale deeply 2-3 times
c) Cough out deeply from the chest
d) Open the container, bring it close to the mouth and bring the sputum out into it
e) Avoid contaminating the outside of the container with the sample
f) Do not give saliva or nasal secretions
g) Close the container firmly.

Early Morning Sample.

a) Give the patient another container
b) Ask the patient to rinse his mouth with plain water before collecting the early morning sample. This will remove any food particles in the mouth.
c) Follow the same instructions for sputum collection mentioned in the spot sample collection above.

Before the patient leaves the laboratory, visually examine the sputum sample for quality. If the sample is not good, ask the patient to cough again until a good sample is obtained. However, for follow-up, a poor-quality sample can be accepted and examined.

Qualities of a Good Sputum Sample:

a) Thick sputum
b) Purulent (yellowish or whitish mucoid)
c) Sufficient in amount (at least 2-5ml)
d) Not saliva or nasal secretions. **Caution: Do not stand in front of the patient when sputum is being collected**

Procedure for collection of Urine for LF LAM Assay

Urogenital areas should be cleaned before collecting urine for LF LAM test.

Mid-stream urine should be collected in a sterile standard urine container. Fresh samples should be tested ideally immediately after collection. If testing cannot be done immediately, urine can be kept at room temperature up to a maximum of 8 hours or at 2 to 8°C for a maximum of 3 days. If kept at 2 to 8°C, samples must be brought to room temperature before testing.

Procedure for collection and handling of Samples for the -IGRA Test

- Whole Blood for IGRA test for detection of Latent TB should be collected in Lithium or sodium heparin tube and handled appropriately to maintain viability of the white blood cells prior to testing.
- Blood collected into lithium or sodium heparin tube may be held at room temperature up to 3 hours after blood collection.
- Blood collected into lithium or sodium heparin tube may be refrigerated (2 to 8°C) up to 48hrs
- Plasma can also be harvested and referred for testing. Plasma can be stored at 2 to 8°C for up to 28 days, and at -20°C or preferably -80°C for longer periods.

Procedure for collecting and handling of Stool samples for GeneXpert testing for TB in Children

- Stool collection should be done by the caregivers or the patients, depending on the age of the child.
- Use a sterile stool container with a small spoon attached to the screw cap (Figure 6) to collect at least 3–5 g of stool.
- As soon as the stool sample has been collected, store the stool container in a clean, cool place (e.g. in a fridge if possible), avoiding exposure to direct sunlight.
- Do not freeze the sample. For transport and storage of stool specimens, the same conditions apply as for transport and storage of sputum specimens for Xpert
- testing. Collected stool can be kept at a maximum of 35 °C for up to 3 days, followed by a maximum of 7 days at 2–8 °C.

Figure 8: Stool container

4.2 Laboratory Request Form.

a) The laboratory should use a standard request form that includes; the patient's name, clinical information, address, Phone contact, reason for examination, examination requested, specimen details.
b) Ensure the request form is filled and complete and indicate if the sputum is for follow up or diagnosis.
c) Record the lab number on the request form after recording in the laboratory register.

HMIS TB 002: REQUEST FORM FOR TB SPECIMEN EXAMINATION

Name of Health Facility: ..... Date: .....

Name of Patient: ..... Age: ..... Sex: F ☐ M☐

A. Address of Patient:

District: ..... Nearest Health Unit: .....

Sub-County: ..... Tel No.: .....

Parish: ..... NIN: | | | | | | | | | | | | | | |

Village: ..... Next of Kin: ..... Tel No.: .....

B. Type of Patient:

☐ New
☐ Previously Treated (R,F,LTF,THU)

HIV STATUS
☐ POS
☐ NEG
☐ HIV Status Unknown

C. Risk Group (tick all options that apply)

☐ Health Care Worker
☐ TB Contact
☐ Prisoner Pregnant
☐ Refugee
☐ Uniformed/Armed Personnel
☐ Fisher Folk
☐ Diabetic
☐ Miner
☐ Tobacco User
☐ Mentally ill:
☐ Others (specify) .....

D. Reason for Examination:

† Diagnosis
† Baseline Tests
† Follow-up Treatment
Month: .....

E. Examination Request:

† Xpert MTB/RIF
† Microscopy
† Culture**
† DST**
† LPA**
† TB LAM

Others (specify): .....

F. Specimen Details

Presumptive No.: ..... Type of Specimen: .....
Unit TB No. ..... Date of Collection: .....
District TB No: ..... Time of collection: .....
Requesters Name & Signature: ..... Tel. No. ..... Email: .....

LABORATORY RESULTS

Lab No.: ..... Receivers Name and Signature: .....
Date Received: ..... Specimen Appearance: .....
Volume: ..... Mls.

Xpert MTB/ RIF: ☐

Circle option that applies :

† T = MTB detected, rifampicin resistance not detected;
† RR = MTB detected, rifampicin resistance detected;
† TI = MTB detected, rifampicin resistance indeterminate;
† TT = MTB Trace Detected RR indeterminate;
† N = MTB not detected;
† I = invalid / no result / error

Microscopy	Date	Specimen No.	Result				
			No AFB.	Exact No.	+	++	+++
		1					
		2					
TB LAM:			Positive:		Negative:		

Examination by (signature): ..... Date: ..... Telephone: .....

Reviewed by (signature): ..... Date: ..... Telephone: .....

***Results can be printed on a separate form*

(Print Version September 2019)

Figure 9: HMIS TB 002 Request Form for TB Specimen Examination

4.2.1 Sample Rejection Criteria

Samples should be rejected in the following situations

a) Broken or leaking sample containers
b) Sample container details do not match the Laboratory Request Form
c) Sample has been collected into a fixative (e.g. formalin)
d) Container unlabelled
e) Sample has been collected in an inappropriate sample container.
f) Empty container

4.3 TB Sample storage and transport

If the health centre is not a laboratory diagnostic TB unit, the sample should be collected and transported to the nearest designated TB diagnostic centre for examination through the hub system.

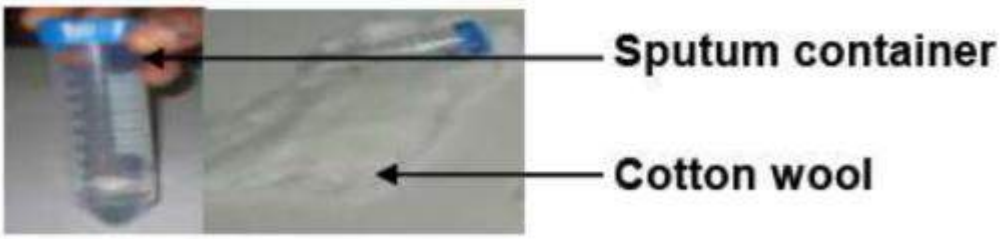
4.3.1 Storage

a) Sputum for microscopy, Truenat, TB LAMP or GeneXpert can be kept at room temperature if no fridge is available for a maximum of 3 days before testing.
b) If the sputum is intended for culture and phenotypic DST, the sample should be kept in a refrigerator (approx. 2 to 8^0C).
c) IGRA testing should be done within 3 hours of blood collection. If testing cannot be done immediately, whole blood can be kept at 2 to 8^0C up to 48hours. Plasma can be harvested and stored at 2 to 8^0C for up to 28 days, or at -20°C preferably at -80°C for extended periods.

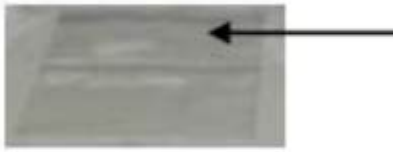
Packaging of samples for transportation

All referral samples should be appropriately packaged to protect the handlers from exposure to infectious agents in case of spillages.

The triple packaging system that consists of three layers should be used, where the primary receptacle is the specimen container, wrapped with enough absorbent material to absorb all fluid in case of leakage due to breakage or improper capping of bottles.



Secondary packaging- water tight, leak proof packaging such as zip lock bag to enclose and protect the primary receptacle.



For cold transportation conditions, ice packs can be placed outside the secondary receptacle in the leak proof container.

Outer packaging-Secondary packages should be placed in the outer shipping package/shipment box.

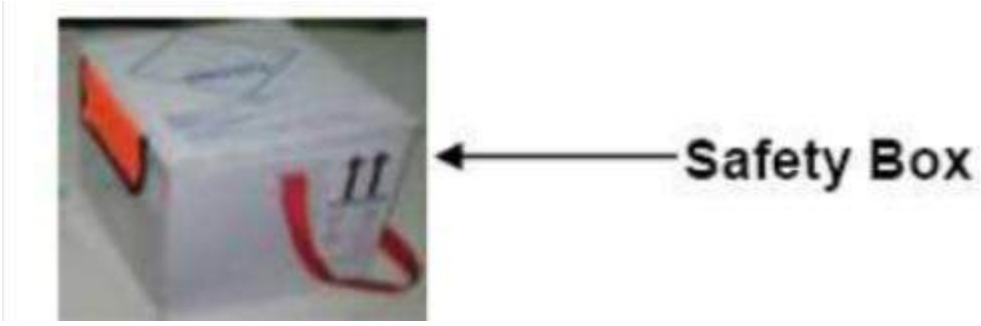


Illustration of the Triple packaging system for biological specimens

4.3.2 Transportation of Samples

a) Samples should be transported to the testing laboratory as soon as possible to avoid over growth of indigenous microbial flora.
b) Sputum for culture should be delivered to the culture laboratory within 72 hours from date of collection.
c) A completed sample request form with patient information must always accompany any sample to be referred.
d) To avoid cross-contamination, as a result of sample leakages, samples should be packaged individually in Ziplock bags.
e) Avoid contamination of the request forms or any paper reports by packaging them in a separate plastic bag.
f) Referral of samples for TB testing should be done through the national sample referral system.

Note: If TB isolates are to be shipped to NTRL for DST, adequate biosafety measures should be observed and isolates should be transported in appropriate media.

4.3.2 Mechanism of Sample transport in the TB laboratory network

Samples and results reports are transported through the national sample and results transport network (NSRTN)/hub system.

4.4 Sample Reception.

Considerations during sample reception;

a) Wear appropriate PPE during receipt and inspection of incoming samples.

b) Inspect delivery box for signs of leakage. Disinfect outside of the box using cotton wool or paper towels saturated with a suitable disinfectant (e.g. 5% phenol)

c) if you observed any sign of leakage.

   - Open the delivery box carefully and check for cracked or broken sample containers.
   - Disinfect cracked or broken sample containers before autoclaving or burning.

d) Check that samples have been adequately labelled with individual Identification numbers or names and that these match with the numbers on the accompanying list/request form.

e) Disinfect the inside of the delivery box with 5% Lysol and 70% Alcohol.

f) Discard gloves appropriately and wash hands after handling sample containers.

g) Record the date and time of sample receipt on the request form.

h) Enter the relevant patient and sample details into the TB laboratory register and assign a laboratory number.

Chapter 5

5.0 Diagnostic Techniques for the Tuberculosis Laboratory Network.

The primary role of the TB network is to provide quality services within the population that will support the NTLP. NHLDS and NTLP have adopted methods, procedures and guidelines recommended by WHO for TB diagnosis in line with the END TB strategy.

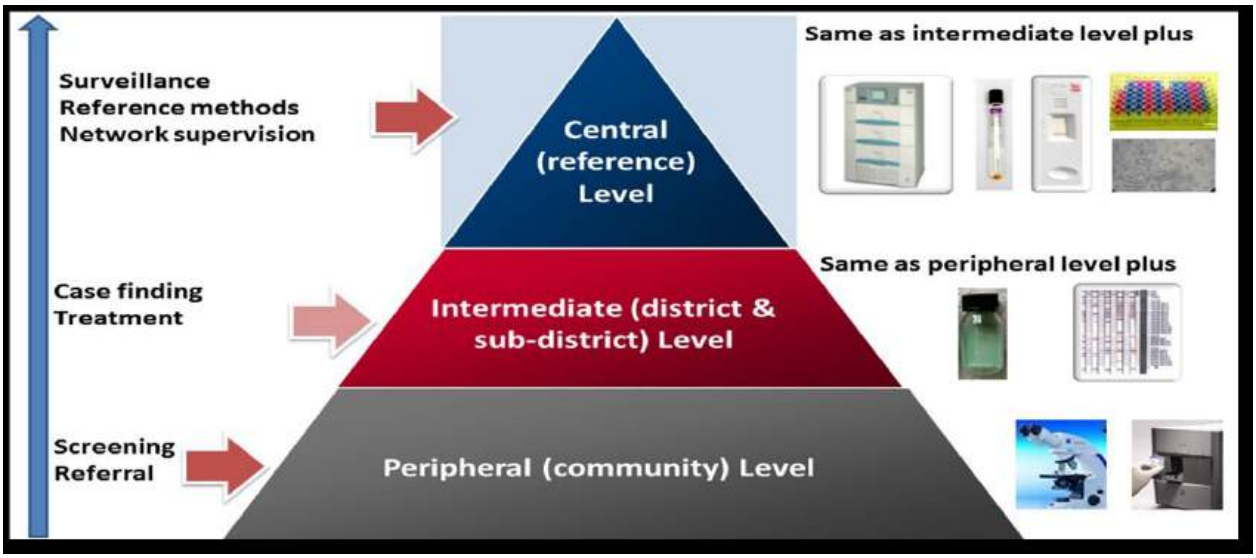


Figure 10: Pyramid showing the diagnostic techniques at each level of the TB network

The network is composed of laboratories with various testing capacities dependent on coverage, infrastructure, and the particular roles and responsibilities assigned to each specific laboratory. The first patient access point for diagnostic testing and treatment monitoring is often at the community or district level, while more sophisticated extensive testing is based at regional or central/ national level facilities.

Below is the full list of tests currently available in the TB laboratory network. The algorithm to guide their use.

a) Ziehl-Neelsen microscopy,
b) Fluorescent microscopy
c) GeneXpert Assay (MTB/ RIF Ultra and MTB/XDR)
d) TB LAM
e) Loop-mediated isothermal Amplification Test (TB LAMP)
f) Truenat
g) Interferon-gamma Release Assay (IGRA)
h) TB Solid and Liquid culture
i) Line Probe Assay (1st and 2nd line)
j) Phenotypic Drug Susceptibility Testing (1st and 2nd line)
k) Genome sequencing (Whole genome and targeted)

Community TB screening and diagnosis using mobile TB clinic

NTLP is implementing Community TB screening outreach activities to support same-day diagnosis and treatment initiation, targeting TB hotspots and communities that are at high-risk for TB. Community TB screening and diagnosis is performed using mobile TB clinic trucks mounted with portable digital X-ray, GeneXpert machine, and a multi-function room for medical consultation. Figure 11: Mobile TB clinic

Figure 11: Mobile TB clinic

5.1.2. Implementation of Mobile TB clinic services

Prioritization of the regions to be considered for community TB screening is determined by the need and the disease burden by mapping the TB hotspots using the available program data.

- Before a community screening outreach, the NTLP communicates with the regional and district teams to plan for the TB screening outreach in the regions.
- The NTLP in collaboration with the regional team and implementing partner identify communities targeted for TB screening outreach in the districts.
- GIS mapping is used to identify the TB hotspots and high-risk communities targeted for TB screening
- The implementing partner mobilizes logistics for the mobile TB clinic team support mobilizing the community members for the activity and conducting TB sensitization meetings in the targeted TB hotspots and high-risk TB communities.
- The screening outreach is carried out by a multi-disciplinary team comprising a technical team from NTLP/NTRL, RTLP and RRH, as well as the host district.

5.1.3. Roles of the mobile TB clinic team

- **Radiographer:** Manage the digital X-ray equipment ensuring radio-safety standards are observed, provide X-ray reports to the clinicians as produced from the machine, or interpret X-ray images in consultation with radiologists/physicians at the health facility in case auto interpretation is not available.

- **Laboratory personnel:** Support sample collection, perform laboratory tests process results for patients tested and manage storage and transportation of samples in case they cannot be examined on-site.
- **Nurse/ clinician:** perform triage, medical consultation for patients being screened and decision to treat TB or not. The clinician also ensures documentation of the whole screening process and reports on the utilization of the X-ray services.

5.2. Acid Fast Microscopy/Sputum microscopy

The purpose of sputum microscopy is to:

a) Diagnose of TB

b) Monitor the progress of treatment.

c) Confirm that cure has been achieved for Drug susceptible TB.

TB smear microscopy requires minimal biosafety requirements, trained staff, standard procedures, use recommended equipment, consumables and reagents, and labs participating in Quality Assurance schemes.

5.3. Fluorescent Microscopy

FM is 10% more sensitive compared to the Ziel Neelsen microscopy technique and has qualitative, operational, cost and workload advantages for all laboratories performing sputum smear microscopy. *Smear Preparation requirements and ZN/FM Staining procedure are illustrated in the appendix 1*

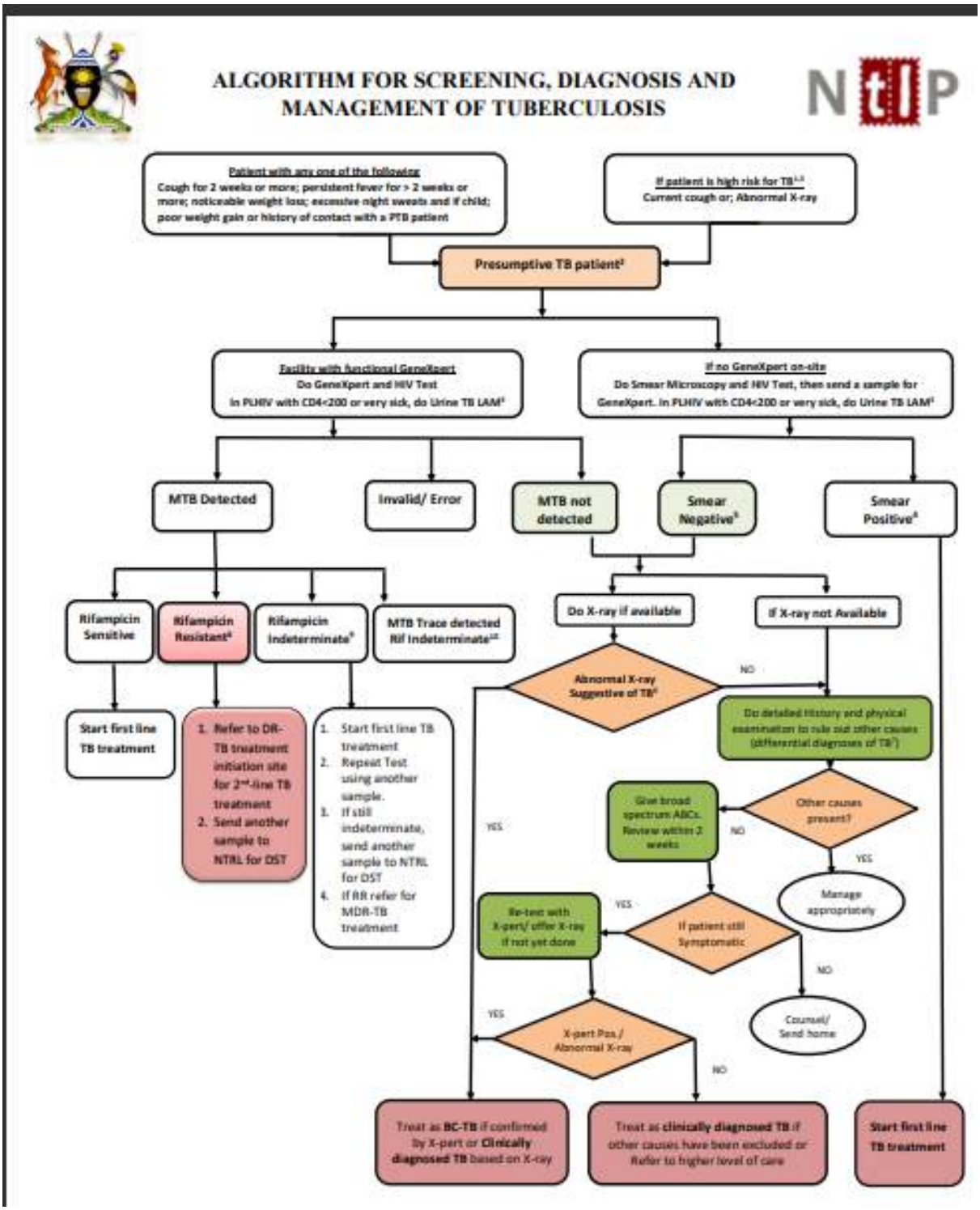
5.4 GeneXpert Assay

GeneXpert is a platform used to test for TB and other diseases, (e.g., HIV-Early infant diagnosis, HIV-Viral Load, Human Papilloma Virus (HPV), COVID-19, Ebola, Hepatitis viral load, *Trichomonas vaginalis* and chlamydia among others.

The Xpert MTB/RIF Ultra is used to test for TB and Rifampicin resistance among presumptive TB patients.

Xpert MTB/XDR Assay is used to test for TB and resistance to Isoniazid. Ethionamide, Fluoroquinolones and Second line injectable drugs. Xpert MTB/XDR assay should **ONLY** be used as a **reflex** test as per the National TB Diagnostic Algorithm.

5.1.1 Eligibility for GeneXpert test: Use algorithm for screening, diagnosis and management of tuberculosis:



1. **Presumptive TB** is presence of any or a combination of the following symptoms; cough≥ 2weeks or current cough if high risk patient, fever, night sweats, history of contact with a TB case, weight loss or poor weight gain for children. Also consider abnormal chest x-ray in a high-risk patient as presumptive TB

2. **HIV positive patients:** Presumptive or diagnosed TB patients who are HIV positive should be offered comprehensive HIV care services. In HIV positive individuals with CD4 ≤ 200 or very sick **(Temperature >39˚C, Respiratory Rate >30 breaths/min, Heart Rate >120 beats/min, New Seizure, Unable to walk without assistance / Bed-ridden)**, do **Urine TB LAM.** If Urine TB LAM is positive, the Patient should be started on TB Treatment and a sputum sample should be collected for GeneXpert testing to rule out Rifampicin resistance**.**
3. **TrueNat** is a molecular test for diagnosis of TB and Rifampicin resistance
4. **Smear positive** (AFB positive): is defined as at least one positive smear
5. **Smear negative**: defined as at least one negative smear. Carry out other investigations such as CXR **if available**. Those with **CXR suggestive of TB** should be treated as clinically diagnosed TB. I**f not available** or CXR not suggestive, do further history, physical examination and other investigations to exclude **other causes of chronic cough, fever and weight loss.**
6. **Differential Diagnoses of TB:** Chronic Obstructive Pulmonary Disease (COPD), heart disease, asthma, bronchiectasis, emphysema; Histoplasma pneumonia, trypanosomiasis, brucellosis; Fungal infection of the lung; Malignancy (lung cancer, lymphoma etc.)
7. **Features of abnormal X-ray suggestive of TB:** Heterogeneous opacities and cavitation in the upper parts of the lung, mediastinal L/nodes, pleural effusion and miliary picture
8. **If MTB detected RIF resistance detected**, Refer patient for MDR-TB treatment. Send another sample to NTRL for culture and DST (Sample should be sent by DR-TB treatment initiation site).
9. **If MTB detected Rifampicin Resistance Indeterminate;** Start first line TB treatment, Repeat Test using another sample. If still indeterminate, send another sample to NTRL for DST.
10. If **MTB TRACE Detected, Rifampicin Resistance Indeterminate ("trace calls"):** Start first line TB treatment if **HIV Positive, child or EPTB;** Among **HIV negative patients**, repeat testing using another specimen. The result of the second test should be used for clinical decisions and patient follow-up. Send another sample to NTRL for culture and DST. If Invalid/error/No result X-pert result, repeat test. Manage as per second result"
11. Repeat Truenat and follow algorithm to interpret. If both tests give indeterminate, treat with 1st line TB regimen and promptly conduct additional investigations to assess resistance to rifampicin e.g DST. Review treatment based on DST result.
12. Continue with 1st line treatment if Xpert results do not show rifampicin resistance. If Rifampicin resistance is detected, refer to DR-TB treatment initiation site for 2nd line treatment
13. For facilities with Xpert MTB XDR assay, treat as MDR (2nd line treatment) where results show rifampicin and Isoniazid resistance. If only MTB detected, initiate 1st line treatment

5.5 Truenat

The Truenat is used to test for TB and Rifampicin resistance among presumptive TB patients.

Refer to appendix 3

5.6 TB Loop-mediated isothermal Amplification (LAMP)

Loop-mediated Isothermal Amplification (LAMP) Assay is a molecular test used for the rapid detection of TB and Not Drug Resistance.

Refer to appendix 4

5.7 Interferon Gamma Release Assay (IGRA)

IGRA test is used for diagnosis of TB infection (Latent TB), IGRA measures a person's immune reactivity to Tuberculosis. Types of IGRAs used for testing include Quanti-Feron TB Gold (QFT), QIA reach, and T-SPOT TB.
Refer to appendix 5

5.8 TB Culture and Identification

At a minimum, a quality-assured culture must be established at the national TB laboratory with the appropriate equipment, biosafety measures, infrastructure, and referral mechanisms in place. TB culture in the network is mainly used as a method for multiplying and identification of viable MTB in qualifying patient samples. Culture is also done to allow for the performance of second-line Phenotypic DST (pDST) and to monitor the response to treatment of patients with MDR-TB and extensively drug-resistant TB (XDR-TB). Cultures are required monthly for patients on DR TB treatment.

At the NTRL both solid (LJ, LJ/Mkit) and liquid (MGIT/BMD) cultures are done to increase the chances of recovery of viable MTB organisms for purposes of performing 2nd line pDST as well as routine follow-up for the DR patients on treatment.

5.10 Line Probe Assay (LPA)

LPAs are used for rapid detection of drug resistance to first- and second-line anti-TB agents. They can be used to test culture isolates, smear microscopy-positive specimens (first-line LPA) and both smear-positive and smear-negative sputum specimens (second-line LPA).

In line with the END TB strategy, the Uganda national TB diagnostic algorithm indicates LPA for second-line drugs for all patient samples with Rifampicin resistant (RR) results using Xpert or Truenat.

5.11Phenotypic Drug Susceptibility Testing (pDST)

The END TB strategy objective for universal access to DST (for both 1stand 2nd line drugs including repurposed drugs), and is mainly done to guide the establishment of treatment regimens for DR-TB patients as well as determine TB Drug resistance patterns for patients not responding to treatment.

Consequently, phenotypic DST is performed for all samples as per the NTLP guidelines.

At the NTRL, phenotypic DST is performed using methods including;

a) Lowen Jensen (LJ)/ proportional method
b) Mycobacterium Growth Indicator Tube (MGIT) method
c) Multiplexing KIT (Mkit) for DST
d) Broth microdilution method (BMD)

Chapter 6

Quality Assurance for Tuberculosis diagnosis

6.1 Laboratory quality management systems (LQMS)

The Ministry of Health through NHLDS has instituted a strategy of implementing a laboratory quality management system for the laboratory network. This strategy will ensure that all National and Regional Referral Hospitals get ISO 15189 accredited, hubs are accredited or certified and all Health Centres with Laboratories implement QMS.

Laboratories that are accredited and those preparing for accreditation will receive proficiency testing panels for all tests in the TB test menu, while labs that are for certification will participate in Blinded rechecking for Microscopy EQA and receive PT for the other tests.

6.2 External Quality Assessment (EQA) activities for the TB Laboratory Network

External quality assessment (EQA is an external evaluation of a laboratory's performance using proficiency testing or random blinded re-checking and on-site evaluation.

EQA or PT programs are intended to allow laboratories or other testing sites to compare the closeness of their output to their peers through statistical evaluation against an expected or target value. Laboratories with unacceptable performance are expected to perform corrective action to improve performance.

a) provides early warning for systematic problems associated with reagents or operations.

b) Allows comparison of performance and results among testing sites.

c) Provides objective evidence of testing quality.

d) Identify problems in laboratory practices, allowing for appropriate corrective actions.

6.2.1 Proficiency Testing

Proficiency testing (PT) is the evaluation of a laboratory's performance against pre-established criteria by means of interlaboratory comparisons. It's used to demonstrate competency and validate a laboratory's measurement process by comparing its results to the results of a reference laboratory and other participant laboratories.

Goals of proficiency testing

- To provide laboratories with support and information in improving the quality of their testing and/or measurements.
- To give laboratories an unbiased method of demonstrating and evaluating the reliability of their data.
- To add an external evaluation of the testing/calibration capabilities of laboratories.
- To boost laboratories' internal quality control processes.

The National Tuberculosis Reference Laboratory has established and maintained a proficiency testing scheme for most TB laboratory diagnostics including conventional microscopy and WHO-recommended molecular diagnostic e.g., GeneXpert. The NTRL provides an annual PT calendar that states the frequency of each scheme to prospective customers so that laboratories can enrol in a scheme of their choice on an annual basis.

Due to the large number of laboratories performing smear microscopy, there is limited capacity to produce microscopy PT for all the laboratories in the network hence, PT for microscopy will only be provided to accredited laboratories and laboratories that are earmarked for accreditation within the stated calendar year.

The NHLDS is establishing the National External Quality Assurance department that will oversee and coordinate all proficiency testing schemes in the country.

6.2.2 Blinded Rechecking of Routine Slides

Blinded rechecking is the EQA process that involves re-reading a statistically valid sample of slides from a laboratory to assess whether that laboratory has an acceptable level of performance.

Blinded rechecking is based on Lot Quality Assurance Sampling (LQAS); randomly selected slides from the routine workload of the laboratories are sent to a higher laboratory (first controller) for validation.

The WHO refers to blinded rechecking as the EQA method that provides reliable assurance that a country has an effective AFB microscopy network because it only functions in through a network. Blinded rechecking;

a) Reflects reality of routine performance by checking:
   - ✓ Sample Quality
   - ✓ Smear technique
   - ✓ Stain performance and
   - ✓ Accuracy of reading
b) Motivates staff

An accurate and practical Blinded rechecking system should ensure that;

a) The sample contains a sufficient number of randomly selected slides to be representative.
b) The first controller (re-checker) must be unaware of the original result of the peripheral laboratory lab personnel to prevent bias, i.e. is “blinded”.
c) Minor errors, representing false positive or false negative interpretations of 1-9 AFB/100 fields, are included with major errors to obtain a smaller sample size. The smaller sample size facilitates the implementation and sustainability of rechecking programs.
d) A second controller resolves discrepant results.
e) There must be a system to provide timely feedback and improvements to the Laboratories that are supervised.

Blinded rechecking in the TB Laboratory network is done for all active diagnostic centres from Health Centre III Level to Regional Hospitals. It is coordinated through the normal NHLDS structures from the National TB Reference Laboratory to the lowest peripheral laboratories. The regionalization strategy that the MOH is adopting is to ensure that the intermediate levels activities are transitioned to the Reginal Referral Hospital with oversight of laboratories at the district levels.

All microscopy laboratories to be rechecked under blinded re-checking should have:

a) Sufficient slide boxes to store all slides for at least one quarter.
b) Tools for permanent labelling of slides for proper identification: either pencils (for slides with frosted ends) or a diamond marker (for plain slides).

c) An NTLP Laboratory Register.
d) Staining facility, microscopes, and reagents.
e) A piece of absorbent paper for example newspaper for use to remove oil from the slides before storing them in the slide box.

First Controllers (DLFP and/or chosen staff in the District Lab) need;

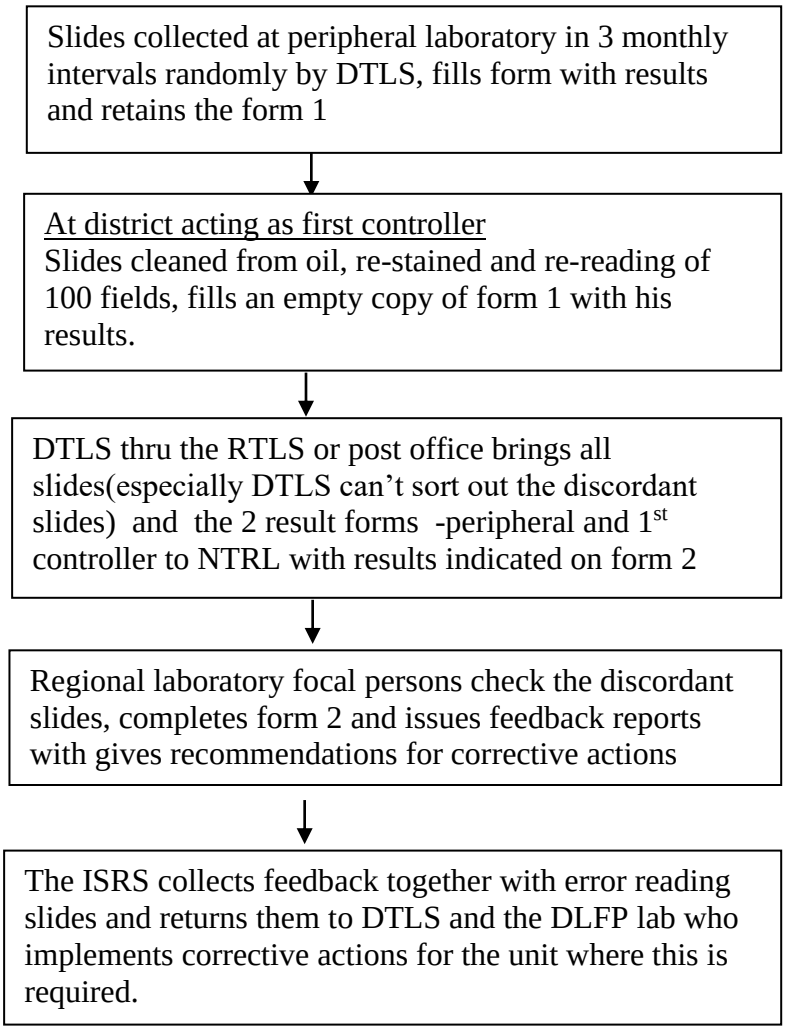
a) A good microscope.
b) Sufficient time.
c) Xylene (for the labs doing rechecking of slides stained by the ZN technique).
d) Staining facilities and equipment.

Second controllers (Regional laboratory supervisors) need

a) A good microscope.
b) Sufficient time.
c) Discordant forms for listing discordant results.
d) A computer and its printer for compiling and printing feedback reports and quarterly summary performance reports for DTLS, regional and NTLP and data entry in the regional analysis sheets.
e) An EQA Excel Database for analysis of District and Zonal data entries.

The flow of activities for blinded rechecking in the Uganda TB lab Network is as demonstrated in the flow chart shown below

6.2.2.4 Flowchart of blinded re-checking activities, NTLP Uganda



Based on the Lot Quality Assurance System (LQAS), Uganda blinded rechecking program randomly samples 10 slides per lab per quarter.

6.2.3 Roles and Responsibilities of different players

6.2.3.1 Lab staff at the periphery

a) Labels the slides with a District code, Unit code, patient Serial number, and sample number i.e., 1, 2 for the first or second sample respectively; use a pencil (frosted-end slides) or diamond marker (plain slides). Do not write the result on slides.

b) After reading the slides, place them on absorbent paper (newspaper, toilet paper) until the end of the day or the following morning when the oil has dried out. Do not use xylene or any other solvent to remove the remaining oil and never rub the smears. Slides examined by the FM technique are stored directly in the slide box since no oil is used for microscopy in this technique.

c) Keeps all slides from all patients, regardless of results in the boxes sequentially according to the slide patient serial numbers in the lab register leaving empty slots in the box for those expected to arrive over the coming few days, until the end of the quarter and sampling has been done. In case of a shortage of slide boxes, roll slides in toilet paper taking care that they don't touch each other.

d) After the sampling has been done discard all the remaining slides and re-use the slide boxes for the storage of the slides in the new quarter.

e) Analyze, summarize, and sends quarterly performance lab reports (number of AFB done, stock situation) to the district.

6.2.3.2District TB/Leprosy Supervisor (DTLS)

a) Ensure that all Laboratories in your district know their District codes and Unit codes. Assign Units codes to New Units or any others without codes.

b) Slides are sampled at the end of every three months according to instructions from the national level. In principle, 10 slides are collected from every laboratory every 3 months; however, if in the previous quarter sampling was not done for any reason, twice as many slides should be collected for rechecking

c) Indicate on the sampling form the period covered by the sample in the sputum smear Lab register. From the register, note the total number of smears examined during this period. Divide this total by the sample size which is 10 slides required for the current visit (rounding down to the next whole number); this yields the *sampling interval*.

d) Fill in a sampling form with details of laboratory identity, the period rechecked, etc. Ensure that a second form is filled with only the *slide identification numbers only* without the results. (see Appendix 10)

e) Use a random number (e.g. last digit of a bank note serial number) to select the first slide of the sample, counting from the first result in the register (slides are selected using the laboratory register and must not be selected from the slide box). Identify the first slide to be collected, and note its identification number and result on the sampling form.

f) From there, start counting results (without differentiating between positive/negative/actual numbers or suspect/follow-up) using the sampling interval to find the second sample slide, noting its identification and result on the form.

g) Continue in this way until the required 10 slides is reached; if the calculation was done well and rounding done in the right way, the sampler will have got all the ten by the end of the of the total smear count in that quarter.

h) Ask the technicians to retrieve the selected slides from the boxes; check these against the list. Note any slides that are missing and replace each with the next slide in the register (even if the result was different), adding its identification and result at the bottom of the form. Ensure that each selected slide is clearly labelled with a unique laboratory number; if it does not, replace it with the next slide that does bear the unique lab number and indicate the one replaced. Write the unique Lab number of the replaced at the bottom of the list and record its result.

i) If none of the selected slides has a positive or scanty AFB result, add an extra recent positive in that same quarter of sampling as an eleventh slide, record its unique Lab number and it results on the same sampling form.

j) Ensure that the remaining slides in that quarter (the quarter being sampled) are all properly discarded, and instruct the technicians to start a new collection in the new quarter.

k) Arrange the slides in a transport box in the order listed on the form and deliver them as soon as possible to the first-level controller, together with the form ***without results***. Retain (the sampler) the form *with results* or give it to the regional laboratory supervisor or the NTRL Zonal Coordinator.

l) Send all slides and forms to the regional Laboratory for 1st control

m) Hand over feedback reports to DLFP and units either before or at sampling for the next quarter. Major error slides should always be returned with the feedback reports for all Labs identified to have errors.

NOTE:

a. It is the role of the DTLS as a sampler to ensure that the reading of slides by the first-level controller (DLFP) is completely blinded. The controller should have *no access whatsoever to the peripheral lab results*, only a list of slide identification numbers should be provided to him.

b. DTLS should also ensure that laboratories with a major error or many minor errors after the rechecking are visited in time by the DLFP for corrective actions.

6.2.3.3District Laboratory Supervisor (DLFP)

a) Ensures that reading of slides by the first-level controller is completely blinded. The controller should have no access to the peripheral lab results, only a list of slide identification numbers.

b) Cleans all oil-immersion examined slides by dipping them in xylene for at least two hours and allow to dry. Slides from labs doing diagnosis by the FM technique need not be dipped in xylene since they have no oil on them.

c) For each slide, makes notes in the comment column of the sampling form (See attached)) about the size, thickness, evenness and background staining of the smear. For smears that

are not re-stained, add notes on AFB colour and artefacts after microscopic examination. (Use the remarks column).

d) **Re-stains all slides** (those examined by ZN technique and also FM technique) to avoid reading false Negative results which may have risen because of fading.

e) For re-staining, he uses the routine staining cycle and a certified quality auramine (FM) or carbol-fuchsin solution (ZN). Do not decolorize first; make a note on the form (Sampling forms) stating that re-staining was done.

f) Checks the smears using the original microscopy system (brightfield for ZN, fluorescence for FM) and standard magnification. Check the number of fields prescribed for routine work in the NTLP guidelines; do not check more fields unless there is doubt about the quantification after one length.

g) Note the first control result on the sampling form in the first controller' column.

h) Add overall notes on smearing and staining at the bottom of the forms, and send them to the local coordinator.

i) Do not recheck slides that lack clear identification or that have severely damaged smears. Report them as "excluded/ID" or "excluded/damaged".

j) After rechecking, the oil on the slides is left to soak into absorbent paper as for routine work (only for the ZN technique), then the slides are returned to their original box.

k) Rechecked FM slides – and ZN slides if fading is a problem – should be kept in a closed box, and in a cool place.

Note: It is expected that about 5-10 % of the slides will be discordant. **The absence of discordant from several centres indicates that re-checking was most likely not blinded.** Controllers should have less FN and almost no HFP for the controls to be valid should be equally divided between canter and first controller.

6.2.3.4Regional laboratory supervisor

a) Copy the first-level controller's results to the original form with the microscopy laboratory results, and/or the microscopy laboratory results to the first-level controller's copy.

b) Identify slides for which the laboratory results and the first-level controller's results are discordant; these will be either positive (or actual number) versus negative, or a quantification difference of at least 2 steps (actual number versus 2+ or 3+; 1+ versus 3+).

c) Discordant results for each microscopy laboratory sampled should be listed on the discordant rechecking form [Appendix 11, Form 2]. Record the name of the laboratory, the identification number of each slide concerned, and the two discordant results. Switch recording both results in the two columns, for some labs recording the laboratory result as "Result 1" and the controller result as "Result 2", but reversing the order for other labs. This ensures blinding of the second controller to the origin of the results and helps to avoid bias, while at the same time guiding the work of the second controller

d) *Do not provide feedback* for samples with discordant at this stage, since errors have not yet been identified.

e) Re-strain all (ZN and FM) slides with discordant results unless they were re-stained at the first control level and have not faded again. Make a note if re-staining is done.

f) Recheck these smears, assuming that AFB are present. Use both laboratory result and first-controller result (results 1 and 2) as a guide to the number of fields to be checked. For negative/clear positive discordance, two lengths are sufficient before concluding that the result is negative; for scanty/negative discordance, examine five lengths before declaring a negative result; for quantification differences examine as many fields as necessary to yield a confident result.

g) Note the quantified result in the column for the second controller; add qualitative remarks. On the list of discordant form.

h) Copy the second control results to the appropriate column on both the original forms. (Sampling form) Using this second controller result as the "gold standard," determine who was responsible for the error and, from Table 1 below, the nature of the error. *Remember that errors may be made by the first controller as well as by the laboratory.*

i) *For results that were not discordant, assume that both the microscopy laborat*ory and the first controller were correct in their conclusions.

j) Fill in the summary tables on the feedback report form with the number of slides re-checked and the errors by both microscopy laboratory and first controller identified. Make a summary of performance and recommendations for corrective action, for a final Feedback report for each lab that submitted slides for rechecking in that quarter.

k) Send through the integrated sample referral system feedback report to the peripheral unit that sent, and copies to the RTLS, the DTLS and the DLFP. Error slides for laboratories identified with such are also sent back with the feedback reports.

6.2.3.5 Zonal TB/ Leprosy Supervisor

a) Transmit rechecking slides and forms in both directions (region, district and national).

b) Supervise districts and regions for correct execution of re-checking, including.

6.2.4 Interpretation of Blinded Rechecking Feedback Reports.

Classification and definition of errors found in rechecking

Registered result being controlled	**Final result**				
	negative	**scanty**	**1+**	**2+**	**3+**
negative	correct	LFN	HFN	HFN	HFN
scanty	LFP	correct	Correct	QE	QE
1+	HFP	correct	Correct	correct	QE
2+	HFP	QE	Correct	correct	correct
3+	HFP	QE	QE	correct	correct

HFP: high false-positive, negative versus clearly positive result

HFN: high false-negative, positive versus totally negative result

LFP: low false-positive, negative versus scanty result

LFN: low false-negative, scanty versus totally negative result

QE: quantification error, at least two steps difference in quantification

Note:

a) Any error detected by LQAS must be viewed as a potential indicator of diminished competency and investigated further.

b) Repeated major errors would almost certainly signal the need for a prompt on-site assessment and or retraining.

c) An occasional minor error (quantification) is unlikely to be a signal of ongoing problems.

d) Whenever a serious error occurs, the peripheral laboratory must be informed as soon as practicable.

The controlling laboratory must give feedback that includes a likely explanation for the discrepancy as well as suggestions for corrective action.

6.2.2.1 On-site Evaluation

This involves an onsite visit by a supervisor to a laboratory to make a realistic evaluation of the condition of the laboratory including checking the functionality of the equipment, the availability and quality of reagents, and the technical skills and competence of the laboratory staff among others.

On-site evaluation should ideally be performed at least once a year by personnel from a higher-level laboratory (regional referral or national referral) to evaluate the overall operating conditions in the laboratory. This provides an opportunity for immediate joint problem-solving with an emphasis on two-way communication between supervisor and supervisee. It promotes managerial accountability, staff support, and development because they are enabled to identify their problems, acknowledge the achievement, and plan joint actions which ultimately improves motivation for better individual and organizational performance.

Each supervisory level ought to plan and budget for onsite evaluation. At the district level, this is done by the district laboratory Focal person (DLFP) or the District TB Leprosy Supervisor at least once every month.

A standardized checklist should be used for uniformity and consistency during the supervisory visits.

Appendix 1- Preparation of TB Smears

Principle of Acid-Fast Bacilli staining (ZN)

The property of acid-fastness of mycobacteria is based on the presence of mycolic acid in their cell wall. Primary stain (fuchsin) binds to cell wall mycolic acids. Intense decolorization (strong acid or acid/alcohol) does not release the primary stain from the cell wall and AFBs retain the red color of fuchsin. Counter-stain (methylene blue) provides a contrasting background. Only very few other bacteria possess the property of acid-fastness, however, their acid-fastness is weak (e.g. Nocardia). AFB found in respiratory specimens of patients from TB high-prevalence countries are almost always TB bacilli.

Non-TB mycobacteria are more commonly found in countries where TB prevalence is low. In high-burden countries, some patients suspected of having MDR-TB may have disease caused by non-TB mycobacteria.

Slide Labelling

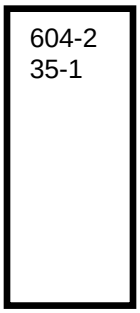
Slides for smear Preparation are Labelled with a district code assigned by NTRL, a facility code assigned by the District TB/ Leprosy supervisor, a Laboratory serial number as given in the TB Laboratory register for every patient and finally a sample number either 1 for the first (spot) and 2 for the second (early morning sample or spot-spot samples). Frosted slides are labelled with a pencil and the un-frosted slides with a diamond Pencil. Proper Labelling is as demonstrated below.

604- District code

2- Unit/Health facility code

35- Lab. Serial Number

1-Sample Number



Note:

- All samples received in the laboratory must be assigned a laboratory number.
- The Laboratory Number begins with 1 or 01 on 1st of January each year and continues serially with each new patient until 31st of December of the same year.
- Do not assign Lab numbers on a monthly or quarterly basis.

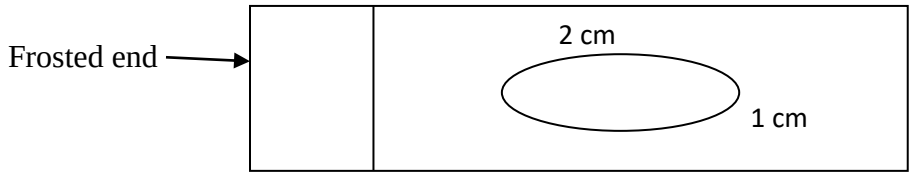
Smear Preparation

Using labelled slides, prepare smears, starting from the center of the slide, outwards making small coil-like movements.

Select a small portion of purulent or muco-purulent material with the applicator stick and transfer it to the slide; if a stick is used, break it in two pieces and use the ragged ends for dissecting

sputum and for smearing.

Spread the material carefully over the area equal to about 2x1 cm using repeated coil-like movements, without touching the margins of the slide and smear should be in the middle of the slide.



Make the smear as even as possible by continuing this process until no thick parts remain. Remove excess material with the second stick and discard in the biohazard bag.

The thickness of the smear should be such that a newspaper can barely be read through the dried smear held about 10 cm above it. (Transluscent)

Air dry the smear on the slide rack

After the smear has dried fix the smears by passing a flame under the slides before staining.

Do not over-heat the smears, or else AFB staining will be poor.

ZN Smear staining Procedure

Place the slides with smear upwards on the staining rack over a sink about 1 cm (a finger) apart. Include a positive (Smear of known grade) and negative control slide on every batch of slides stained. Always filter carbo fuchsin before staining.

Prepare a cotton plug and pour alcohol/spirit (for burning purposes) and light it.

Heat all slides by passing the flame gently under the slides until steam arises; make sure that the stain does not boil since this may disintegrate the bacteria or crack the smear or slide. Heating should be done two more times at an interval of 5 minutes such that the total exposure time of carbol fuchsin is 15 minutes. Tilt each slide to drain off the staining solution.

Rinse the slides well with clean running water e.g from a beaker; DO NOT splash water onto adjacent slides.

Pour the decolourizer (25% Sulphuric acid or 3% acid alcohol) over the smears covering them completely.

Allow to act for 5 minutes.

Tilt each slide to drain off the decolourizer; then gently rinse each slide again with clean water; DO NOT splash water onto adjacent slides.

If needed, repeat covering by decolourizer and rinsing until all macroscopically visible stain has been washed away.

Flood smear with 0.3% methylene blue solution for 30 seconds-1 minute.

Tilt each slide draining off the methylene blue solution. Wash with clean water; DO NOT splash water onto adjacent slides.

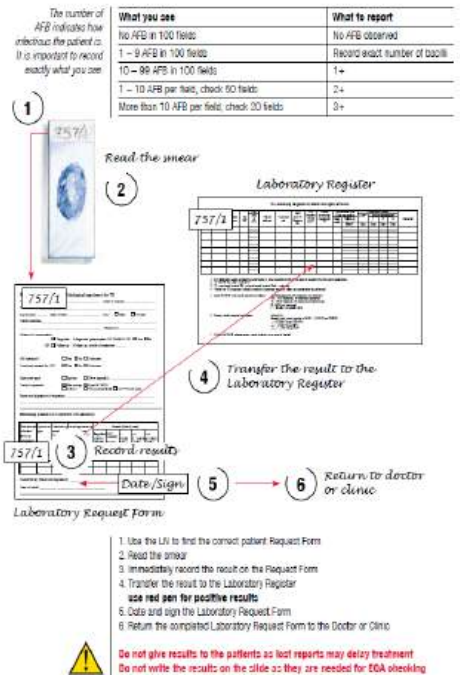
Take the slides from the rack, drain off the water and stand each slide on its edge to air dry on the drying rack

Note: The stained smear should show a light blue colour from methylene blue. If the smear is dark blue, it usually indicates that the smear is too thick or prolonged/over exposure with methylene blue.

If the smear is purple of pink, it means poor decolourization. (See guide for a good smear preparation (WHO Chart).

3 Examination

1. Fill in the smear microscopy worksheet as you start to examine slides.
2. Make sure you examine the control slides first and record results on the worksheet. The control slides should give the expected results before proceeding to examine patient slides.
3. Use the objective 100x and a x10 eye piece that collectively give a x1000 magnification.
4. Apply one drop of synthetic immersion oil to one edge of the stained smear. Mount on a microscope for examination.
5. Scan the stained smear systematically from left to right side or vice versa, covering one length (100 microscopic high-power fields, the length of the smear 2 cm). This is the minimum to be scanned before reporting a negative result, and should take about 5 minutes. Count AFB in positive smears for quantification.
6. Always search for useful areas, i.e. those containing mucoid threads and pus cells; do this by moving up or down when arriving at an almost empty area, till another useful zone has been found, then continue moving to the right.

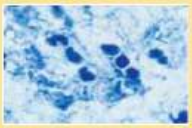


Note: Acid-fast bacilli appear bright red against the background material counterstained in blue. Report as positive for AFB when the background is bluish and at least one red acid-fast bacillus was seen in a well stained smear, even if you think they might be other mycobacteria other than tubercle bacilli. Tubercle bacilli are quite variable in shape, from very short fragments to elongated types. They may be uniformly stained or with one or many gaps, or even granular. They occur singly or in small groups (coded), and rarely in large clumps. The typical appearance of bacilli are usually rather long and slender, straight or slightly curved rods.

Ziehl-Neelsen Reporting

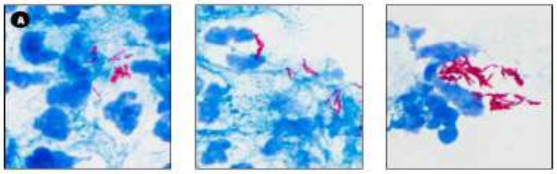
Common Problems and Remedies at Microscopy

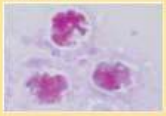
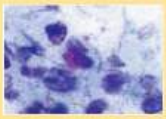
Problem	Cause	Remedy
Pale acid-fast bacilli 	CF prepared from poor quality reagents	Use reagents from reputable manufacturer For in-house reagents, recheck preparation and QC results
	CF insufficiently heated	Heat CF to steaming
	CF staining time less than 10 minutes	Stain for a minimum of 10 minutes
	Smear overheated during preparation or staining	Pass over flame 3 times; 1-2 seconds each time Stop heating when CF steams
	CF reagent has expired or stored in direct sunlight	Replace reagent Store stain bottle in the dark

Problem	Cause	Remedy
Counterstain too dark 	Excessive counterstaining time	Do not exceed 60 seconds
	Inadequate washing step after counterstaining	Extend washing step
	Methylene blue concentration too strong	For commercial reagents, check with NTP For in-house reagents, recheck preparation and QC results
	Smear too thick	Prepare new smear

Problem	Cause	Remedy
Deposit on slide 	Stains not filtered	Filter stains
	Soot deposit on underside of smear	Clean with a moist tissue paper

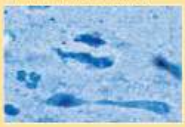
Correctly stained slides



Problem	Cause	Remedy
Smear too pink   	Insufficient decolourisation	Decolourise for longer
	Acid concentration very low, or applied for too short a time	For commercial reagents, check with NTP For in-house reagents, recheck stain preparation and QC results
	Carbol fuchsin (CF) has dried on smear	Check smears are level over sink Add sufficient CF
	Smear too thick	Prepare new smear
		When correctly stained this slide looks like **A** above

Problem	Cause	Remedy
Light flickers or does not turn on	Loose plug or connection	Check wall sockets, transformer, power supply
	Loose light bulb	Reinstall the bulb – Do not touch bulb with fingers
	Dirty bulb contacts	Clean contacts with 70% alcohol and retry or replace bulb
	Erratic voltage supply	Use a voltage stabiliser
	Faulty on-off switch	Replace the switch
	Fuse blown or transformer blown	Replace the fuse
	Discoloured bulb/burnt out	Replace the bulb – Do not touch bulb with fingers

Problem	Cause	Remedy
Uneven illumination 	Field of view partially blocked	Rotate the nose-piece until it clicks into position
	Iris diaphragm is almost closed or condenser is not aligned	Recalibrate microscope
	Dirty lenses	Gently wipe the lenses with lens paper/soft cloth. If the trouble persists clean with lens paper soaked in the recommended lens cleaning fluid (see page 38)
	Heavy fungal growth on lenses	Clean the lens using lens cleaning fluid as recommended by the manufacturer

Problem	Cause	Remedy
Excessive image contrast 	Iris diaphragm is almost closed	Open diaphragm

Fluorescent Microscopy Staining

Principle

The property of acid-fastness of mycobacteria is based on the presence of mycolic acid in their cell wall. Primary stain (auramine O) binds cell wall mycolic acids. Intense decolorization (strong acid) does not release primary stain from the cell wall easily and AFBs keep the fluorescent bright yellow color of auramine. Counterstain (Methylene blue or Potassium Permanganate) provides a contrasting background. Fluorescent stains are usually organic substances that absorb ultraviolet light and remit part of the energy as light of a longer wavelength which can be observed through the eyepiece as fluorescence. When exposed to ultraviolet light, the fluorescent bacilli are perceived as brightly colored organisms against a dark background.

Procedure for FM staining

1. Place the slides on the staining rack. **It is a must** to keep a distance of at least **1cm** between every slide. Otherwise, there is a possibility that acid-fast bacilli may cross-contaminate the negative smears due to over-flooding or splashes from a positive smear to a negative smear.
2. Cover the smears completely with filtered 0.1% auramine solution **Do not heat**
3. Leave for 15 minutes
4. Wash the slides well with distilled or running water
5. Pour the acid alcohol solution over the slides
6. Allow to act for 3 minutes.
7. Gently rinse each slide with distilled or running water
8. Repeat decolorization if macroscopically visible stains are still visible.
9. Flood smear with 0.5% Potassium permanganate counterstain solution for 1 minute. Time is critical because counterstaining for a longer time may quench the acid-fast bacilli fluorescence.
10. Gently wash off the counter stain with distilled or running water
11. Stand the slide on its edge to drain, and air dry on the slide rack out of the direct sunlight and examine immediately.

Examination

1. Keep stained smears in the dark (box or folder) till reading, and read as soon as possible (within 24 hours) since fluorescence fades quickly out of the box.
2. Use 20x for scanning and 50x for confirmation. Scan the stained smear systematically from one side to the other.
3. One length has to be scanned before reporting a negative, (note that the surface observed by 20x will correspond to 3 streaks by the high power objective if using ZN and by 50x will correspond to 2 streaks by the high power objective if using ZN)
4. Acid-fast bacilli appear bright yellow against the dark background material.
5. With good staining (always check first a freshly stained positive control), there may still be fluorescing (sometimes green) artifacts, which do not have the typical shape. Also, non-fluorescing bacillary shapes must be considered as artifacts. In case of doubt, change to a higher magnification than that used for scanning (i.e., use 50X objective).
6. Store the slides in a slide box according to the Laboratory Number as they will be needed for EQA.

Note: Acid-fast bacilli appear bright yellow against a dark background. Report as positive for AFB if at least one acid-fast bacillus was seen in a well-stained smear, even if you think they might be mycobacteria other than tubercle bacilli. Tubercle bacilli are quite variable in shape,

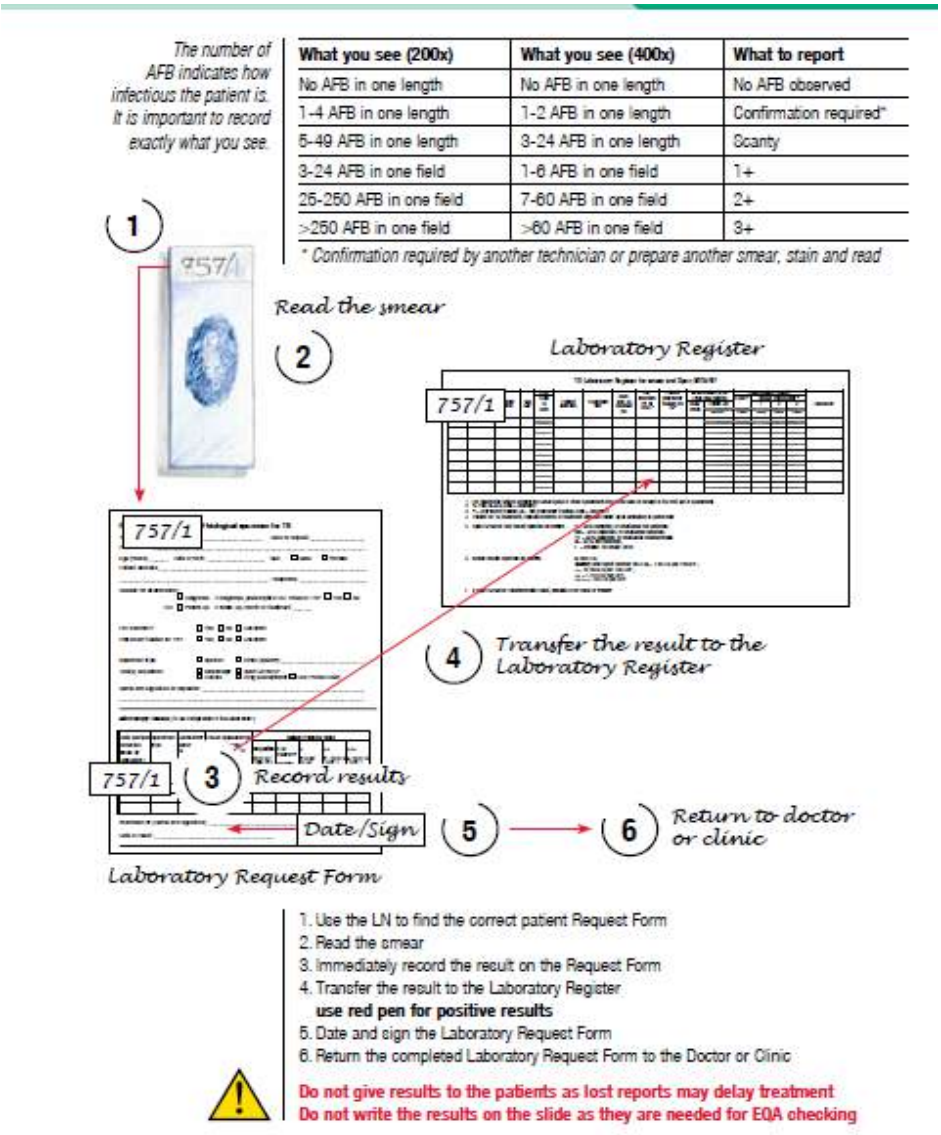
from very short fragments to elongated types. They may be uniformly stained with one or many gaps, or even granular. They occur singly or in small groups (coded), and rarely in large clumps. The typical appearance of bacilli is usually rather long and slender, straight or slightly curved rods.

Reporting Fluorescent Microscopy Results

AFB in one length at 200 x magnifications, or 1-2 in one length at 400 x magnifications should be confirmed by:

a) Viewing additional fields.
b) Have another technician check the AFB morphology or collect another sputum sample.

Confirmation of FM low-positive smears by re-staining with ZN should not be done



Quality control and evaluation of smear quality

The purpose of quality control (QC) is to ensure the accuracy and reliability of test results. Quality control checks the quality of staining solutions, the absence of reagent contamination, and the accuracy of the examination procedure.

Technicians preparing new staining solutions are responsible for QC before using positive and negative control smears. Validate the control smears (both positive and negative).

Internal QC of freshly made staining solutions

a) Prepare batches of control slides from suitable sputum samples homogenized after liquefaction by standing overnight at room temperature. These are negatives that have been thoroughly examined, and low positive (1+, 10–99 AFB/100 fields).

Prepare at least 20 smears of each batch of control (positive and negative) smears, as nearly identical in size and thickness as possible, giving each series the same QC identification number. Check 2–3 smears of each batch after good staining.

Check every newly prepared staining solution with unstained control smears, using at least one positive, with a known approximate number of AFBs, and one negative slide.

b) Unacceptable control results include the following:
    - Positive control AFB are not stained bright yellow or are too few.
    - Negative control remains bright yellow after decolorization.
    - The background is too dark or contains too many fluorescent artifacts.
    - Negative control shows AFB.

Internal QC indicators

Monitor laboratory performance by monthly counts, plotted on a graph of:

a) Number of smears,
b) Positivity rate,

These indicators provide an early warning of problems and signal the need for corrective actions.

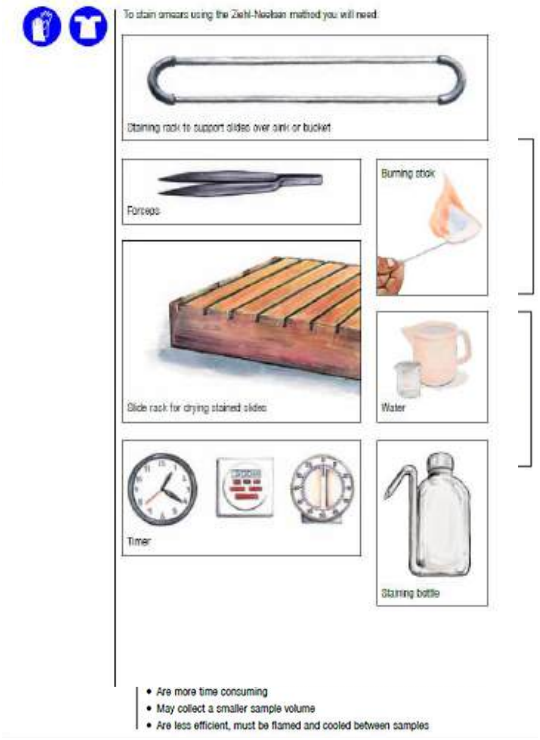
Prevention of false-positive results:

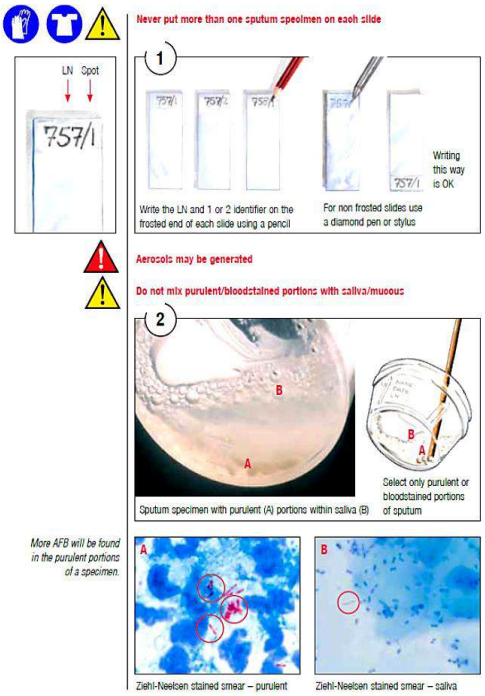
a) Do not re-use of sample containers or positive slides
b) Do not use contaminated water for preparing stains. Water may contain environmental mycobacteria
c) Do not use scratched slides
d) Wipe off objective lens between examination of adjacent slides;
e) Decolorize adequately
f) Do not confuse artifacts or poorly filtered stains for AFB
g) Do not use microscope in poor condition or poorly adjusted: interpreting glitter as AFB;

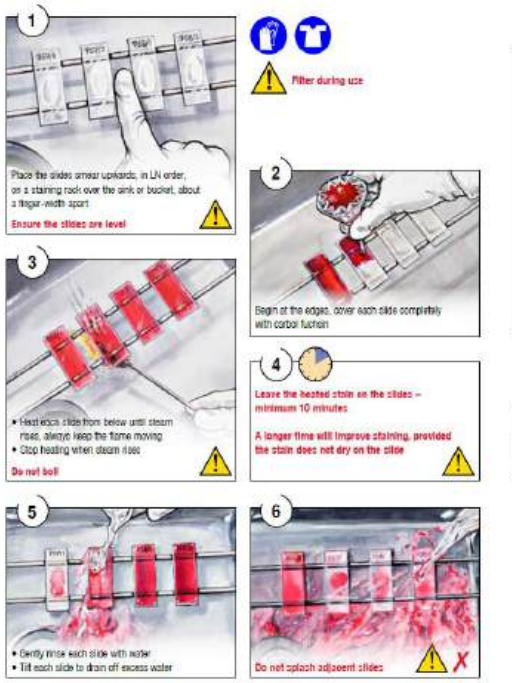
Prevention of False-negative results

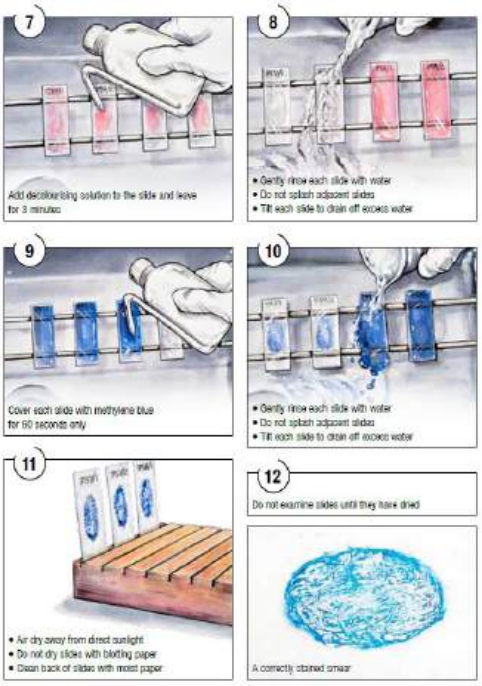
a) Do not use poor-quality of sample
b) Take a proper portion of the sample for smear preparation
c) Do not excessively decolorize
d) Do not use poorly prepared stain solution
e) Do not over/understain the smears
f) Do not overheat while fixing
g) Do not read less than one length
h) Do not expose the slides to daylight for too long
i) Do not spend too long an interval between staining and reading, particularly if slides were poorly stained or not kept in the dark

Smear Preparation requirements, ZN Staining Procedure are all described in the pictures shown below









Smears must be consistently and systematically examined to ensure a representative area of the smear is reported.

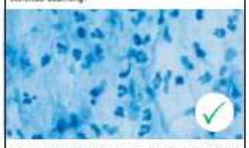
1

- Check the smear is facing upwards
- Apply one drop of immersion oil
- The drop must fall freely onto the smear so that the oil applicator does not become contaminated with TB organisms

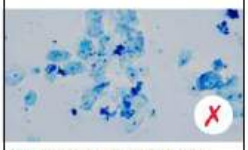
Never allow the oil applicator to touch the slide

2

Use the 10X objective to focus the first smear, avoiding the oil drop. Scan the smear, looking for purulent or mucoid material. Where the smear is too thick, too thin, or contains epithelial cells only, move up or down to find purulent or mucoid material, continue scanning.



Inflammatory cells (high power) – look for areas like this



Avoid areas containing epithelial cells (low power)

3

Carefully rotate the 100X oil objective lens over the slide

4

Carefully adjust the fine focus until cells are sharp

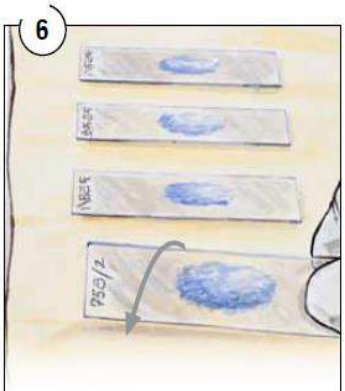
Never allow the lens to touch the glass slide

5

Direction of traversing the stained slide

Examine at least 100 high power fields (one length) before recording a negative result
You should take approximately 5 minutes to read a negative smear

6



Place the slides smear down on a clean piece of paper, leave overnight

Avoid contamination, always use a clean piece of toilet paper

7

✓ Store the slides in LN order in a closed box. They will be needed for EQA

✗ Do not write the result on the slide

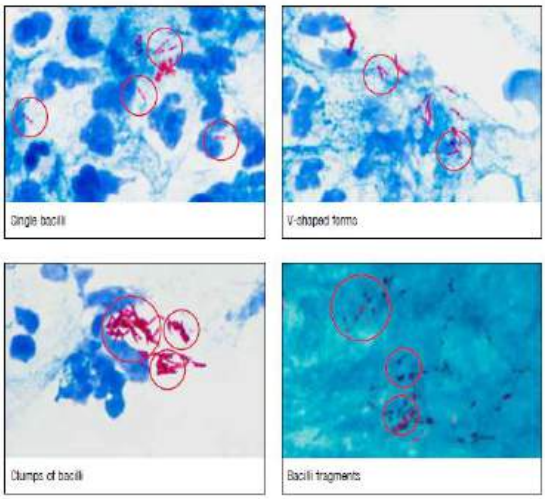
✗ Do not treat slides with xylene

8

Wipe the microscope lens gently with tissue paper to remove immersion oil after each positive slide and when you have finished examining a batch of slides (for cleaning agents see page 38)

- Viewed with an oil immersion lens, AFB are red, slender rods, sometimes with one or more granules
- Tubercle bacilli may occur singly, as V-shaped forms, or as clumps of bacilli
- Report fragments of bacilli – often seen during treatment

Typical morphological characteristics of *Mycobacterium tuberculosis*



Where possible, all positive smears should be reviewed by another technician

Appendix 2: Preparation of Ziehl-Neelsen stains

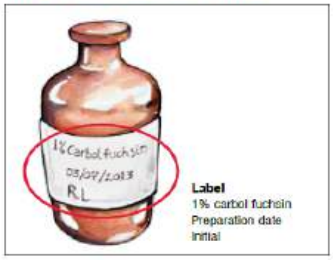
Stain

Carbol fuchsin – 1.0%		**Grade**
Basic fuchsin powder	10g	Certified
Ethanol (or methanol)	100ml	Technical
Phenol crystals*	50g* *use colourless not tinted crystals*	Analytical
Distilled water	900ml	

Phenol crystals and vapour are corrosive, toxic and may cause burns Use care, prepare in a well ventilated area

Preparation
1. Add 100ml of ethanol (or methanol) to a one litre glass flask
2. Add 50g of phenol crystals and dissolve
3. Add 10g of basic fuchsin powder
4. Mix well until dissolved
5. Add distilled water to make one litre
6. Label the bottle – **"1% carbol fuchsin", date and initial**
8. Store in a dark bottle in a cupboard at room temperature (expiry 12 months)

Wash your hands after preparing reagents



Label
1% carbol fuchsin
Preparation date
Initial

Perform a Quality Control check and record results in the QA log book
Filter solution at time of use

Decolourising solution

Always add the acid to ethanol or water. Solutions will generate heat.

25% H_2SO_4		**Grade**
Concentrated sulphuric acid (H_2SO_4)	250ml	Technical
Distilled water	750ml	

Preparation
1. Carefully add the H_2SO_4 to the water
2. Label the bottle **"25% H_2SO_4", date and initial**
3. Store in a dark bottle in a cupboard at room temperature (expiry 12 months)

3% HCl in ethanol (acid alcohol)		**Grade**
Fuming hydrochloric acid (HCl)	30ml	Technical
95% ethanol	970ml	Technical

Preparation
1. Carefully add the HCl to the ethanol
2. Label the bottle **"3% HCl in ethanol", date and initial**
3. Store in a dark bottle in a cupboard at room temperature (expiry 12 months)

6% HCl		**Grade**
Fuming hydrochloric acid (HCl)	60ml	Technical
Distilled water	940ml	

Preparation
1. Carefully add the HCl to the water
2. Label the bottle **"6% HCl", date and initial**
3. Store in a dark bottle in a cupboard at room temperature (expiry 12 months)

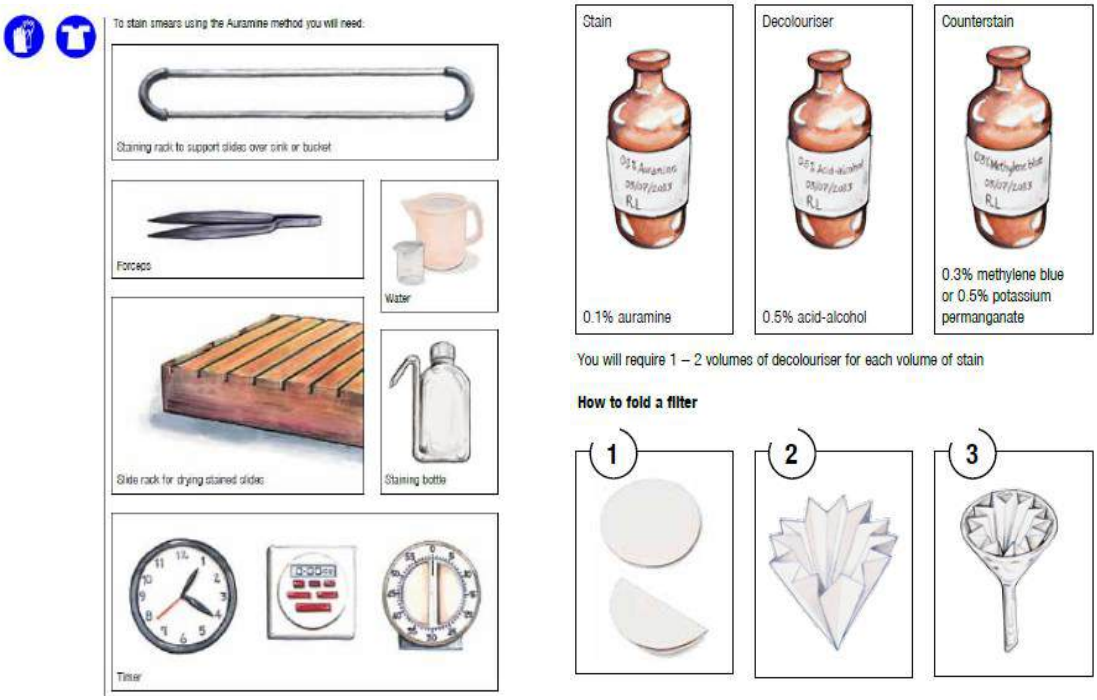
Counterstain

0.1% methylene blue	
Methylene blue chloride	1.0g
Distilled water	1000ml

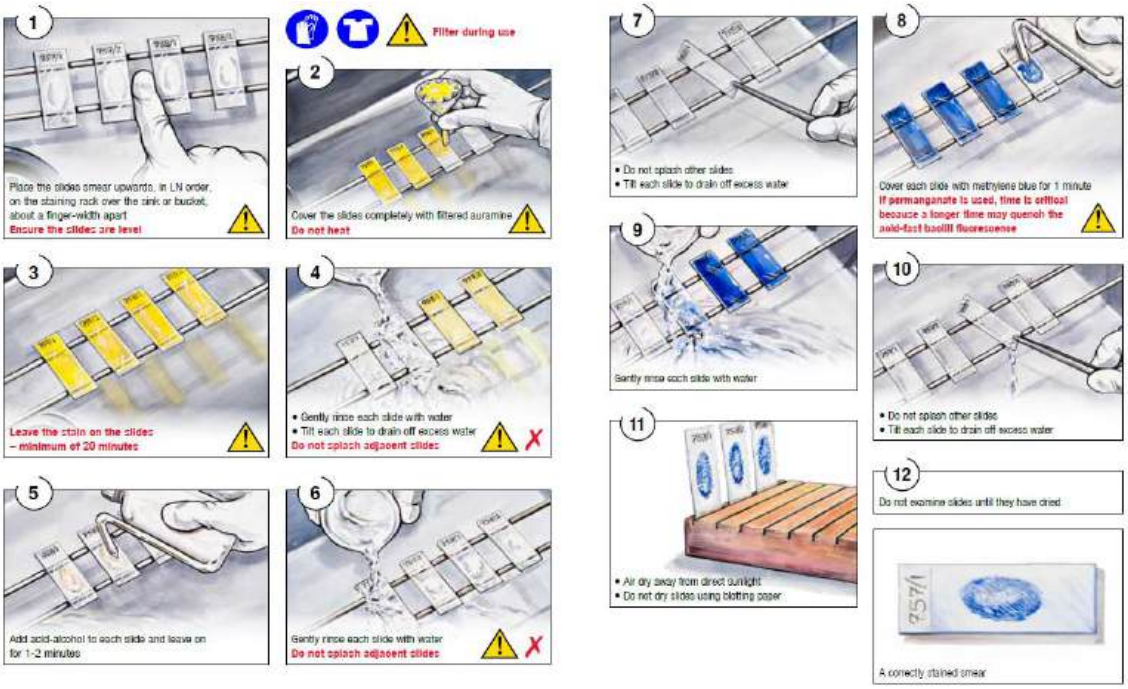
Preparation
1. Dissolve the methylene blue chloride in distilled water
2. Label the bottle – **"0.1% methylene blue", date and initial**
4. Store in a dark bottle in a cupboard at room temperature (expiry 12 months)

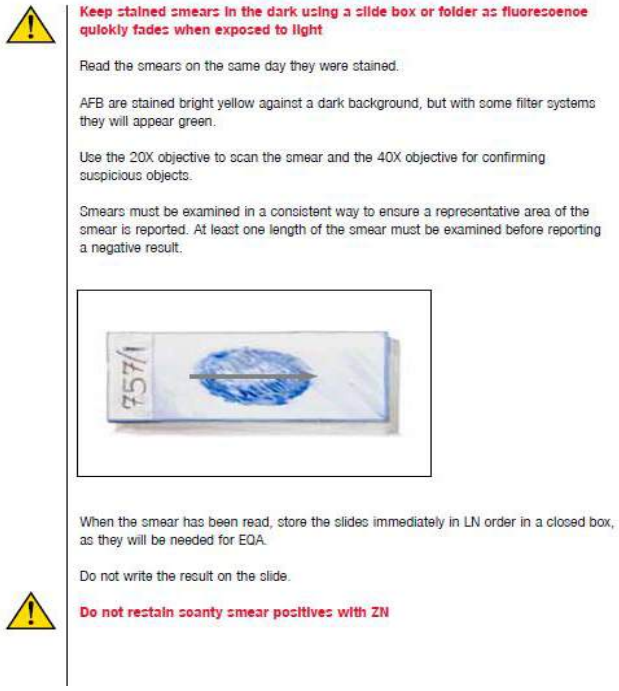
Perform a Quality Control check and record results in the QA log book.

Appendix3 Fluorescent Microscopy Staining



Auramine Staining method





Keep stained smears in the dark using a slide box or folder as fluorescence quickly fades when exposed to light.

Read the smears on the same day they were stained.

AFB are stained bright yellow against a dark background, but with some filter systems they will appear green.

Use the 20X objective to scan the smear and the 40X objective for confirming suspicious objects.

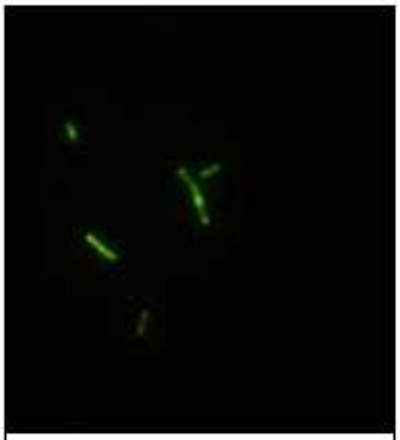
Smears must be examined in a consistent way to ensure a representative area of the smear is reported. At least one length of the smear must be examined before reporting a negative result.

When the smear has been read; store the slides immediately in LN order in a closed box, as they will be needed for EQA.

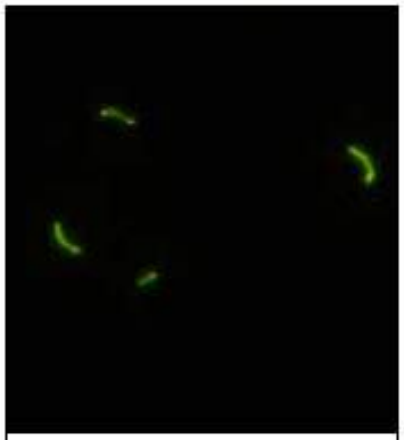
Do not write the result on the slide.

The typical appearance of AFB is a long, slender, slightly curved rod, but variable in shape and staining intensity

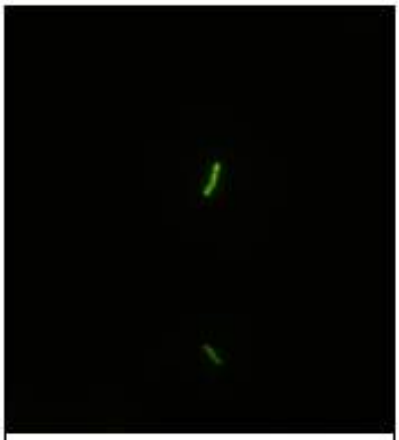
They may be uniformly stained or may contain one or more gaps, or have a granular appearance. AFB occur singly, in small groups containing a few bacilli, or more rarely, as large clumps.



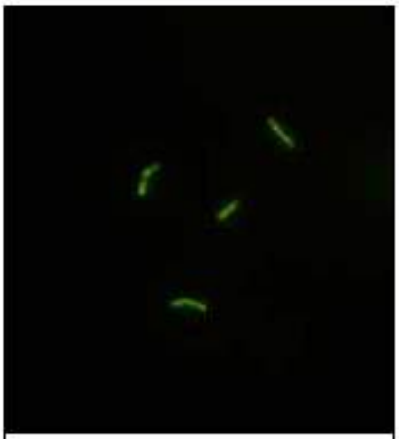
Long and slender



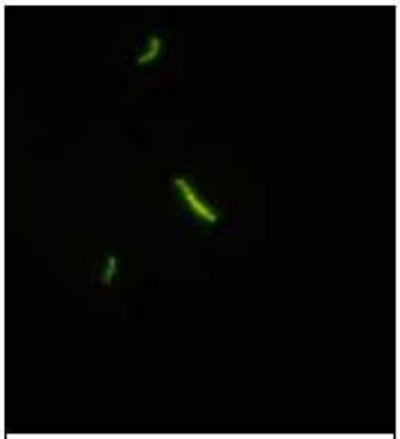
Slightly curved



Uniformly stained



One or more gaps



Granular

Appendix 4 : Preparation of Fluorescent Microscopy stains

Stain

Auramine is a potential cancer causing agent – always wear gloves and clean any spills immediately

Phenol crystals and vapour are corrosive, toxic, and may cause burns; avoid contact with skin and mucous membranes, prepare in a well ventilated area

0.1% Auramine		Grade
Auramine	10.0g	Certified
Ethanol (denatured) or methanol	1000ml	Technical
Phenol crystals*	30g* *use colourless not tinted crystals*	Analytical
Distilled water	900ml	

Preparation
To ensure solutions are fresh, laboratories examining low numbers of smears should prepare smaller volumes.

Solution A
1. Add 1000ml of ethanol (or methanol) to a one-litre glass flask
2. Add 10.0g of auramine powder, mix until dissolved completely

Do not use heat since this can inactivate the auramine
3. Label **"1.0% auramine in alcohol", date and initial**
4. Store in a dark bottle in a cupboard at room temperature (expiry 12 months)

Solution B
1. Dissolve 30g of phenol crystals in 900ml distilled water, mix
2. Label the bottle **"3% phenolic solution for auramine", date and initial**
3. Store in a dark bottle in a cupboard at room temperature (expiry 12 months)

Preparation of 0.1% auramine solution
1. Add 50ml of solution A (1% auramine in alcohol) to a 500ml dark glass bottle
2. Add 450ml of solution B (phenolic solution for auramine) and mix
3. Label the bottle **"0.1% auramine", date and initial**
4. Store in a cupboard at room temperature (expiry 2 months)

Filter auramine solution when applying to smears or filling bulk staining containers.

Wash your hands after preparing reagents

Perform a Quality Control check and record results in the QA log book.

Correctly prepared auramine is a rich golden colour – discard if pale

Decolouriser

Always add the acid to ethanol. Solutions will generate heat

0.5% acid-alcohol		**Grade**
Fuming hydrochloric acid	5ml	Technical
Ethanol (denatured) or methanol	1000ml	Technical

Preparation

1. Carefully add the hydrochloric acid to the alcohol
2. Label the bottle **"0.5% acid alcohol"**, **date and initial**
3. Store in a dark bottle in a cupboard at room temperature (expiry 12 months)
4. Perform QC and record in the QA log book

1% HCl in 10% alcohol in water		**Grade**
Fuming hydrochloric acid	10ml	Technical
Ethanol (denatured) or methanol	100ml	Technical
Distilled water	890ml	

Preparation

1. Carefully add the alcohol (or methanol) to the distilled water
2. Carefully add the hydrochloric acid to the 10% alcohol (or methanol) in water
3. Label the bottle **"1% HCl in 10% alcohol in water"**, date and initial
4. Store in a dark bottle in a cupboard at room temperature (expiry 12 months)
5. Perform QC and record in the QA log book

Counterstain

Two counterstains are described; 0.3% methylene blue (preferred) is a true counterstain, whilst 0.5% potassium permanganate acts as a quenching agent.

The choice of counterstaining solution depends on the microscope system used: permanganate produces a very dark background in some systems, making it hard to keep focus. If this occurs, then 0.3% methylene blue is a better choice counterstain, although there is slightly less contrast.

0.3% methylene blue		**Grade**
Methylene blue	3.0g	Analytical
Distilled water	1000ml	

Preparation

1. Add the methylene blue to the distilled water
2. Label the bottle **"0.3% methylene blue"**, **date and initial**
3. Store in a dark bottle in a cupboard at room temperature (expiry 12 months)
4. Perform QC and record in the QA log book

Appendix 5: GeneXpert Testing Standard Operating Procedure

For Processing Sputum

M Principle

The GeneXpert platform is a fully automated nested real-time PCR system, that detects MTB complex DNA and Rifampicin resistance-associated mutations in the rpoB gene.

To improve assay sensitivity for the detection of M. tuberculosis, the Ultra assay incorporates two different multi-copy amplification targets (IS6110 and IS1081).

It also has a larger DNA reaction chamber than Xpert MTB/RIF (50 PCR reaction in Ultra versus 25 µl in Xpert MTB/RIF).

Ultra also incorporates fully nested nucleic acid amplification, more rapid thermal cycling, and improved fluidics and enzymes.

This has resulted in Ultra having a limit of detection (LOD) of 16 bacterial colony-forming units (cfu) per ml

To improve the accuracy of rifampicin resistance detection, the Ultra incorporates melting temperature-based analysis instead of real-time PCR. Specifically, four probes identify rifampicin resistance mutations in the rifampicin resistance determining region of the rpoB gene by shifting the melting temperature away from the wild-type reference value.

Mycobacterium tuberculosis (MTB) complex detection with Ultra is defined as one or both of the probes that detect the multi-copy targets are positive with cycle thresholds (Cts) less than 37 cycles and at least two rpoB probes with Cts less than 40 cycles.

Ultra uses semi-quantitative categories such as high, medium, low and very low as well as the addition of another semi-quantitative category "*trace*" that corresponds to the lowest bacillary burden for MTB detection.

The "MTB Detected, trace", is referred to as a "trace call".

MTB is reported as NOT detected if neither of the multi-copy target probes are positive and the sample processing control is positive with a Ct less than 35 cycles.

For rifampicin resistance detection, rifampicin resistance is considered absent if MTB is detected (not trace) and all four rpoB probes have identifiable melt temperature ® peaks in the wild-type profile.

Rifampicin resistance is detected if MTB is detected (not trace) and all four rpoB probes have identifiable Tms and at least one of the rpoB probes has a Tm mutant profile.

If MTB is detected with a "trace call", then no interpretation can be made regarding rifampicin resistance and results are reported as MTB detected, trace, RIF indeterminate.

Following a 3-step sample preparation procedure in the laboratory, the sample is transferred into the Xpert cartridge and entered the GeneXpert instrument. By starting the test on the system software, the GeneXpert automates all the following steps, including sample work-up, nucleic acid amplification, detection of the target sequence and result interpretation.

The primers in the Xpert MTB/RIFUltra assay amplify a portion of the rpoB gene containing the 81-base pair "core" region. The probes can differentiate between the conserved wild-type sequence and mutations in the core region that are associated with RIF resistance. Furthermore, the assay includes on-board internal controls for each sample and they are; sample processing control (SPC) and *Probe Check Control (PCC). SPC is used* to control for adequate processing

of the target bacteria and to monitor the presence of inhibitor(s) in the PCR reaction. A Probe Check Control (PCC) verifies reagent rehydration, PCR tube filling in the cartridge, probe integrity, and dye stability.

It is important to monitor errors ®nd invalid results to ensure timely corrective actions. The MTB/RIF Ultra test is recommended for testing of sputum, stool, and other samples such as Aspirates, Gastric lavage, and Cerebrospinal fluid (CSF).

The sample reagent used for the preparation of samples for testing liquefies the sample and completely deactivates the bacilli.

Method

a) Start-up the GeneXpert instrument:
b) Before start processing the sample, check that the GeneXpert instrument is functioning and the modules are available
c) Turn on the computer, and then turn on the GeneXpert Dx instrument.
d) On the Windows desktop, double-click the GeneXpert Dx shortcut icon.
e) Log on to the GeneXpert Dx System software using your user's name and password.

Preparation of sputum for testing

a) Disinfect the working area.
b) Label each Xpert MTB/RIF Ultra cartridge with the sample ID. Do not put the label on the lid of the cartridge or obstruct the existing 2D barcode on the cartridge. Write on the sides of the cartridge or affix ID label.
a) Leave the sample in the leak-proof sputum collection container.
b) Unscrew the lid of the sputum collection container, add Sample Reagent 2:1 (v/v) to the sample and close the lid again.
c) Shake vigorousl– 10 - 20 times.

Note: One back-and-forth movement is a single shake.

d) Incubate for 5 minutes at room temperature.
e) Shake the sample again vigorously 10 – 20 times.
f) Continue incubation for another 10 minutes.

Note: Samples should be liquefied with no visible clumps of sputum. If there are still clumps of sputum, shake again vigorously, and incubate for another 5 min .

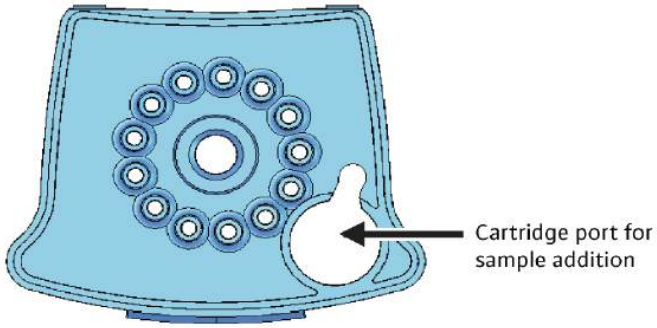


Figure 1: Xpert MTB/RIF Ultra (Top view)

Preparing the Cartridge

a) Start the test within 30 minutes of adding the sample to the cartridge.
b) Using the sterile transfer pipette provided, aspirate the liquefied sample into the transfer pipette until the meniscus is above the minimum mark (= 2ml).
c) Open the cartridge lid.
d) Transfer the sample into the open port of the Xpert MTB/RIF Ultra cartridge (Fig. 1).
e) No bubbles must be created when transferring the sample into the cartridge as this can lead to an error (no result).
a) Dispense slowly to minimize the risk of aerosol formation.

b) Close the cartridge lid. Make sure the lid snaps firmly into place.
c) The remaining liquefied sample may be kept for up to 12 h at 2-8 C should repeat testing be required (e.g. no results or error results).

Start the test on the GeneXpert instrument

a) In the GeneXpert Dx System window, click "CREATE TEST." The Scan Cartridge Barcode dialog box appears.
b) Scan the barcode on the Xpert MTB/RIF Ultra cartridge.
c) The Create Test window appears.
d) Using the barcode information, the software automatically fills the boxes for the following fields: Select Assay, Reagent Lot ID, Cartridge SN, and Expiration Date.
e) In the Sample ID box, scan or type the sample ID. Make sure you type the correct sample ID. The sample ID is associated with the test results and is shown in the "View Results" window and all the reports.
f) Click "Start Test."
g) In the dialog box that appears, type your password.
h) Open the instrument module door with the blinking green light and load the cartridge.
i) Close the door.
j) The test starts and the green light stops blinking.
k) When the test is finished, the light turns off?
l) Wait until the system releases the door lock at the end of the run, then open the module door and remove the cartridge.
m) Dispose of used cartridges in the appropriate sample waste containers according to your institution's standard practices.

Reading, recording and reporting

a) In the GeneXpert Dx System window, click "VIEW RESULTS" on the menu bar. The View Results window appears.
b) If the software reports "Error", "Invalid" or "No result", repeat the test following the SOP.
c) Should the test again show "Error", "Invalid" or "No result", proceed to the troubleshooting manual.

Recording and Reporting

a) Results must be recorded in a TB laboratory register (HMIS TB 010) following the guide in the register. Use red ink for positive results. Result reports must be provided *as soon as possible*.
b) Reporting of Xpert MTB/RIF Ultra results should be reported through DHIS-2 using reports such as; HMIS 033b, HMIS 105 and HMIS 106a. Additionally, LabXpert DS, is used to automatically report Xpert MTB/RIF Ultra data.
c) GeneXpert utilization: The target for monitoring GeneXpert utilization is 3 samples (runs) per module per day. Consider 5 days in a week, 21 days in a month, 63 days in a quarter and 252 days in a year.

Troubleshooting: Check chapter 11 in the GeneXpert operator manual provided on the desktop of the GeneXpert computer.

Maintenance and Service of GeneXpert Instrument

a) Proper functioning and safe operating of the GeneXpert system requires regular disinfection of the instrument surfaces and such parts of it that potentially encounter

infectious materials as the cartridge bay interior. Check the module performance with Xpert®Check on an annual basis. Required materials for maintenance

- 1:10 dilution of household chlorine bleach* (used within 1 day of preparation)
- 70% ethanol solution
- Lint-free wipes / non-cotton swabs
- Brushes (when provided with system/available upon request)
- Disposable gloves
- Clean water
- Eye protection
- Replacement fan filters (available upon request)

Frequency of maintenance

a) *Daily maintenance:* Discard cartridges, clean the bench work and keep the doors upright
b) *Weekly maintenance:* Reboot the system i.e Power down the GeneXpert computer, Power down the GeneXpert Instrument, turn on the GeneXpert Instrument and Turn on the on GeneXpert computer. Note: Whenever the GeneXpert system is not used: Switch it off, cover the GeneXpert with the GeneXpert dust cover (provided with GX-IV only)
c) *Monthly maintenance*: clean instrument surfaces, cartridge bays, plunger rods, module PCR tube slots, fan filter and archive and purge(delete) run
d) *Yearly maintenance* Check the module performance with Xpert®Check

Disinfection of the instrument surfaces

a) Perform cleaning monthly
b) Moisten a lint-free wipe with 70% ethanol solution
c) Wipe all outside surfaces of the instrument
d) Wipe table surfaces around the instrument

Note: Use one lint-free wipe for the instrument and one for the work area. Discard used wipes according to your standard laboratory procedure

Disinfection of the cartridge bay

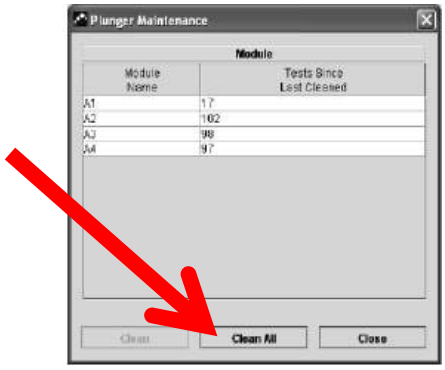
a) Perform cleaning monthly
b) Moisten a lint-free wipe with a 1:10 solution of household chlorine bleach
c) Wipe the inside of the cartridge bay, the inside of the door and the top lip of the door
d) Wait 2 minutes
e) Repeat step a to c 3 times
f) Moisten a lint-free wipe with 70% ethanol solution
g) Wipe the parts described above with the ethanol solution

Note: Change lint-free wipes frequently while wiping. Discard used wipes according to your standard laboratory procedure

Disinfection of the plunger rod

h) Perform cleaning monthly
i) Remove cartridges from the modules you want to clean
j) In the GeneXpert Dx System window, click Maintenance on the menu bar. The Maintenance window appears:

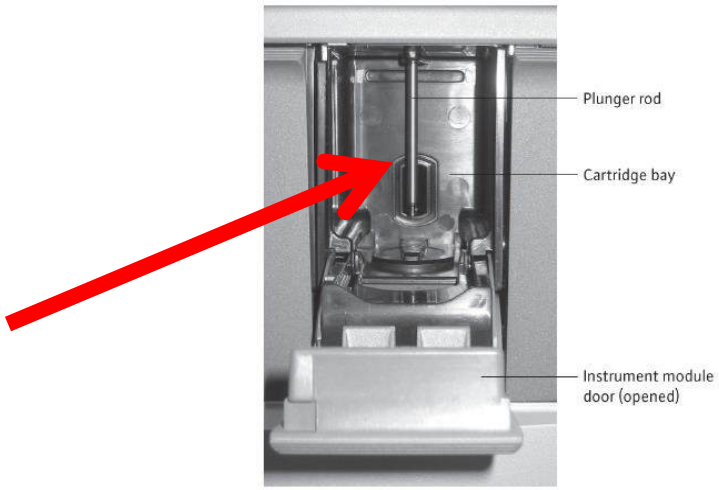
k) On the Maintenance menu, click Plunger Maintenance. The Plunger Maintenance; the dialog box appears:



l) In the Module table, select the module you want to clean, and then click Clean or select Clean All to clean all modules simultaneously. The Plunger Cleaning dialog box appears:

m) Follow the directions in the Plunger Cleaning dialog box, then click OK

n) In the Plunger Maintenance dialog box, the *clean* button changes to Move Up (if you clicked Clean All button, it changes to Move Up All). In the instrument, the plunger rod in the selected module (or all modules if you clicked Clean All button) lowers into the cartridge bay:



o) Moisten a lint-free wipe with a 1:10 solution of household chlorine bleach
p) After the plunger rods are lowered, gently wipe the plunger rods
q) Wait 2 minutes (never longer than 5 minutes)
r) Repeat step o to q 3 times
s) Moisten a lint-free wipe with 70% ethanol solution
t) Wipe the plunger rod with the ethanol solution

Note: Change lint-free wipes frequently while wiping. Discard used wipes according to your standard laboratory procedure

u) In the Plunger Maintenance dialog box, click Move Up (or Move Up All). The plunger rod moves back up to its resting position
v) Click Close to dismiss the Plunger Maintenance dialog box
w) After cleaning the plungers, clean module PCR slots (Get dry PCR slot cleaning brush, make sure that all the bristles are fully inserted (up to the shoulder of the plastic shank of the brush), brush the inside of the slot with up and down movements, Rotate the brush abellipprox. 180^0 and back, then repeat the previous step 2 times, Clean each module for at least 30 sec).

Cleaning of the filter (only for GX with white cover)

a) Unplug the machine (Internet and power supply cables)
b) When filter is positioned behind the back panel: Turn off the System and remove the 4 screws on the rear grey panel, Tilt back the panel. When filter is positioned on the outside: Turn off the System, Unclip 4 clips one by one
c) Remove the filter (sponge)
d) Gently, wash in soapy water
e) Rinse in running water
f) Dry the filter (e.g. pressing in between absorbent paper/filter paper)
g) Place it back on the back cover.
h) Place back the back cover and pay attention to the position of the filter (fan should not be visible)
i) Screw the cover

Yearly Maintenance

j) Check the module performance with Xpert®Check
k) All modules must be verified every year with Xpert Check cartridges
l) Performed by end users or by Cepheid approved Service Engineers

Archival of results

a) Test data should be archived as often as possible or according to your lab protocols. This will help to keep the GeneXpert® Dx software efficient.
b) In the GeneXpert® Dx software, click on the Database menu.
c) On the dropdown list, click on "Archive test." An archive test dialog box appears.
d) Highlight the tests you want to archive and proceed to do so.

Standard operating procedure for processing extra-pulmonary specimens (CSF, stool, lymph nodes and other tissues) for Xpert MTB/Rif Ultra assay

Lymph nodes and other tissues

a) Using a sterile pair of forceps and scissors, cut the tissue specimen into small pieces in a sterile mortar (or homogenizer or tissue grinder).
b) Add approximately 2 ml of sterile phosphate buffer (PBS).
c) Grind the solution of tissue and PBS using a mortar and pestle (or homogenizer or tissue grinder) until a homogeneous suspension has been obtained.
d) Use a transfer pipette to transfer approximately 0.7 ml of the homogenized tissue specimen to a sterile, conical screw-capped tube.
e) NOTE: Avoid transferring any clumps of tissue that have not been properly homogenized.
f) Use a transfer pipette to add a double volume of the MTB/RIF Ultra Sample Reagent (1.4 ml) to 0.7 ml of homogenized tissue.
g) Vigorously shake the tube 10 to 20 times or vortex for at least 10 seconds.
h) Incubate for 10 minutes at room temperature, and then shake the specimen vigorously again for another 10–20 times or vortex for at least 10 seconds.
i) Incubate the specimen at room temperature for an additional 5 minutes.
j) Using a fresh transfer pipette, transfer 2 ml of the processed sample to the MTB/RIF Ultra cartridge.
k) Load the cartridge into the GeneXpert instrument following the manufac'urer's instructions.

Cerebrospinal Fluid (CSF)

a) The preferred processing method for CSF in MTB/RIF Ultra depends on the volume of specimen available for testing.

If there is more than 5 ml of CSF

a) Transfer all the specimens to a conical centrifuge tube, and concentrate the specimen at 3000 g for 15 minutes.
b) Carefully pour off the supernatant through a funnel into a discard can containing 5% phenol or other mycobacterial disinfectant.
c) NOTE: Concentrated CSF should be decanted within a biological safety cabinet
d) Re-suspend the deposit to a final volume of 2 ml by adding the MTB/RIF Ultra sample reagent.
e) Label an Xpert/MTB/RIF cartridge with the spe'imen's identification number.
f) Using a fresh transfer pipette, transfer 2 ml of the concentrated CSF specimen to the MTB/RIF Ultra cartridge.
g) Load the cartridge into the GeneXpert instrument following the manufac'urer's instructions.

If there is 1–5 ml of CSF

b) Add an equal volume of sample reagent to the CSF.
c) Add 2 ml of the sample mixture directly to the MTB/RIF Ultra cartridge.
d) Load the cartridge into the GeneXpert instrument following the manufac'urer's instructions.
e) If there is 0.1–1ml of CSF
f) Re-suspend the CSF to a final volume of 2 ml by adding the MTB/RIF Ultra sample reagent.
g) Add 2 ml of the sample mixture directly to the MTB/RIF Ultra cartridge.
h) Load the cartridge into the GeneXpert instrument following the manufac'urer's instructions.

If there is less than 0.1 ml

a) This is an insufficient sample for testing using the MTB/RIF Ultra assay.

Stool (Based on Simple One Step stool processing method)

Use the Bristol Stool Chart to categorise stool from Type 1 to Type 7

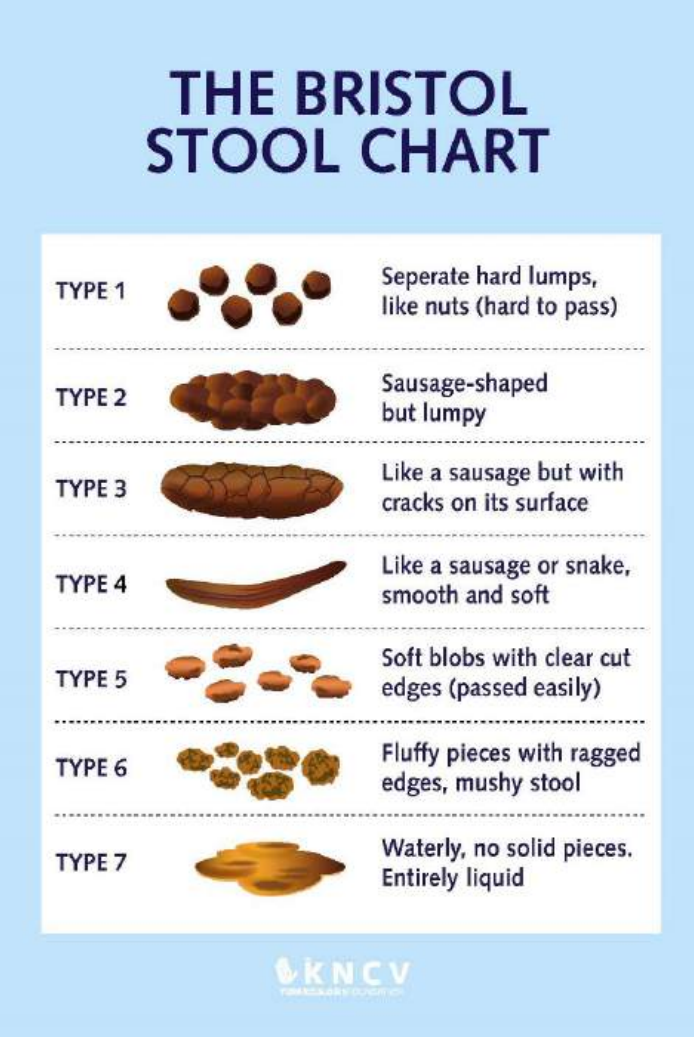
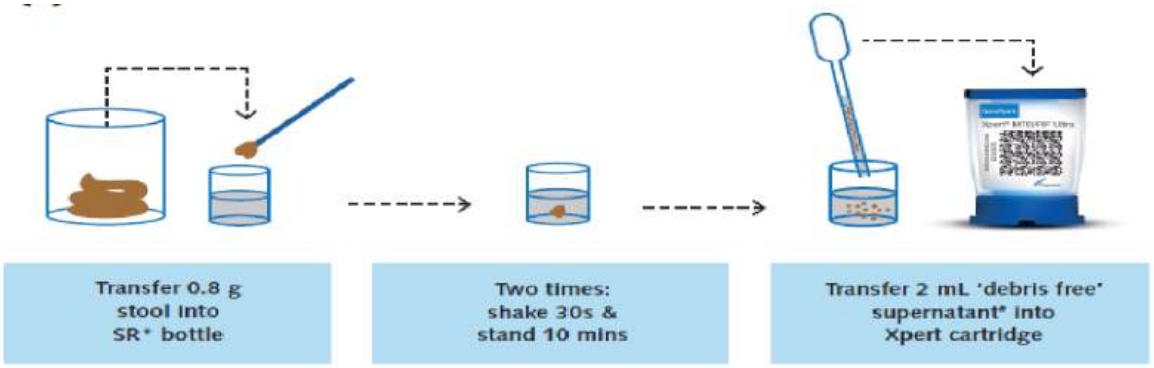
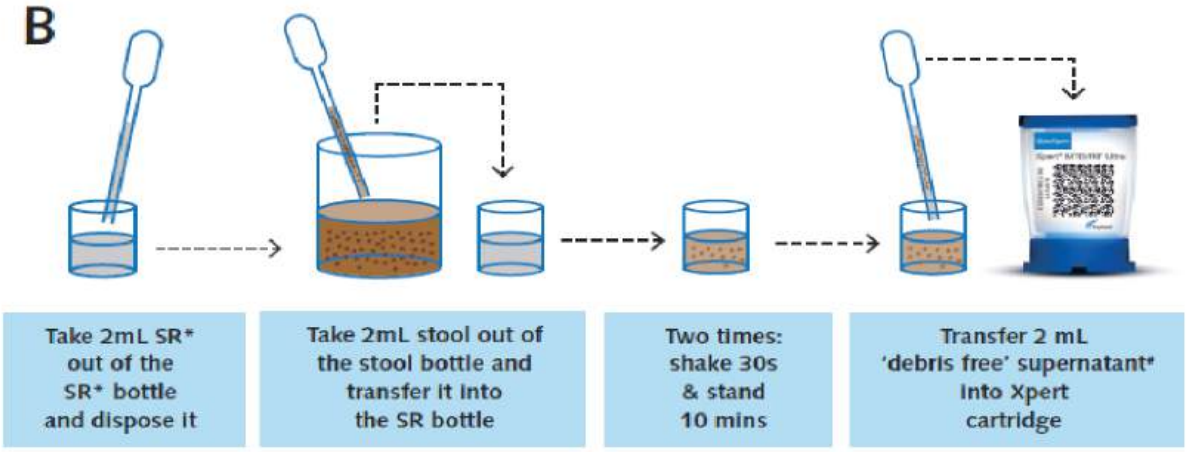


Figure 2: The Bristol Stool Scale, named after the University of Bristol where it was first described by Lewis and Heaton 21; from type 1, being the most solid, to type 7, being the most liquid.

Hard and soft stool



Liquid stool



Nasopharyngeal aspirate-same protocol as sputum

Appendix 3: Truenat testing standard operating procedure (sop) for processing sputum

Principle

The system uses room-temperature stable reagents and Truenat micro-PCR chips

Available chips:

a) **MTB:** amplifies a portion of the ribonucleoside-diphosphate reductase gene, nrdZ, and has a limit of detection (LOD) of about 100 colony forming units (CFU)/ml sputum sample
b) **MTB Plus (more sensitive):** amplifies a portion of the nrdZ gene as well as a portion of the multicopy IS6110 element and has a LOD of about 30 CFU/ml.
c) **MTB-RIF Dx:** If the Truenat MTB or MTB Plus test result is positive, an aliquot of the already extracted DNA should be loaded onto a MTB-RIF Dx chip. Mutations associated with RIF resistance are detected by a probe melt analysis of the real-time PCR products

Step 1: Sample preparation using the liquefaction and lysis buffers (Trueprep AUTO MTB Sample Pre-treatment Pack)
Step 2: Extraction and purification of DNA using the Trueprep AUTO v2 Universal Cartridge Based Sample Prep Kit and the Trueprep AUTO v2 Universal Cartridge Based Sample Prep Device

Step 3: PCR amplification and fluorescent probe-based detection of MTB using Truenat chips, a Truepet® 6µl Precision Micropipette and the Truelab micro–PCR Analyzer

Equipment

a) Trueprep® AUTO v2 Universal Cartridge Based Sample Prep Device.
b) Truelab Real Time micro–PCR Analyzer (can be Truelab® Uno Dx, Truelab® Duo or Truelab® Quattro)
c) Truelab micro–PCR Printer

Reagents and Consumables

Three packets include the reagents and consumables needed to run Truenat

a) Trueprep® AUTO MTB Sample Pre-treatment Pack: Its content are;
   - Graduated transfer pipettes (1 ml)
   - Lysis buffer bottle (2.5 ml of buffer)
   - Liquefaction buffer bottle
b) Trueprep® AUTO v2 Universal Cartridge Based Sample Prep Kit (for 25 or 50 tests): Its content are;
   - Reagent pack
   - Transfer pipettes (3 ml)
   - Cartridge pouch
     - Cartridge
     - Elute collection tube (ECT)
     - Transfer pipette
     - Label for ECT
c) **Truenat Chip Pack (for 5, 20, or 50 tests): The contents are;**
   - Truenat chip pouches
   - Microtube containing freeze-dried PCR reagents
   - Filter barrier pipette tip
   - Desiccant pouch

   Note: Truenat has three types of chips, so three different types of chip packs (For MTB, MTB Plus and MTB-RIF Dx)

Eligibility for Truenat test: See TB diagnostic algorithm

Type of Sample

Collect a minimum of 1 ml of sputum.

Storage of Samples

Same as for Xpert MTB/RIF Ultra

Method

Prepare Sample and Extract DNA.

Materials: Trueprep Auto v2, Trueprep® AUTO MTB Sample Pretreatment Pack\ and Supplies (Sample Prep Kit)

a) Wear the appropriate gloves for specimen handling
b) Collect 2-5ml adult pulmonary sputum sample in sputum cup and label with patient details
c) Add 2 drops of liquefaction buffer to the sputum cup
d) Close the cap and swirl gently to mix
e) Incubate for 10 minutes at room temperature. If the sample is not pipetteable after 10 minutes, incubate for another 5 minutes while swirling at 2-minute intervals
f) Transfer 0.5 ml of liquefied sputum sample into the lysis buffer bottle using a 1 ml transfer pipette
g) Add 2 drops of liquefication buffer into the lysis buffer bottle, swirl gently to mix and incubate for 3-5 minutes

h) Remove the cartridge from the pouch, label it and place it on the cartridge stand. Take out the elute collection tube (ECT) and label it appropriately. Keep it aside for later use. Keep the elute transfer pipette in the pouch for later use.
i) Transfer the entire contents of the lysis buffer tube to the sample chamber (black cap) of cartridge using 3 ml transfer pipette
j) Switch "on" the Trueprep® AUTO v2 device. Press "eject" button to open and gently pull out the cartridge holder
k) Place the cartridge in the tray in the orientation shown (Figure 7), and gently push to close the cartridge holder. Press "start."
l) The device will beep at the end of the DNA extraction process (20 minutes), and the cartridge holder will eject automatically.
m) Gently pull out the cartridge holder, remove cartridge, and place it on the cartridge stand.
n) Carefully pierce the elute chamber with the provided elute transfer pipette and transfer the entire elute into the ECT. Discard the transfer pipette and cartridge.

Note: 50 T Reagent Pack Replacement: change TruePrep Auto V2 reagent pack after completion of 50 extractions.

TRUENAT TEST PROCEDURES: Run a PCR TB Test

Materials: Truelab micro-PCR Analyzer (Uno, Duo or Quattro), Supplies (Chip pack)

a) Switch "on" the Truelab micro-PCR analyzer by pressing the red button in the back right corner for 2 seconds. LED will glow in Green (Figure 9). Wait for 30-50 seconds for "boot-up screen" to appear followed by "home screen."
b) Select USER ID, enter password. Press "Sign in" to Log in
c) Select test profile "MTB" or "MTB Plus". To confirm selection tap "PROCEED" and enter patient details (referred by, patient ID, gender, patient name & age)
d) Select sample type (sputum).
e) Press "START TEST" on the screen. Chip tray opens. "Please Load Sample" will appear. (Don't press "YES" until chip loading is complete.}
f) Open a TRUENAT™ MTB Plus chip pouch. Pull out the orange desiccant pouch and confirm that it is orange in color.
g) Gently take out the chip without touching white well portion and place it on the chip tray by aligning it in the slot provided
h) Open the master mix tube, discard the stopper, and place the tube in the microtube stand. Check for white cake at the bottom of the microtube.
i) Attach the 6ul micro tip provided in the pouch to the single push pipette.
j) Transfer 6ul of the elute from ECT into the master mix tube
k) Allow the master mix to stand for 30 SECONDS to get a clear solution. Do not mix by tapping, shaking or reverse pipette. Do not discard the pipette tip.
l) Transfer the elute from the master mix tube to the white reaction well of the chip. Avoid spillage of the clear solution outside the white reaction well. Discard the pipette tip and master mix tube.
m) Click "YES" on the device screen to start the test. The PCR will be completed in 35 minutes.
n) Tap the "Open/Close Tray" button to eject the chip tray and discard the used chip immediately after the reaction.
o) If MTB is detected (Figure 17) test the same elute for RIF resistance using the Truenat MTB RIF Dx chip as a follow-on test. The test takes about 55 minutes.
p) Optional: Press "Print" to print result page using Truelab® microPCR printer.

TRUENAT TEST PROCEDURES – Run a RIF Resistance Test

Materials: Truelab micro–PCR Analyzer (Uno, Duo, or Quattro), Supplies (Chip pack)

a) If MTB is detected in a sample, you should run a RIF resistance test.
b) A portion of the same DNA eluate can be used to test for RIF resistance using a Truenat MTB-RIF Dx chip.
c) Start by returning to Step 3 in the PCR TB test process and repeat for RIF-resistance
d) Select "MTB RIF" as the test type in the Truelab micro–PCR Analyzer.
e) RIF-resistance testing takes an additional 60 minutes

Reading, recording and reporting

If TB test result is MTB detected MTB detected results appear as follows: Truenat MTB: 'detected, Truenat MTB Plus: 'detected high,' 'medium,' 'low,' or 'very low and next Step is to conduct RIF resistance testing with Truenat MTB-RIF-Dx.MTB-RIF Dx test result can be RIF Resistance Detected or RIF resistance not detected

a) If the software reports "Error," "Invalid" or "No result," repeat the test following your SOP.
b) Should the test again show "Error," "Invalid" or "No result," proceed to troubleshooting manual.

Recording and Reporting

a) Results must be recorded in a TB laboratory register (HMIS TB 010) following the guide in the register. Use red ink for positive results. Result reports must be provided *as soon as possible*.
b) Reporting of Xpert MTB/RIF Ultra results should be reported through DHIS-2 using reports such as; HMIS 033b, HMIS 105 and HMIS 106a. Additionally, Data Connectivity solutions such as LabXpert DS, electronic case-based surveillance system (eCBSS) may be used to report Xpert MTB/RIF Ultra data.
c) Truenat utilization: The target for monitoring Truenat utilization is 3 samples (runs) per sample bay per day. Consider 5 days in a week, 21 days in a month, 63 days in a quarter and 252 days in a year.

Troubleshooting: Check the Troubleshooting, Alerts and Errors handout for Trueprep and Truelab

Quality control

- Maintain the instrument according to SOP.
- Validate results prior to reporting
- Monitor the errors and invalid results
- Waste management
- Manage waste as in Xpert MTB/RIF Ultra

Maintenance and Service of Truenat Equipment

Daily maintenance

a) Clean work area using 0.5% Hypochlorite.
b) Discard used chips and cartridges in 0.5% Hypochlorite solution.

Monthly maintenance

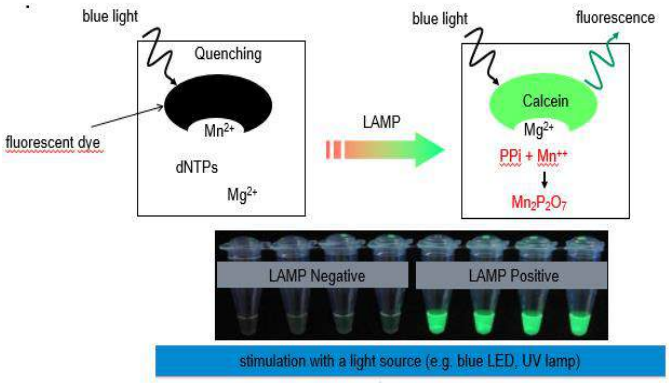
a) Disinfect instrument surfaces with 0.5% Hypo.
b) Clean Truelab bays with 70%IPA and allow to dry by keeping the bays open.
c) Temperature calibration using Calibration Chip provided for Truelab
d) Verification of the fixed 6µl pipette

As necessary

a) Flush protocol for the Trueprep instrument
b) Spillage tray replacement in Trueprep
c) Slider glass replacement in Truelab -indicate bay

Appendix 4: TB Loop-Mediated Isothermal Amplification (LAMP) testing standard operating procedure for processing sputum

Principle



The LAMP reaction begins with short single-stranded molecules called oligonucleotides or primers. These are designed to bind to the target DNA sequ–nce - in our case, regions in the DNA gyrase subunit B (gyrB) and Insertion sequence IS6110 of the MTBC genome. If mycobacteria DNA is present, one of the specially designed LAMP primers can anneal (bind or stick) to the complementary or matching DNA from the mycobacteria. This can occur because DNA is in dynamic equilibrium (constantly unfolding and refolding) when it reaches the reaction temperature. Primer binding initiates the process of DNA synthesis, whereby the Bst DNA polymerase enzyme generates new DNA that matches the mycobacteria DNA. As the DNA synthesis continues, some of the new DNA folds back on itself to fo“m a "stem”loop" structure that looks like a dumbbell. This structure is the starting point for the amplification cycle of LAMP. The loops on the dumbbell structure now act as additional binding sites for primers to initiate DNA synthesis. As more loops are created, there are more starting points for DNA synthesis as the LAMP reaction continues. The creation of multiple loops of DNA, which provide a starting point for additional DNA synthesis, is one reason LAMP is much faster than traditional PCR.

The visual detection under ultraviolet light is based on the presence of calcein. Before DNA amplification, calcein contained in the reagent is in its quenched state as it is bound to manganese ions. Upon the start of DNA amplification, pyrophosphate ions bind to manganese ions and calcein is released producing fluorescence. If we see fluorescence, the LAMP result is positive; if there is no fluorescence, the result is negative.

Figure 3: TB-LAMP showing fluorescence of positive samples

Equipment

a) HumaLoop T
b) HuMax ITA -optional

\

Figure 4: Image showing parts of the HumaLoop T

Reagents and Consumables

a) Extraction Kit: Contains; Heating Tubes (Sample lysis), Adsorbent Tubes (Removal of inhibitors) and Injection Caps (Elution of DNA) with shelf life of 24 months from

production, Storag at room temperature (2-30 °C) or refrigerator (2-8 °C) and operating temperature of 2-30 °C.

b) Detection Kits- Loopamp™ MTBC Detection Kit: Contains; Positive control (plasmid solution containing target sequences) & negative controls (water), LAMP reaction tubes with dried reagents (BST* DNA polymerase, deoxy nucleotid triphosphates (dATP, dCTP, dGTP,dTTP) magnesium sulfat, calcein, manganese chloride, Loopamp™ primers)and 30µl droppers

c) Pipette-60

Eligibility for Truenat test: See diagnostic algorithm

Type of Sample

Collect a minimum of 1ml of sputum. The technique uses 60µL of sputum collected in a sputum container. Sample can be transported at room temperature and send it to the laboratory on the day of sampling. The Sputum sample can be stored at +4°C for 3 days if the analysis is delayed.

Method

DNA extraction with PURE method

Materials

a) Permanent Marker
b) Disposable examination gloves (2 pairs per run)
c) 0.5% Sodium hypochlorite
d) Pipette-60 Set
e) LoopampTM PURE DNA Extraction Kit
f) Plastic Bag with zip

Preparation of workspace

a) Turn on HumaLoop T
b) Clean the bench with 0.5% sodium hypochlorite (both sample transfer area and amplification area)
c) Spread the solution on the surface and incubate for 5 minutes
d) Wipe the surface with dry paper, then wipe again with water to remove remaining sodium hypochlorite
e) All required materials must be placed into the cleaned area

Sputum transfer

a) Start working at amplification area!
b) Remove a sufficient number of Heating Tubes (Number of samples plus one for Negative Control) from the aluminium pouch by pouring. Do not grab into the bag! Place them into Heating Tube stand.
c) Label the lids of the Heating Tubes
d) Transfer the Heating Tube stand to sample transfer area
e) Open lid of the Heating Tube and place the lid on the tube. Tilt the sputum container diagonally. The sputum must be collected at one spot at the side of the container.
f) Confirm the 1st ^{an}d 2nd Stop of the plunger of Pipette 60- Set
g) Slowly aspirate 60µL of sputum (> 5sec). Use the most purulent portion of sputum, whenever possible. Check if the volume is within the indicated range. In case of sputum strands, rub the pipette tip against the bottom of the cup to cut the strands.

h) Transfer 60µL of sputum into the respective Heating Tube. Beware of sputum strands! Wash the pipette tip once with the Heating Tube solution, then check if the sputum has been released completely into the solution. Transfer 60µL of NC into the respective Heating Tube
i) Mix the Heating Tubes 3-5 times. Flick the Tube to collect the solution at the bottom of the tube.
j) Discard the first pair of gloves
k) Transfer the Heating Tube stand to the amplification area
l) Verify that the Heating block temperature has reached 90°C.

PURE Treatment

a) Verify that the Heating block temperature has reached 90°C.

b) Load the Heating Tubes into the heating block of HumaLoop T and close the lid. Press the Start/Stop button, to start 5 minutes of incubation.

c) Remove the Heating Tubes immediately after the incubation has finished. This is indicated by an audible signal. Let them cool down for 2 minutes, then mix again 3-5 times.

d) Tap the Adsorbent Tubes to loosen the white powder. Remove the cap of the Adsorbent Tube. DO NOT discard the cap! You can place it into the Heating Tube stand.

e) Connect the Heating Tube to the Adsorbent Tube by screwing to release the solution into the Adsorbent Tube.

f) Mix thoroughly! Powder remains, as well as pink or blue color indicate improper mixing. In this case, continue shaking and place them on the adsorbent tube tray.

g) Attach the cap to the nozzle of the Injection Cap. Avoid touching the nozzle!

h) Hold the Adsorbent Tube upside-down and connect the Injection Cap by pushing once and then screwing.

DNA amplification by MTBC Detection Kit

a) Place a sufficient amount of Reaction Tubes into the reaction tube stand. (One Reaction Tube per Adsorbent Tube plus one for the Positive Control). Use scissors for the transfer of the tubes and place them on the rack. After removing the Reaction Tubes close the aluminium pouch tightly!
b) Flick the tube to collect the solution at the bottom of the tube. Place it into the squeezing tool.
c) Remove the cap. Insert the complete nozzle into the Reaction Tube. Squeeze the Adsorbent Tube and dispense 30µL of DNA directly into the Reaction Tube. The liquid level should be within the two lines on the Reaction Tube. After that, re-connect the cap again to avoid touching the nozzle.
d) Close the lid of the Reaction Tube and mark it. Repeat the last five steps from attaching the cap to the nozzle to closing the lid of the Reaction Tubes for all samples and the NC.
e) Dispense 30µL of PC into a Reaction Tube. Close the cap and mark it respectively. Use the provided dropper for the transfer (The black line on the dropper shows 30µL). Close the PC immediately.
f) Reconstitute the dried reagent in the cap by inverting the tube. All liquid must be in the cap. Make sure that incubate upside-down for 2 minutes at room temperature. As an alternative, you could also use the HuMax ITA.

g) Invert the reaction tubes 5 times to mix the content. Make sure that all liquid is in the bottom of the tube at the end.
h) Make sure that the temperature of the reaction block has reached 67°C.
i) Place the tubes into the reaction block and close the lid. Press the Start/Stop button. The entire amplification takes 45 minutes including the enzyme inactivation at 80°C for 5 minutes.
j) After the audible signal indicates that the amplification is finished, take the Reaction Tubes out of the reaction block and put them into the fluorescence detection unit.
k) Turn on the UV Light by pushing the red button.
l) Confirm the validity of the run:

Positive Control (PC) -green fluorescence; brighter than Negative Control (NC)

- Negative Control –NC) - no fluorescence
- Positive sample -green fluorescence
- Negative sample -no fluorescence

m) Document the results.
n) Then discard the Reaction Tubes as highly infectious waste.
o) The plastic bag with zip is recommended to reduce the risk of contamination by leaking the LAMP product.
p) Never do autoclave processing! Never open the Reaction Tubes after amplification

Reading, recording and reporting

a) If you get "Invalid" results, repeat the test following the SOP.
b) Should the test again Invalid" proceed to troubleshoot using the manual.

Recording and Reporting

a) Results must be recorded in a TB laboratory register (HMIS TB 010) following the guide in the register. Use red ink for positive results. Result reports must be provided as soon as possible.
b) Reporting of Xpert MTB/RIF Ultra results should be reported through DHIS-2 using reports such as; HMIS 033b, HMIS 105 and HMIS 106a. Additionally, Data Connectivity solutions such as LabXpert DS, electronic case-based surveillance system (eCBSS) may be used to report TB-LAMP data.
c) Truenat utilization: The target for monitoring Truenat utilization is 3 samples (runs) per sample well. Consider 5 days in a week, 21 days in a month, 63 days in a quarter and 252 days in a year.

Troubleshooting: Check the manual.

Quality control

d) Maintain the instrument according to SOP.
e) Validate results prior to reporting (Positive and negative controls must pass)
f) Monitor the invalid results

Waste management: Manage waste as in Xpert MTB/RIF Ultra

Maintenance and Service of TB-LAMP Equipment

Frequency	Task
Daily	Clean and disinfect working area Clean all accessory parts Disposal of wastes
Weekly	Clean surface of the analyzer Clean isolation cover, reaction and Heating Block Clean the Fluorescence Detection Unit
Monthly	Check brightness of the Fluorescence Detection Unit
Yearly	Temperature check on Heating Block Temperature check on Reaction Block

Daily Maintenance

a) Switch off the device
b) Unplug the power cord
c) Disconnect the fluorescence module. NB: **!Do not forget to wear gloves and lab coat!**

Clean and disinfect working areas

a) Wipe the work area with 0.5% Sodium Hypochlorite (bleaching solution)
b) Wait for 5 minutes
c) Wipe the work area to eliminate the bleaching solution
d) Clean the work area with DI water
e) Wipe until the work area is clean and dry
f) Wipe the work area with 0.5% Sodium Hypochlorite (bleaching solution)
g) Wait for 5 minutes
h) Wipe the work area to eliminate the bleaching solution
i) Clean the work area with DI water
j) Wipe until the work area is clean and dry

Clean all accessories

a) Clean all accessory parts such as 'heating- and adsorbent tube stand', scissors, stylus etc. with 0,5% Sodium Hypochlorite (bleaching solution)
b) After 5 minutes of incubation time clean them with DI water
c) Wipe of the liquid by using a lint-free paper tissue

Disposal of waste

a) Upon completion, remove any reaction tubes or probe material from the heating- and reaction block and fluorescence module
b) Dispose of reaction tubes or probe material in the appropriate biohazardous waste container

Weekly Maintenance

Clean the surface of Huma Loop

a) Clean the instrument frequently in order to ensure a proper and reliable operation
b) Use a lint-free paper tissue and wipe the surface of the instrument by using 0,5% of Sodium Hypochlorite
c) After 5 minutes of wait, clean up the surface with a lint-free paper tissue moistened with DI water.

Note! **When cleaning the wells of the Heating and Reaction block, prevent any cleaning agents dropping into the wells!**

Cleaning of isolation cover, reaction and heating block

a) Open both cover of the heating and reaction block
b) Use a cotton swab moistened with DI water and remove the dirt and dust from the inside of the cover and incubation blocks
c) Use 0,5% Sodium Hypochlorite solution if the concerned parts are contaminated with biohazard material
d) Let it stay for 5 minutes, then use a lint-free paper tissue moistened with DI water and clean up the concerned parts
e) Clean the Fluorescence Module
f) Use a lint-free paper tissue moistened with de-ionised water and clean the entire unit especially the viewing window carefully
g) Wipe the unit with a soft dry paper tissue.

Note! **Do not use Ethanol, Hypochlorite or other bleach solution to clean the fluorescence unit!**

Monthly Maintenance

Check the Fluorescence Detection Unit

a) Make sure the Fluorescence Detection Unit is connected to the backside of the instrument
b) Switch on the instrument
c) Press the red button on the right side of the Fluorescence Detection Unit
d) Check the LED light
e) You can adjust the brightness of the light by turning the screw at the backside of the instrument
f) Reduce the brightness by turning the screw clockwise

Annual Maintenance

Tools required to perform the yearly maintenance:

a) A calibrated temperature measurement device with temperature sensor cable
b) Temperature measurement probe set
c) Pipette with capacity of 1 ml
d) Maintenance cover for Heating Block

Check temperature on Heating Block

a) After the HumaLoop T became switched on, the temperature heats up to 90°C.
b) The LED (incubation) is flashing and the START / STOP button is off
c) This approximately takes up to 15 minutes

d) Once the display shows 89.9°C, add 1ml of DI water to position 1, 5 and 16
e) Use the Maintenance cover to close the Heating Block
f) Wait another 5 minutes for the liquid to be heated
g) Insert the temperature sensor cable through the small hole of the measuring cover into position 1
h) The tip of the sensor cable should touch the bottom inside the well
i) The temperature displayed on the measurement unit may reach 90°C
j) A tolerance of +/- 1.0°C is allowed
k) Check the temperature on position 5 and 16 too
l) Note! The temperature measurement unit and the sensor cable have to be calibrated!
m) Check temperature on Reaction Block for amplification
n) Once the temperature displays 67°C on the screen of the instrument, insert the temperature sensor to position 5
o) Wait a couple of minutes until the temperature gets stable
p) The temperature displayed on the measurement unit should reach 67°C
q) A tolerance of +/- 0.5°C is acceptable
r) Check the temperature on position 1 and 16 too

Check temperature on Reaction Block for inactivation

a) After 40 minutes of incubating, the temperature automatically heats up to 80°C in order to stop the reaction process
b) This may takes up to 4 minutes
c) When 80°C were reached, the LED (Inactivation) turns on
d) During the next 5 minutes the external measurement unit
e) should measure 80°C with a tolerance of +/- 0,5°C
f) If the temperature is out of the tolerance, a calibration is required

Certificate: When the execution of the preventive maintenance has been done, send the signed maintenance checklist to www.my.human.de and a certificate will be assigned.

Appendix 5: Quantiferon® - TB Gold Plus (QFT®-Plus) Testing Procedure

Objectives and scope

Quantiferon-TB Gold plus(QFT-Plus) is an in vitro diagnostic test that uses peptide derivatives including ESAT-6 and CFP- 10 proteins (all absent from all BCG strains and from most common non-tuberculosis mycobacteria) to stimulate immunological cells in heparinized whole blood. Immunological response by sensitized cells produces interferon gamma (IFN-γ), which is quantitatively detected by Enzyme-linked Immunosorbent assay (ELISA) to correlate the in vitro responses to peptide derivatives that are associated with Mycobacterium tuberculosis infection.

Principle of the assay

The QFT-Plus assay uses specialized collection tubes to collect whole blood, containing peptide antigens derivatives that simulate M. tuberculosis proteins. The QFT-Plus test is performed in two stages.

Whole blood is collected into each of the QFT-Plus Blood Collection Tubes, which include a Nil tube, TB1 tube, TB2 tube and a Mitogen tube. Alternatively, blood may be collected in a single blood collection tube that contains an anticoagulant, lithium-heparin, and then transferred to QFT-Plus Blood Collection Tubes.

The QFT-Plus Blood Collection Tubes are shaken to mix antigen with the blood and are

Incubated at 37°C ± 1°C for 16 to 24 hours of collection. Following incubation, the tubes are centrifuged at 2000 to 3000RCF (g) for 15 minutes, the plasma is harvested and presence of IFN-γ produced in response to the peptide antigen derivatives is quantified by ELISA. The QFT-Plus ELISA uses a recombinant human IFN-γ standard, which has been assayed against a reference IFN-γ preparation (NIH Ref: Gxg01- 902-535). Results for test samples are reported in International Units per ml (IU/ml) relative to a standard curve prepared by testing dilutions of the standard supplied with the kit.

Hand washing and careful techniques are appropriate practices and should be followed.

Wear protective disposable gloves and laboratory coats during the technique to reduce on the risk of acquiring body fluid borne pathogens like hepatitis B virus and HIV. Used gloves have to be changed at every interruption of activity and discarded as contaminated material in the biohazard waste bin.

Discard unused reagents and biologically infectious material in their respective biohazard bins.

Waste management

At the end of each day, any contaminated material used is closed in a biohazard bag and autoclaved before being transferred for disposal out of the laboratory

Procedure

- ELISA Kit components
- Microplate strips (12 x 8 wells) coated with murine anti-human IFN-γ monoclonal antibody
- IFN-γ Standard, lyophilized (contains recombinant human IFN-γ, bovine casein, 0.01% w/v Thimerosal)
- Green Diluent (contains bovine casein, normal mouse serum, 0.01% w/v Thimerosal) Conjugate 100x Concentrate, lyophilized (murine anti-human IFN-γ HRP, 0.01% Thimerosal)
- Wash Buffer 20x Concentrate (pH 7.2, contains 0.05% v/v ProClin® 300)
- Enzyme Substrate Solution (contains H2O2, 3, 3', 5, 5' Tetramethylbenzidine)
- Enzyme Stopping Solution (contains 0.5 M H_2SO_4)

Equipment

- 37°C ± 1°C incubator (with or without CO2)
- Calibrated variable-volume pipets for delivery of 10 µl to 1000 µl with disposable tips Calibrated multichannel pipet capable of delivering 50 µl to 100 µl with disposable tips
- Deionized or distilled water, 2 litres
- Optional: 1 ml micro tubes with caps in 96-well format racks or uncoated microplates with Plastic seals for plasma storage (22 patients/rack or plate)
- Centrifuge capable of centrifuging the blood tubes at least to 3000 RCF (g)
- Microplate shaker capable of speeds between 500 and 1000 rpm Microplate washer (for safety in handling plasma samples)
- Microplate reader fitted with 450 nm filter and 620 nm to 650 nm reference filter
- Variable speed vortex
- Timer
- 50ml and 25ml Falcon tubes
- Graduated cylinder, 1 litre or 2 litre

- Reagent reservoirs
- Plate lid
- Small Plastic biohazard bag for waste disposal

Work procedure

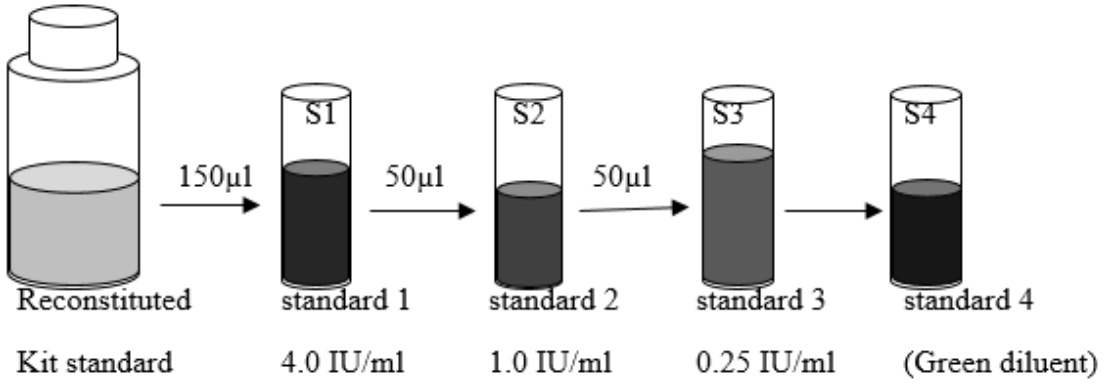
- Harvesting of plasma
- Prior to harvesting plasma, the QFT-plus blood collection tubes should have been incubated at 37℃ for 16-24 hours.
- QFT-plus Blood collection tubes may be held between 4℃ and 27℃ after incubation (37℃ ±1℃) for up to 3 days prior to centrifugation.
- The blood collection tubes are centrifuged at 2000 to 3000 RCF (g) for 15 minutes. The gel plug will separate the cells from the plasma.
- After centrifugation Plasma is harvested used a pipette and mixing of plasma up and down or by any means should be minimised. Don't disturb the materials on the surface of the gel.
- An adequate volume of at least 150µl of plasma is preferably required.

Note:

- It's possible to harvest plasma without centrifugation, however additional care is required to remove the plasma without disturbing the cells.
- Storage of blood and plasma
- Plasma (in QFT-plus blood collection and harvested samples) can be stored for up to 28 days at 2℃ to 8℃ . Harvested samples can be stored at -20℃ (preferably -70℃) for extended periods.

Preparation of reagents

- Plasma samples and reagents with exception of conjugate 100X Concentrate must be brought to room temperature (22℃ ± 5℃ [71.6℉ ±9℉]) before usage. Allow at least 60 minutes for equilibration.
- Note: The conjugate 100x concentrate should be returned to a refrigerator at 2℃ to 8℃.
- ELISA strips not required for use are removed from the frame, resealed in foil pouch and returned to the refrigerator for storage. At least one strip for QFT-plus Standards and sufficient strips for plasma samples to be assayed.
- Note: Retain frame and lid for subsequent use with the remaining strips.
- Reconstitute IFN-y standard with a volume of deionized or distilled water (1.68ml) indicated on the label of the vial. Mix gently to minimize frothing and ensure content of the vial is completely dissolved (Reconstitution with the recommended volume gives a solution with a concentration of 8.0 IU/ml).
- Use the reconstituted standard to prepare a dilution series of 4 IFN – concentrations.



Note:

The standards must be assayed at least in duplicate.

Always prepare fresh dilutions of the kit standard for each ELISA session.

Dilution for duplicate standards.

Procedure for serial dilution of duplicate standards	
A	Label 4 tubes: S1, S2, S3, S4
B	Add 150 µl of Green Diluent (GD) to S1, S2, S3, S4
C	Add 150 µl of the kit standard to S1 and mix thoroughly
D	Transfer 50 µl from S1 to S2 and mix thoroughly
E	Transfer 50 µl from S2 to S3 and mix thoroughly
F	Green diluent serves as the zero standard(S4)

Reconstitute lyophilized conjugate 100x concentrate with 0.3ml of deionized or distilled water, mix gently to minimize frothing and ensure the contents in the vials are completed dissolved.

Working strength conjugate is prepared by diluting the required amount of the conjugate 100x Concentrate with its corresponding green diluent volume as below

Number of strips	Volume of conjugate (100x concentrate)	Volume of Green Diluent
2	10 µl	1.0 ml
3	15 µl	1.5 ml
4	20 µl	2.0 ml
5	25 µl	2.5 ml
6	30 µl	3.0 ml
7	35 µl	3.5 ml
8	40 µl	4.0 ml

9	45 µl	4.5 ml
10	50 µl	5.0 ml
11	55 µl	5.5 ml
12	60 µl	6.0 ml

Note:

Return any unused Conjugate 100X Concentrate to 2℃ to 8℃ immediately after use.

For Plasma samples that were harvested from blood collection tubes and subsequently stored, thorough mixing is required before addition to the ELISA well.

Important: plasma samples to be added to the ELISA plate from the Centrifuged QFT-Plus Blood Collection Tubes, should not be mixed at any moment to prevent disturbing of the material on the surface of the gel.

50 µl of freshly prepared working strength conjugate is added to each ELISA plate well.

50 µl of test plasma sample is added to appropriate wells (check ELISA plate layout not to put in wells reserved for the duplicate standards).

Finally, add 50 µl of each standard 1 to 4 to the appropriate plate wells (assayed in duplicate).

	1	2	3	4	5	6	7	8	9	10	11	12
A	1N	3 N	5N	7N	9N	S1	S1	13N	15N	17N	19N	21N
B	1 TB1	3 TB 1	5TB1	7TB1	9TB1	S1	S2	13TB1	15TB1	17TB1	19TB1	21TB1
C	1 TB2	3 TB 2	5TB2	7TB2	9TB2	S3	S3	13TB2	15TB2	17TB2	19TB2	21TB2
D	1M	3M	5M	7M	9M	S4	S4	13M	15M	17M	19M	21M
E	2 N	4 N	6N	8N	10N	11N	12N	14N	16N	18N	20N	22N
F	2 TB 1	4 TB1	6TB1	8TB1	10TB1	11M	12TB1	14TB1	16TB1	18TB1	20TB1	22TB1
G	2TB 2	4 TB2	6TB2	8TB2	10TB2	11M	12TB2	14TB2	16 TB2	18TB2	20TB2	22TB2
H	2 M	4 M	6M	8M	10M	11M	12M	14M	16M	18 M	20M	22M

Recommended ELISA plate layout. S1 Standard 1), S2 (Standard 2), S3 (Standard 3), S4 (Standard 4). 1N (Sample 1. Nil Control plasma), 1 TB 1 (Sample1. TB1 Plasma), 1 TB2 (Sample 1. TB2 plasma), 1M (Sample 1. mitogen)

Cover ELISA plate and mix the conjugate and plasma samples / standards thoroughly using a microplate shaker for 1 minute at 500 to 1000 rpm. Avoid splashing.

The ELISA plate after mixing is incubated at room temperature (22℃ ± 5℃[71.6℉ ±9℉]) for 120 ± 5 minutes. Avoid exposure of the ELISA plate to direct sunlight during incubation.

Note: Deviation from the specified temperature range can lead to erroneous results.
During the incubation of the ELISA plate, prepare working strength wash buffer. Dilute one part wash Buffer 20x Concentrate with 19parts (1:20) deionized or distilled water and mix thoroughly (Sufficient Wash Buffer 20x Concentrate is provided to prepare 2 litres of working strength wash Buffer).

After incubation, wash each ELISA plate wells with 400 µl of working strength wash buffer. Perform wash steps at least 6 times (An automated plate washer is recommended for safety reasons when handling plasma samples).

Note: Thorough washing is very important and make sure each well is filled with wash buffer to the top of the well for each wash cycle. A soak period of at least 5 seconds between each cycle is recommended.

Standard laboratory disinfectant should be added to the effluent reservoir and established procedures for decontamination of potentially infectious material should be set up.

Tap ELISA plate face down on absorbent (low-lint) towel to remove residual wash buffer. Add 100µl of Enzyme Substrate Solution to each plate well, cover and mix thoroughly for 1 minute at 500 to 1000 rpm using a micro plate shaker.

Cover ELISA plate and incubate at room temperature (22°C ±5°C[71.6°F ±9°F]) for 30 minutes (Don't expose ELISA plate to direct sunlight).

Following 30minute incubation, add 50 µl of Enzyme Stopping Solution to each plate well in the same order as the substrate was added, mix thoroughly at 500 to 1000 rpm using a micro plate shaker.

Measure the optical Density (OD) of ELISA plate wells within 5 minutes of stopping the reaction using a micro plate reader fitted with 450 nm filter and with a 620 nm reference filter. OD values are used to calculate results.

Calculations and Test Interpretation

QFT-Plus Analysis Software can be used to analyse raw data and calculate results. It is available at www.qiagen.com. Please make sure that the most current version of the QFT-Plus Analysis Software is used.

The software performs a Quality Control assessment of the assay, generates a standard curve and provides a test result for each subject. Software reports all concentrations greater than 10 IU/ml as ">10" as such values fall beyond the validated linear range of the ELISA.

As an alternative to using the QFT-Plus Analysis Software, results can be determined according to the following method.

Generation of standard curve and sample values

If QFT-Plus Analysis Software is not used

Determination of the standard curve and determination of sample IU/ml values require a spreadsheet program, such as Microsoft® Excel®, if the QFT-Plus software is not used.

Using a spreadsheet program:

1. Determine the mean OD values of the kit standard replicates on each plate.

2. Construct ®o®)–log(e) standard curve by plott® the log(e) of the mean OD (y axis) a®nst the log(e) of the IFN-γ concentration of the standards in IU/ml (x axis), omitting the zero standard

from these calculations. Calculate the line of best fit for the standard curve by regression analysis.

3. Use the standard curve to determine the IFN-γ concentration (IU/ml) for each of the test plasma samples, using the OD value of each sample.

4. These calculations can be performed using software packages available with Microplate readers, and standard spreadsheet or statistical software (such as Microsoft Excel). It is recommended that these packages be used to calculate the regression analysis, the coefficient of variation (%CV) for the standards, and the correla®n coefficient (r) of the standard curve.

Quality control of the test

The accuracy of test results is dependent on the generation of an accurate standard curve. Therefore, results derived from the standards must be examined before test sample results can be interpreted.

For the ELISA to be valid:

The mean OD value for Standard 1 must be ≥0.600. λ

The %CV for Standard 1 and Standard 2 replicate values must be ≤15%. λ Replicate OD values for Standard 3 and Standard 4 must not vary by more than 0.040 optical density units from their mean.

The corr®tion coefficient (r) calculated from the mean absorbance values of the standards must be ≥0.98.

If the above criteria are not met, the run is invalid and must be repeated. λ The mean OD value for the Zero Standard (Green Diluent) should be ≤0.150. If the mean OD value is > 0.150, the plate washing procedure should be investigated. The QFT-Plus Analysis Software calculates and reports these quality control parameters.

Each laboratory should determine appropriate types of control materials and frequency of testing in accordance with Local, State, Federal, or other applicable accrediting organizations. External quality assessment and alternative validation procedures should be considered.

Note: Plasmas spiked with recombinant IFN-γ have shown reductions of up to 50% in concentration when stored at either 2°C to 8°C and –20°C. Recombinant IFN-γ is not recommended for establishing control standards.

Interpretation of results

Important: Diagnosing or excluding tuberculosis disease, and assessing the probability of LTBI,

Nil (IU/ml)	TB1 minus Nil (IU/ml)	TB 2 minus Nil (IU/ml)	Mitogen minus Nil (IU/ml)	QFT-Plus Result	Report/ interpretation
≤8.0	≥0.35 and ≥25% of Nil	Any	Any	Positive^	M. tuberculosis infection likely
	Any	≥0.35 and ≥25% of Nil			
	<0.35 or ≥0.35 and <25% of Nil	<0.35 or ≥0.35 and <25% of Nil	≥0.50	Negative	M. tuberculosis infection NOT likely
	<0.35 or ≥0.35 and <25% of Nil	<0.35 or ≥0.35 and <25% of Nil	<0.50	Indeterminate&	Likelihood of M. tuberculosis infection cannot be determined
>8.0$	Any				

requires a combination of epidemiological, historical, medical, and diagnostic findings that should be considered when interpreting QFT-Plus results. See general guidance on the diagnosis and treatment of TB disease and LTBI at https://www.cdc.gov/tb/default.htm provided under section “US Centres for Disease Control and Prevention (CDC) Guidelines” below.

QFT-Plus results are interpreted using the following criteria:

Responses to the Mitogen positive control (and occasionally TB Antigen) can be outside the range of the Microplate reader. This has no impact on test results. Values >10 IU/ml are reported by the QFT-Plus software as >10 IU/ml.

Where M. tuberculosis infection is not suspected, initially positive results can be confirmed by retesting the original plasma samples in duplicate in the QFT-Plus ELISA. If repeat testing of one or both replicates is positive, the test result is considered positive.

&Indeterminate results are uncommon and may relate to the immune status of the individual being tested, but may also be related to a number of technical factors (e.g., inappropriate handling/storage of blood collection tubes, incomplete ELISA plate washing) if the above instructions for use are not followed.

In clinical studies, less than 0.25% of subjects had IFN-γ levels of >8.0 IU/ml for the Nil value.

The magnitude of the measured IFN-γ level cannot be correlated to stage or degree of infection, level of immune responsiveness, or likelihood for progression to active disease. A positive TB response in persons who are negative to Mitogen is rare, but has been seen in patients with TB disease. This indicates the IFN-γ response to TB antigens is greater than that to Mitogen, which is possible as the level of Mitogen does not maximally stimulate IFN-γ production by lymphocytes.

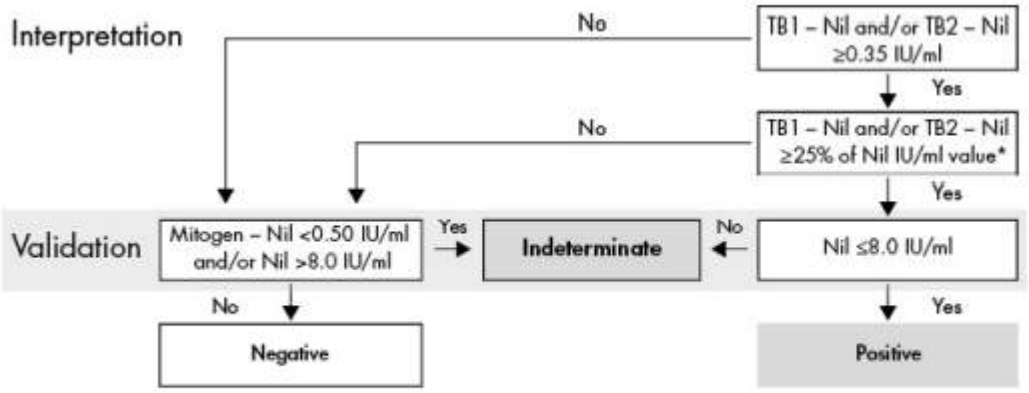


Figure 6. QFT-Plus test interpretation. *For TB1 minus Nil or TB2 minus Nil value to be valid, the ≥25% of Nil IU/ml value must be from the same tube as the original ≥0.35 IU/ml result.

Specificity and S–nsitivity of QFT Gold - plus

Specificity = 97.27%

Sensitivity = 94.09%

Limitations

Results from QFT-Plus testing must be used in conjunction with everyone's epidemiological history, current medical status, and other diagnostic evaluations.

Individuals with Nil values greater than 8 IU/ml are classed as "Indeterminate" because a 25% higher response to TB Antigens may be outside the assay measurement range.

The predictive value of a positive QFT-Plus result in diagnosing M. tuberculosis infection depends on the probability of infection, which is assessed by historical, epidemiological, diagnostic, and other findings.

A diagnosis of LTBI requires that tuberculosis disease must be excluded by medical evaluation including an assessment of current medical and diagnostic tests for disease as indicated.

A negative result must be considered with the individual's medical and historical data relevant to probability of M. tuberculosis infection and potential risk of progression to tuberculosis disease, particularly for individuals with impaired immune function. Negative predictive values are likely to be low for persons suspected to have M. tuberculosis disease and should not be relied on to exclude disease.

Unreliable or indeterminate results may occur due to:

- Deviations from the procedure described in the package insert
- Incorrect transport/handling of blood specimen
- Elevated levels of circulating IFN-γ or presence of heterophile antibodies
- Exceeding validated blood times from blood specimen draw to incubation

Blood samples collected directly into QFT-Plus Blood Collection Tubes stored longer than 16 hours at room temperature (17–25°C).

Blood samples collected in lithium-heparin tube stored longer than 12 hours at room temperature (17–25°C) prior to transfer to QFT-Plus Blood Collection Tubes.

Reasons to repeat the assay

If technical issues are suspected with the reagent storage, blood collection or handling of the blood samples, repeat the entire QFT-Plus test with new blood specimens. Repeating the ELISA testing of stimulated plasmas can be performed if inadequate washing or other procedural deviation with the ELISA test is suspected. Physicians may choose to redraw a specimen or perform other procedures as appropriate.

Technical information

- Clotted plasma samples

Should fibrin clots occur with long-term storage of plasma samples, centrifuge samples to sediment clotted material and facilitate pipetting of plasma.

- Lipemic plasma samples

Care should be exercised when pipetting lipemic samples as fatty deposits can block pipette tips

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see the technical information provided at www.qiagen.com.

Comments and suggestions	
Nonspecific color development	
Incomplete washing of the plate	Wash the plate at least 6 times with 400 µl/well of wash buffer. More than 6 washing cycles may be required depending on the washer being used. A soak time of at least 5 seconds between cycles should be used.
Cross-contamination of ELISA wells	Take care while pipetting and mixing sample to minimize risk.
Kit/components have expired	Ensure kit is used before the expiry date. Ensure reconstituted standard and Conjugate 100X Concentrate are used within three months of the reconstitution date.
Enzyme Substrate Solution is contaminated.	Discard substrate if blue coloration exists. Ensure clean reagent reservoirs are used.
Mixing of plasma in in QFTPlus Blood Collection Tubes before harvesting.	After centrifugation, avoid pipetting up and down or mixing plasma by any means prior to harvesting. At all times, take care not to disturb material on the surface of the gel.
Low optical density readings for standards	
Standard dilution error	Ensure dilutions of the Kit Standard are prepared correctly as per this Package Insert.
Pipetting error	Ensure pipets are calibrated and used according to manufacturer's instructions.
Incubation temperature too low	Incubation of the ELISA should be performed at room temperature (22°C ± 5°C).
Incubation time too short	Incubation of the plate with the conjugate, standards and samples should be for 120 ± 5 minutes. The Enzyme Substrate Solution should be incubated on the plate for 30 minutes.
Incorrect plate reader filter used	Plate should be read at 450 nm with a reference filter of between 620 and 650 nm.
Reagents are too cold	All reagents, except for the Conjugate 100X Concentrate, must be brought to room temperature prior to commencing the assay. This takes approximately 1 hour.
Kit/components have expired	Ensure that the kit is used before the expiry date. Ensure reconstituted Standard and Conjugate 100X Concentrate are used within 3 months of the reconstitution date.
High background	
incomplete washing of the plate	Wash the plate at least 6 times with 400 µl/well of wash buffer. More than 6

	washing cycles may be required. A soak time of at least 5 seconds between cycles should be used.
Incubation temperature too high	Incubation of the ELISA should be performed at room temperature (22°C ± 5°C).
Kit/components have expired	Ensure that the kit is used within the expiry date. Ensure reconstituted standard and Conjugate 100X Concentrate are used within three months of the reconstitution date.
Enzyme Substrate Solution is contaminated	Discard substrate if blue coloration exists. Ensure clean reagent reservoirs are used.
Nonlinear standard curve and duplicate variability	
Incomplete washing of the plate	Wash the plate at least 6 times with 400 µl/well of wash buffer. More than 6 washing cycles may be required. A soak time of at least 5 seconds between cycles should be used.
Standard dilution error	Ensure dilutions of the standard are prepared correctly as per this Package Insert.
Poor mixing	Mix reagents thoroughly by inversion or gentle vertexing prior to their addition to the plate.
Inconsistent pipetting technique or interruption during assay setup	Sample and standard addition should be performed in a continuous manner. All reagents should be prepared prior to commencing the assay.

Note:

a) The standards must be assayed at least in duplicate.
b) Always prepare fresh dilutions of the kit standard for each ELISA session.

Dilution for duplicate standards.

Procedure for serial dilution of duplicate standards	
A	Label 4 tubes: S1, S2, S3, S4
B	Add 150 µl of Green Diluent (GD) to S1, S2, S3, S4
C	Add 150 µl of the kit standard to S1 and mix thoroughly
D	Transfer 50 µl from S1 to S2 and mix thoroughly
E	Transfer 50 µl from S2 to S3 and mix thoroughly
F	Green diluent serves as the zero standard(S4)

Reconstitute lyophilized conjugate 100x concentrate with 0.3ml of deionized or distilled water, mix gently to minimize frothing and ensure the contents in the vials are completed dissolved.

Working strength conjugate is prepared by diluting the required amount of the conjugate 100x Concentrate with its corresponding Green diluent volume as below

Number of strips	Volume of conjugate (100x concentrate)	Volume of Green Diluent
2	10 µl	1.0 ml
3	15 µl	1.5 ml
4	20 µl	2.0 ml
5	25 µl	2.5 ml
6	30 µl	3.0 ml
7	35 µl	3.5 ml
8	40 µl	4.0 ml
9	45 µl	4.5 ml
10	50 µl	5.0 ml
11	55 µl	5.5 ml
12	60 µl	6.0 ml

Note:

a) Return any unused Conjugate 100X Concentrate to 2℃ to 8℃ immediately after use.
b) For Plasma samples that were harvested from blood collection tubes and subsequently stored, thorough mixing is required before addition to the ELISA well.

Important: plasma samples to be added to the ELISA plate from the Centrifuged QFT-Plus Blood Collection Tubes, should not be mixed at any moment to prevent disturbing of the material on the surface of the gel.

1. 50 µl of freshly prepared working strength conjugate is added to each ELISA plate well.
2. 50 µl of test plasma sample is added to appropriate wells (check ELISA plate layout not to put in wells reserved for the duplicate standards).
3. Finally, add 50 µl of each standard 1 to 4 to the appropriate plate wells (assayed in duplicate).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	1N	3 N	5N	7N	9N	**S1**	**S1**	13N	15N	17N	19N	21N
B	1 TB1	3 TB 1	5TB1	7TB1	9TB1	**S1**	**S2**	13TB1	15TB1	17TB1	19TB1	21TB1
C	1 TB2	3 TB 2	5TB2	7TB2	9TB2	**S3**	**S3**	13TB2	15TB2	17TB2	19TB2	21TB2
D	1M	3M	5M	7M	9M	S4	S4	13M	15M	17M	19M	21M
E	2 N	4 N	6N	8N	10N	11N	12N	14N	16N	18N	20N	22N
F	2 TB 1	4 TB1	6TB1	8TB1	10TB1	11M	12TB1	14TB1	16TB1	18TB1	20TB1	22TB1
G	2TB 2	4 TB2	6TB2	8TB2	10TB2	11M	12TB2	14TB2	16 TB2	18TB2	20TB2	22TB2
H	2 M	4 M	6M	8M	10M	11M	12M	14M	16M	18 M	20M	22M

Recommended ELISA plate layout. S1 Standard 1), S2 (Standard 2), S3 (Standard 3), S4 (Standard 4). 1N (Sample 1. Nil Control plasma), 1 TB 1 (Sample1. TB1 Plasma), 1 TB2 (Sample 1. TB2 Plasma), 1M (Sample 1. mitogen)

4. Cover ELISA plate and mix the conjugate and plasma samples / standards thoroughly using a microplate shaker for 1 minute at 500 to 1000 rpm. Avoid splashing.
5. The ELISA plate after mixing is incubated at room temperature (22°C ± 5°C[71.6°F ±9°F]) for 120 ± 5 minutes. Avoid exposure of the ELISA plate to direct sunlight during incubation.

Note: Deviation from the specified temperature range can lead to erroneous results.

6. During the incubation of the ELISA plate, prepare working strength wash buffer. Dilute **one part** wash Buffer 20x Concentrate with **19parts (1:20)** deionized or distilled water and mix thoroughly (Sufficient Wash Buffer 20x Concentrate is provided to prepare 2 liters of working strength wash Buffer).
7. After incubation, wash each ELISA plate wells with 400 µl of working strength wash buffer. Perform wash steps at least 6 times (An automated plate washer is recommended for safety reasons when handling plasma samples).

Note: Thorough washing is very important and make sure each well is filled with wash buffer to the top of the well for each wash cycle. A soak period of at least 5 seconds between each cycle is recommended.
Standard laboratory disinfectant should be added to the effluent reservoir and established procedures for decontamination of potentially infectious material should be set up.

8. Tap ELISA plate face down on absorbent (low-lint) towel to remove residual wash buffer. Add 100µl of Enzyme Substrate Solution to each plate well, cover and mix thoroughly for 1 minute at 500 to 1000 rpm using a micro plate shaker.
9. Cover ELISA plate and incubate at room temperature (22°C ±5°C[71.6°F ±9°F]) for 30 minutes (Don't expose ELISA plate to direct sunlight).
10. Following 30-minute incubation, add 50 µl of Enzyme Stopping Solution to each plate well in the same order as the substrate was added, mix thoroughly at 500 to 1000 rpm using a micro plate shaker.
11. Measure the optical Density (OD) of ELISA plate wells within 5 minutes of stopping the reaction using a micro plate reader fitted with 450 nm filter and with a 620 nm reference filter. OD values are used to calculate results.

Calculations and Test Interpretation

QFT-Plus Analysis Software can be used to analyse raw data and calculate results. It is available at **www.qiagen.com**.
Please make sure that the most current version of the QFT-Plus Analysis Software is used.
The software performs a Quality Control assessment of the assay, generates a standard curve and provides a test result for each subject. Software reports all concentrations greater than 10 IU/ml as ">10" as such values fall beyond the validated linear range of the ELISA.

As an alternative to using the QFT-Plus Analysis Software, results can be determined according to the following method.

Generation of standard curve and sample values

If QFT-Plus Analysis Software is not used

Determination of the standard curve and determination of sample IU/ml values require a spreadsheet program, such as Microsoft® Excel®, if the QFT-Plus software is not used.

Using a spreadsheet program:

1. Determine the mean OD values of the kit standard replicates on each pla®
2. Construct a log(e)–log(e) standard®rve by plotting the log(e) of the mea®D (y axis) against the log(e) of the IFN-γ concentration of the standards in IU/ml (x axis), omitting the zero standard from these calculations. Calculate the line of best fit for the standard curve by regression analysis.
3. Use the standard curve to determine the IFN-γ concentration (IU/ml) for each of the test plasma samples, using the OD value of each sample.
4. These calculations can be performed using software packages available with Microplate readers, and standard spreadsheet or statistical software (such as Microsoft Excel). It is recommended that these packages be used to calculate the regression analysis, the coefficient of variation (%CV) for the standards, ® the correlation coefficient (r) of the standard curve.

Quality control of the test

The accuracy of test results is dependent on the generation of an accurate standard curve. Therefore, results derived from the standards must be examined before test sample results can be interpreted.

For the ELISA to be **valid:**

- The mean OD value for Standard 1 must be ≥0.600. λ
- The %CV for Standard 1 and Standard 2 replicate values must be ≤15%. λ
- Replicate OD values for Standard 3 and Standard 4 must not vary by more than 0.040 optical density units from their ®n.
- The correlation coefficient (r) calculated from the mean absorbance values of the standards must be ≥0.98.

If the above criteria are not met, the run is invalid and must be repeated.

The mean OD value for the Zero Standard (Green Diluent) should be ≤0.150. If the mean OD value is > 0.150, the plate washing procedure should be investigated.

Note: The QFT-Plus Analysis Software calculates and reports these quality control parameters.

Note: Plasmas spiked with recombinant IFN-γ have shown reductions of up to 50% in concentration when stored at either 2°C to 8°C and –20°C. Recombinant IFN-γ is not recommended for establishing control standards.

Interpretation of results

Important: Diagnosing or excluding tuberculosis disease, and assessing the probability of LTBI, requires a combination of epidemiological, historical, medical, and diagnostic findings that should be taken into account when interpreting QFT-Plus results. See general guidance on the diagnosis and treatment of TB disease and LTBI at https://www.cdc.gov/tb/default.htm provided under section “US Centers for Disease Control and Prevention (CDC) Guidelines”.

Nil (IU/ml)	TB1 minus Nil (IU/ml)	TB 2 minus Nil (IU/ml)	Mitogen minus Nil(IU/ml)	QFT-Plus Result	Report/ interpretation
≤8.0	≥0.35 and ≥25% of Nil	Any	Any	Positive^	M. tuberculosis infection likely
	Any	≥0.35 and ≥25% of Nil			
	<0.35 or ≥0.35 and <25% of Nil	<0.35 or ≥0.35 and <25% of Nil	≥0.50	Negative	M. tuberculosis infection NOT likely
	<0.35 or ≥0.35 and <25% of Nil	<0.35 or ≥0.35 and <25% of Nil	<0.50	Indeterminate	Likelihood of M. tuberculosis infection cannot be determined
>8.0[$]	Any				

Table 4: Reporting and interpretation of Results

QFT-Plus results are interpreted using the following criteria:

Responses to the Mitogen positive control (and occasionally TB Antigen) can be outside the range of the Microplate reader. This has no impact on test results. Values >10 IU/ml are reported by the QFT-Plus software as >10 IU/ml.

Where M. tuberculosis infection is not suspected, initially positive results can be confirmed by retesting the original plasma samples in duplicate in the QFT-Plus ELISA. If repeat testing of one or both replicates is positive, the test result is considered positive.

Indeterminate results are uncommon and may relate to the immune status of the individual being tested, but may also be related to a number of technical factors (e.g., inappropriate handling/storage of blood collection tubes, incomplete ELISA plate washing) if the above instructions for use are not followed.

In clinical studies, less than 0.25% of subjects had IFN-γ levels of >8.0 IU/ml for the Nil value.

The magnitude of the measured IFN-γ level cannot be correlated to stage or degree of infection, level of immune responsiveness, or likelihood for progression to active disease. A positive TB response in persons who are negative to Mitogen is rare, but has been seen in patients with TB disease. This indicates the IFN-γ response to TB antigens is greater than that to Mitogen, which is possible as the level of Mitogen does not maximally stimulate IFN-γ production by lymphocytes.

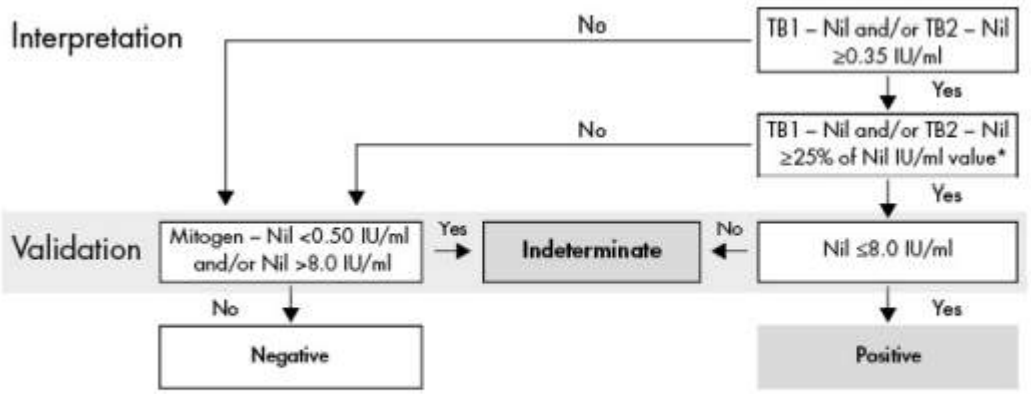


Figure 6. QFT-Plus test interpretation. *For TB1 minus Nil or TB2 minus Nil value to be valid, the ≥25% of Nil IU/ml value must be from the same tube as the original ≥0.35 IU/ml result.

Sp–cificity and Sensitivity of QFT Gold - plus

Specificity = 97.27%
Sensitivity = 94.09%

Limitations

Results from QFT-Plus testing must be used in conjunction with each individual's epidemiological history, current medical status, and other diagnostic evaluations.

Individuals with Nil values greater than 8 IU/ml are classed as "Indeterminate" because a 25% higher response to TB Antigens may be outside the assay measurement range.

- The predictive value of a positive QFT-Plus result in diagnosing M. tuberculosis infection depends on the probability of infection, which is assessed by historical, epidemiological, diagnostic, and other findings.
- A diagnosis of LTBI requires that tuberculosis disease must be excluded by medical evaluation including an assessment of current medical and diagnostic tests for disease as indicated.
- A negative result must be considered with the individual's medical and historical data relevant to probability of M. tuberculosis infection and potential risk of progression to tuberculosis disease, particularly for individuals with impaired immune function. Negative predictive values are likely to be low for persons suspected to have M. tuberculosis disease and should not be relied on to exclude disease.

Unreliable or indeterminate results may occur due to:

- Deviations from the procedure described in the package insert
- Incorrect transport/handling of blood specimen
- Elevated levels of circulating IFN-γ or presence of heterophile antibodies

Exceeding validated blood times from blood specimen draw to incubation:

- Blood samples collected directly into QFT-Plus Blood Collection Tubes stored longer than 16 hours at room temperature (17–25°C).
- Blood samples collected in lithium-heparin tube stored longer than 12 hours at room temperature (17–25°C) prior to transfer to QFT-Plus Blood Collection Tubes.

Comments and suggestions	
Nonspecific color development	
a. Incomplete washing of the plate	Wash the plate at least 6 times with 400 µl/well of wash buffer. More than 6 washing cycles may be required depending on the washer being used. A soak time of at least 5 seconds between cycles should be used.
b. Cross-contamination of ELISA wells	Take care while pipetting and mixing sample to minimize risk.
c. Kit/components have expired	Ensure kit is used before the expiry date. Ensure reconstituted standard and Conjugate 100X Concentrate are used within three months of the reconstitution date.
d. Enzyme Substrate Solution is contaminated.	Discard substrate if blue coloration exists. Ensure clean reagent reservoirs are used.
e. Mixing of plasma in in QFTPlus Blood Collection Tubes before harvesting.	After centrifugation, avoid pipetting up and down or mixing plasma by any means prior to harvesting. At all times, take care not to disturb material on the surface of the gel.
Low optical density readings for standards	
a. Standard dilution error	Ensure dilutions of the Kit Standard are prepared correctly as per this Package Insert.
b. Pipetting error	Ensure pipets are calibrated and used according to manufacturer's instructions.
c. Incubation temperature too low	Incubation of the ELISA should be performed at room temperature (22°C ± 5°C).
d. Incubation time too short	Incubation of the plate with the conjugate, standards and samples should be for 120 ± 5 minutes. The Enzyme Substrate Solution should be incubated on the plate for 30 minutes.
e. Incorrect plate reader filter used	Plate should be read at 450 nm with a reference filter of between 620 and 650 nm.
f. Reagents are too cold	All reagents, with the exception of the Conjugate 100X Concentrate, must be brought to room temperature prior to commencing the assay. This takes approximately 1 hour.
g. Kit/components have expired	Ensure that the kit is used before the expiry date. Ensure reconstituted Standard and Conjugate 100X Concentrate are used within 3 months of the reconstitution date.
High background	
a. incomplete washing of the plate	Wash the plate at least 6 times with 400 µl/well of wash buffer. More than 6 washing cycles may be required. A soak time of at least 5 seconds between cycles

	should be used.
b. Incubation temperature too high	Incubation of the ELISA should be performed at room temperature (22°C ± 5°C).
c. Kit/components have expired	Ensure that the kit is used within the expiry date. Ensure reconstituted standard and Conjugate 100X Concentrate are used within three months of the reconstitution date.
d. Enzyme Substrate Solution is contaminated	Discard substrate if blue coloration exists. Ensure clean reagent reservoirs are used.
Nonlinear standard curve and duplicate variability	
a. Incomplete washing of the plate	Wash the plate at least 6 times with 400 µl/well of wash buffer. More than 6 washing cycles may be required. A soak time of at least 5 seconds between cycles should be used.
b. Standard dilution error	Ensure dilutions of the standard are prepared correctly as per this Package Insert.
c. Poor mixing	Mix reagents thoroughly by inversion or gentle vortexing prior to their addition to the plate.
d. Inconsistent pipetting technique or interruption during assay setup	Sample and standard addition should be performed in a continuous manner. All reagents should be prepared prior to commencing the assay.

Reasons to repeat the assay

If technical issues are suspected with the reagent storage, blood collection or handling of the blood samples, repeat the entire QFT-Plus test with new blood specimens. Repeating the ELISA testing of stimulated plasmas can be performed if inadequate washing or other procedural deviation with the ELISA test is suspected. Physicians may choose to redraw a specimen or perform other procedures as appropriate.

Technical information

1. **Clotted plasma samples**

Should fibrin clots occur with long-term storage of plasma samples, centrifuge samples to sediment clotted material and facilitate pipetting of plasma.

2. **Lipemic plasma samples**

Care should be exercised when pipetting lipemic samples as fatty deposits can block pipette tips

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see the technical information provided at www.qiagen.com.

Appendix 8: Common causes for errors in EQA by Blinded Slide Rechecking

Type of error	Possible causes	Suggested actions
False positive	• Artifact (e.g. stain deposits or crystals) incorrectly interpreted as AFB • AFB carried over in immersion oil from a previous positive smear • Stained AFB have faded since original report	• **Visit the lab and assess the knowledge of technique and possible cause of error. Do on site mentorship for correction of the error** • **Re-stain false positives and re-examine**
False Negative	• Insufficient time spent in scanning smear • Incorrect microscopy technique • Problems with staining (pale AFB, insufficient contrast in background) • Defective microscope	• **Visit the lab and assess the knowledge of technique and possible cause of error. Do on site mentorship for correction of the error** • **Prepare new staining reagents** • **On-site check of microscope performance**
Quantification error (minor)	• Insufficient time spent scanning smear • Lack of understanding of scoring system	• **Mentor the Lab personnel at a visit to the Lab**
Quantification error (major)	• Lack of understanding of scoring system • Incorrect microscopy technique • Defective microscope	• **Visit the lab and assess the knowledge of technique and possible cause of error. Do on site mentorship for correction of the error** • **On-site check of microscope performance**

Appendix 9: EQA Error Investigation Table and suggestions of Possible Trouble shooting for Root causes:

Patterns of errors	Possible causes	Checks to be done
Numerous HFP and HFN	• Unusable microscope • Lab staff does not know AFB or • Does not examine properly	• Examine a 3+ using that microscope • Test with clear-cut positive/negatives and good microscopes • Exclude other causes
Single HFP	• Administrative mistake	• Check lab register whether clerical errors
Few HFP with/without LFP	• Poor registration • No re-staining before QC • Not quite clear on what is AFB	• Check whether lab-register i abellingdate, use of request form and labeling of sputum pots, • Re-stain and re-examine HFP

		• Look for inconsistent results of suspects in lab register: regularly isolated pos/scanty
Many LFP with/without low-grade HFP	• Poor controls and countercheck • Not quite clear on what is AFB • Contaminated carbol-fuchsin stain	• Feedback FP slides to centres: can they show AFB? • Test stain on known negative smears/ central lab records on stain QA? • Cross-check special scanty smears
Single HFN	• Administrative errors as single HFP • Very thick smears and/or poor light • Not looked at all	• Exclude other causes, check registration • Look at thickness, colour of recent smears, sufficient light of microscope (condenser and diaphragm)
Many HFN and many LFN	• Bad stain or poor staining • Poor smearing technique • Problems with microscope • Careless microscopy • Contaminated methylene blue or rinsing water • Heavy workload	• Check colour of carbol-fuchsin • Check if AFB have solid, strong red colour • Observe staining procedure, time, and heating? • Check microscope • Use methylene be blue (distilled for rinsing) on known negatives in repeat staining-cycles, then check to atypical AFB
QE (difference of at least 2 grades) serious)	• Little experience, poor work, poor equipment, • Often points to staining problems	• Check staining process, microscope, • Re-stain

Appendix 10

(one page for entire Excel selection)

Appendix 11

(one page for entire Excel selection)

References

1. Laboratory Diagnosis of Tuberculosis by sputum Microscopy. The Handbook- Global Edition (2013).
2. Cepheid GeneXpert GXMTB/RIF-10. Package inserts. 300-6252 Rev. D, September 2010.
3. Cepheid GeneXpertDx System. Operator Manual. Software version 4.0. 300–7607, Rev. C.1.
4. Indira Soundiram, Cepheid Technical Advisor. Personal communication.
5. Procedure for use and maintenance of personal protective equipment.
6. Procedure for spill management.
7. Procedure for disinfection and decontamination.
8. Procedure for waste management.
9. The laboratory safety manual.
10. The WHO TB laboratory safety manual, 2013.
11. http://apps.who.int/iris/bitstream/10665/77949/1/9789241504638_eng.pdf
12. MSDS for the reagents and solutions used in the procedure.
13. Laboratory Diagnosis of Tuberculosis by sputum Microscopy. The Handbook- Global Edition (2013).
14. Cepheid GeneXpert GXMTB/RIF-10. Package inserts. 300-6252 Rev. D, September 2010.
15. Cepheid GeneXpertDx System. Operator Manual. Software version 4.0. 300–7607, Rev. C.1.
16. Indira Soundiram, Cepheid Technical Advisor. Personal communication.
17. Procedure for use and maintenance of personal protective equipment.
18. Procedure for spill management.
19. Procedure for disinfection and decontamination.
20. Procedure for waste management.
21. The laboratory safety manual.
22. The WHO TB laboratory safety manual, 2013.
23. http://apps.who.int/iris/bitstream/10665/77949/1/9789241504638_eng.pdf
24. MSDS for the reagents and solutions used in the procedure.