TECHNICAL REPORT

Mastering the basics of TB control

Development of a handbook on TB diagnostic methods

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ECDC TECHNICAL REPORT

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Abbreviations

EPTB	Extrapulmonary tuberculosis
ERLN-TB	European Reference Laboratory Network for Tuberculosis
EU/EEA	European Union/European Economic Area
IGRA	Interferon-γ release assays
LTBI	Latent TB infections
NRL	National reference laboratory
TB	Tuberculosis
TST	Tuberculin skin test

Executive summary

Tuberculosis (TB) remains to be a major cause of morbidity and mortality in Europe. To improve both the prognosis of TB patients and TB disease surveillance, care of patients with TB should always start with a quality-assured laboratory diagnosis.

In 2007, a survey (2) on current mycobacterial laboratory services indicated that a European laboratory network on TB would add value for Europe. As a result of this survey, the European Reference Laboratory Network for Tuberculosis (ERLN-TB) was launched in January 2010. One of the main goals of this network is to support the harmonisation of methods in the EU/EEA. A key deliverable of 2010 was the 'Handbook of TB diagnostic methods', which is presented here.

The handbook consists of nine chapters describing the most relevant and reliable laboratory diagnostic methods. It covers biosafety, quality assurance, latent TB infection, smear microscopy, identification of *M. tuberculosis* and drug resistance, culture, drug susceptibility testing, molecular methods, and information related to the diagnosis of TB for physicians.

This first edition is intended to contribute to the harmonisation of methods for TB diagnosis and therefore also covers issues of comparability of TB diagnosis within the EU/EEA. ECDC will continue to work with the ERLN-TB to evaluate the impact of this collaborative technical report and make further provisions for the development of the document.

The experience gained from writing this technical report on diagnostic methods could be applied to other European reference laboratory networks, which would certainly benefit from the developed body of knowledge. There are also several tools available that would support interaction and collaboration between reference laboratories and laboratory experts, particularly in respect to improving the implementation of diagnostic techniques and further training/capacity building. This is exactly where ECDC's added value lies, as outlined in the ECDC founding regulation (1): to prevent and control communicable diseases in Europe by, among other measures, fostering the collaboration between experts and reference laboratories.

Introduction

Tuberculosis (TB) is a major cause of morbidity and mortality in Europe. High-quality laboratory diagnosis of TB is the basis for both individual patient treatment and surveillance.

In 2007, Drobniewski et al. (2) assessed existing mycobacterial laboratory services and quality control practices throughout the EU and confirmed the key role of national reference laboratories (NRL) for TB and their services. The main conclusion of the survey (2) was that a network of reference laboratories for tuberculosis could contribute to improve the performance of mycobacterial laboratories in Europe.

Based on these results, the European Reference Laboratory Network for Tuberculosis (ERLN-TB) was launched in January 2010, with the aim of strengthening TB diagnostics in the European Union. ERLN-TB is funded and coordinated by ECDC. One or two officially nominated reference laboratories from each Member State represent the Network, including EEA countries and candidate countries.

The three main goals of the network are: to support the harmonisation of methods within the EU/EEA; develop External Quality Assurance (EQA) schemes; and provide training activities within the Network to ensure EU-wide capacity-building for TB diagnostics. One of the main activities conducted during the Network's first year was the development of a handbook of key diagnostic methods for tuberculosis (Annex 1), the results of which you are reviewing now.

The aim of this handbook is to provide Network members, as well as other laboratories involved in the diagnosis of tuberculosis, with an agreed list of key diagnostic methods and their protocols in various areas of TB diagnosis, ranging from microbiological diagnosis of active TB to the diagnosis of LTBI. This handbook aims to provide a single source of reference by compiling all methods, with a strong focus on standard (reference) and evidence-based methods.

This handbook also wants to contribute to the improvement of disease surveillance data for Europe: data sent to ECDC's TESSy (The European Surveillance System) or other surveillance systems should be robust and backed by quality laboratory diagnostics.

How this report relates to other available work in this field

This technical report presents a compilation of methods currently applied in Europe. It describes common work carried out and endorsed by European laboratory experts. It also features methods and procedures developed or refined by ERLN-TB network partners.

What this document is/is not

This document is a technical report about developing a handbook of agreed methods in the field of TB diagnostics for laboratories serving reference functions in Europe. In Annex 1 it provides a comprehensive compilation of key methods for the diagnosis of TB. Relevant stakeholders should use this compilation as a basis for the validation, development, updating, and dissemination of information.

The current document is not an official handbook of methods.

Intended use and users

The report will be of interest to public health professionals and policy makers in the field of global TB control, particularly those involved in European initiatives which foster the progress towards elimination of TB. For laboratory experts, this document provides the basis for developing a future handbook of basic- and reference-level methods (to be agreed and validated) for the diagnosis of TB.

Material and methods

The first annual meeting of the ERLN-TB was held in 2010 in Stockholm. During this meeting, the Network partners came to a consensus agreement about the relevant topics to be included in a handbook on (reference) laboratory methods for the diagnosis of TB. The approach was to include several standardised and reliable methods, rather than to focus on one single method. A dedicated writing committee was formed to compile the first draft of these methods, using a handbook format. Each chapter includes descriptions of standardised diagnostic methods and highlights key considerations regarding biosafety and quality assurance.

A draft of the handbook was sent out to all ERLN-TB network partners for review and endorsement. An approved version was submitted to ECDC in September 2010. The current version of the handbook underwent final

consultation and received endorsement for publication by the ERLN-TB during its second annual meeting in January 2011.

Results

The first edition of the ERLN-TB handbook (Annex 1) consists of nine chapters, each with a list of relevant references. Below is a summary of each chapter.

1 Biosafety in clinical laboratory diagnosis of tuberculosis

M. tuberculosis can cause laboratory-acquired infections (LAI). In order to ensure effective infection control, it is crucial that a comprehensive and strict biosafety policy is developed and followed. Such a policy should include standardised rules and regulations for containment, personal protective equipment (PPE), standardised operating procedures (SOP) for the different laboratory tasks, and a transparent structure for regulating safe working conditions in diagnostic TB laboratories.

2 Quality assurance

National tuberculosis programmes should be supported by laboratories that provide reliable and quality-assured results. The chapter provides a comprehensive overview on existing International Organization for Standardization (ISO) standards relevant for the laboratory diagnosis of TB and describes internal and external quality assurance procedures.

3 Latent TB infections

Currently, two types of tests for the diagnosis of latent TB infections (LTBI) are used: tuberculin skin tests (TSTs) and Interferon-γ release assays (IGRAs). The chapter describes the two commercially available IGRAs for the detection of latent TB infection in detail and provides support for the interpretation and reporting of test results.

4 Smear microscopy

Two types of staining are most commonly used for the detection of mycobacteria: carbol-fuchsin staining (Ziehl-Neelsen, Kinyoun) and fluorochrome staining (auramine, auramine-rhodamine). The chapter describes the preparation of the required reagents and the sputum smear samples, as well as the staining procedures and the system of reporting results.

5 Culture for **Mycobacterium tuberculosis** *complex*

The use of cultures improves the sensitivity and specificity of TB tests, particularly at the early stages of the disease, in cases of treatment failures, and in extrapulmonary tuberculosis (EPTB). The chapter provides an overview of key principles for sampling and transportation of clinical specimens, processing of sputum and other specimens before inoculation to solid and liquid culture media, culture incubation and examination. The issue of contaminations is addressed as well as measurements necessary to be taken to prevent laboratory-acquired TB infections.

6 Identification of **Mycobacterium tuberculosis** *and drug resistance in cultures and sputum using molecular assays*

Growth of mycobacteria in culture media can take up to eight weeks as *M. tuberculosis* and many other mycobacteria grow very slowly. This can lead to a delay in the treatment of patients. Molecular assays can speed up mycobacterium identification and drug susceptibility testing, and thus lead to faster and more specific treatment. The chapter describes methods for the identification of mycobacteria and drug sensitivities from culture and clinical specimens and gives suggestions for the interpretation and reporting of results.

7 First- and second-line drug susceptibility testing for **Mycobacterium tuberculosis** *complex*

The main objectives of drug susceptibility testing are individual treatment management of tuberculosis cases and drug-resistance surveillance at the scale of a hospital, city, region, or country. The chapter describes three different methods for drug susceptibility testing for first- and second-line drugs on solid and liquid media.

8 Molecular typing of **Mycobacterium tuberculosis** *complex isolates*

Currently, various DNA fingerprinting methods are available that serve different purposes and have variable characteristics for specific applications. The three most important and widely applied methods (spoligotyping, VNTR typing, IS6110 RFLP typing) as well as the application of these methods are described in this chapter.

9 Information for physicians: the laboratory diagnosis of tuberculosis – first steps

The process of collecting material for the diagnosis of mycobacteria requires great care, and each step can influence the diagnosis. This chapter provides physicians with general information on the diagnosis of TB and gives specific instructions on the collection and treatment of clinical samples.

Discussion and conclusions

This report/handbook represents the first edition of a publication on the most reliable TB diagnostic methods, agreed by the members of the European Network of Reference Laboratories for TB (ERLN-TB). This publication is a compilation of protocols for the laboratory diagnosis of TB, targeted at public health professionals and laboratory experts. It was compiled to contribute to the harmonisation of methods in the field of TB diagnosis in the EU/EEA, with the goal of ensuring comparability of TB diagnoses within the EU/EEA, making sure that TB patients receive the best care possible, based on a quality-assured diagnosis. This publication can also support laboratories in establishing a safe working environment for the staff by minimising the risk of exposure to *M. tuberculosis.*

This first edition of the handbook will be published as an ECDC Technical Report in order to rapidly disseminate the work and foster further collaborations with the reference laboratories in Europe and other laboratory experts in the field of TB diagnostics.

The description of methods described here will need further validation before this publication can be released as an official handbook of methods. As new evidence and new diagnostic tools become available, it will also be vital to update the handbook for future editions. Over time, reader feedback might point out areas that are underrepresented in the current edition. The following sections need further work:

- Biosafety. This chapter will be expanded by a group of European biosafety professionals in order to cover additional issues such as biorisks and biorisk management in the handling of TB. This revision will be based on results of a scientific study carried out by a dedicated expert group on biosafety: ECDC BioRisk Initiative for Capacity Building and Knowledge Base Development (BRICK) (see: http://ecdc.europa.eu/en/activities/microbiology/biosafety/Documents/101117_Bioriskproject_Description.pdf). The study will be funded by ECDC and conducted in collaboration with expert partners and partner laboratories of the ERLN-TB in 2011.
- New tools. ECDC is currently working on several evidence-based guidance documents regarding the implementation of new tools (IGRA, molecular-based methods), which will be included in the next edition of this handbook.
- Molecular typing. It is planned to incorporate protocols on the most relevant methods in the area: spoligotyping, MIRU-VNTR, and RFLP. This is directly connected to another ECDC-funded project dedicated to molecular surveillance of MDR-TB (Molecular surveillance of MDR-TB in the EU, available from: http://ecdc.europa.eu/en/activities/microbiology/projects/Pages/projects_2010.aspx).

A second edition of this handbook will be published at a later stage. There are also plans to further develop this handbook into a more comprehensive communication on mastering the basics of TB control (diagnosis, treatment, prevention) in the European context, taking into account TB epidemiology, the strategic aim of TB elimination, and the promotion of quality and equity in TB care for vulnerable populations.

ECDC will continue to work with the ERLN-TB to evaluate the impact of this collaborative technical report and plan for the continued development of the document.

The experience gained from writing this technical report on diagnostic methods could be applied to other European reference laboratory networks, which would certainly benefit from the developed body of knowledge. There are also several other tools available that could support interaction and collaboration between reference laboratories and laboratory experts, particularly in respect to the implementation of diagnostic techniques and further training/capacity building. This is exactly where ECDC's added value lies, as outlined in the ECDC founding regulation (1): to prevent and control communicable diseases in Europe by, among other measures, fostering the collaboration between experts and reference laboratories.

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Annex 1.
Handbook of TB diagnostic methods

This annex includes a table of content and nine technical chapters on TB diagnostic methods, written by different authors. Each chapter has its own references.

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List of abbreviations

AFB	Acid-fast bacilli
BCG	Bacillus Calmette-Guérin
BD	Becton, Dickinson and Company
BSC	Biological safety cabinet
BSL	Biosafety level
CSF	Cerebral spinal fluid
CXR	Chest X-ray
DNA	Deoxyribonucleic acid
DST	Drug susceptibility testing
EQA	External quality assurance
EQC	External quality control
EU	European Union
HEPA filter	High-efficiency particulate air filter
HPF	High-power field
HVAC	Heating, ventilating and air-conditioning
IFN-γγ	Interferon-gamma
IGRA	Interferon-gamma release assay
IQC	Internal quality control
ISO	International Organization for Standardization
LAI	Laboratory-acquired infections
LED	Light-emitting diode
LJ medium	Löwenstein-Jensen medium
LTBI	Latent TB infection
MGIT	Mycobacteria growth indicator tube
MTBC	*Mycobacterium tuberculosis* complex
NTM	Non-tuberculous mycobacteria
PBMC	Peripheral blood mononuclear cells
PPD	Purified protein derivative
PPE	Personnel protective equipment
QA	Quality assurance
QC	Quality control
RFLP	Restriction fragment length polymorphism
SOP	Standard operating procedures
TST	Tuberculin skin tests
VNTR	Variable number tandem repeat

1. Biosafety in the clinical laboratory diagnosis of tuberculosis

Maryse Fauville-Dufaux, Vincent Jarlier, Dick van Soolingen, Sven Hoffner

Note: This chapter consists largely of a summary of principles and procedures previously published by the World Health Organization (4). It focuses on aspects relevant to the infrastructure of European diagnostic laboratories for TB. For a more comprehensive view, please refer to the publications listed at the end of this chapter.

1.1 Background and principles

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), is classified as a risk group 3 agent, which calls for a Biosafety Level 3 laboratory (BSL3) for culture, drug susceptibility testing and other studies. Access to the safety laboratory should be restricted to staff members and accredited visitors[1].

It is well documented that *M. tuberculosis* can cause laboratory-acquired infections (LAI). *M. tuberculosis* is commonly found in the top-ten list of hazardous agents for laboratory staff, and only in a minority of cases can the source of the TB infection be traced to a specific laboratory accident[2,3,5]. The most important route for LAIs are aerosols. Thus, infection control efforts need to focus on limiting the generation of aerosols during laboratory work, for example through safe centrifugation and by using pipetting devices. The potential risks depend on the type of techniques used. For example, needlestick injuries are rather rare as in most laboratories the BD Bactec 460 system has been replaced by the MGIT 960 system, which does not use needles for the inoculation of test vials.

For effective infection control it is crucial that a comprehensive and strict biosafety policy is developed and followed. The policy should include standardised rules and regulations for containment, personal protective equipment (PPE), a set of standardised operating procedures (SOPs) for all the different laboratory tasks, as well as transparent and clearly defined levels of responsibility for establishing and maintaining safe working conditions in the diagnostic TB laboratory[1,2,4].

When assessing the risk of laboratory infections due to *M. tuberculosis,* it should be kept in mind that the risk of infection is related to the concentration of bacteria and the presence of infectious aerosols. Clinical specimens such as sputum for smear examination pose a lower biohazard than cultures of the bacteria, e.g. for susceptibility testing. Also, working with extracted DNA for molecular characterisation is much less risky than the culture and extraction process. Therefore, sputum smear microscopy, treatment of specimens before culture, and DNA extraction from clinical specimens for molecular techniques (DNA identification, detection of gene mutations related to drug resistance or possibly genotyping) might be carried out in a biological safety cabinet (BSC) in a BSL2 laboratory setting. Conversely, techniques performed on mycobacterial cultures, such as the extraction of DNA from positive cultures as well as conventional drug susceptibility testing, require a higher degree of biosafety and the use of a BSL3 containment laboratory. Molecular techniques on previously extracted DNA do not require a biosafety level standard[1,2,4].

Before initiating a new test, task or method in a laboratory, a risk assessment is obligatory. This will identify possible risks and allow for proper infection control measurements.

Overall infection control is based on four main components:

- administrative component;
- environmental (engineering) component;
- personal protective equipment; and
- technical expertise and training.

If correctly implemented, each of these parts will contribute to decreasing the risk of exposing laboratory personnel to the pathogen.

A scheme for biosafety training of all laboratory staff is a useful tool for reducing/avoiding the risk of laboratory infections. A policy for the medical examination of new staff members as well as the regular examination of all laboratory staff offers a way to document effective infection control measures and allows for the timely detection of possible LAIs.

The different sections of the biosafety policy and guidelines should be based on national and international regulations and recommendations/guidelines as well as on a local risk assessment for each step of the clinical diagnostic work. Since international recommendations might change over time, it is of great importance to always base new biosafety instructions on the most recent sources and to implement a system for the regular update of the biosafety policy[1,4].

This chapter is based on available biosafety recommendations at the time of its writing (summer 2010), and should not be seen as a replacement of other international recommendations. A new recommended standard for biosafety in TB laboratories is expected from the WHO by the end of 2010. ECDC is also working with a group of biosafety professionals[6] who in 2011 will take a risk-based approach for a scientific study that will analyse overall work practices in TB laboratories in Europe and make some general recommendations to the ERLN-TB. This chapter reflects the views of the authors and does not imply an endorsement by ECDC.

1.2 The containment laboratory (biosafety level 3)

1.2.1. General considerations

The international biohazard warning symbol and other relevant signs displayed on the laboratory access doors must identify the biosafety level and the name of the laboratory supervisor who controls access. Signs must also indicate conditions for access, for example the use of respirators and immunisation requirements.

Figure 1.1. Biohazard warning sign for laboratory doors

Source: World Health Organization. Laboratory biosafety manual. 3rd edition. Geneva; 2004[4]

Inside the laboratory, protective clothing must be used according to local regulations. It is recommended that non-permeable/waterproof laboratory coats be used, either with a solid front or a wrap-around cut. Where appropriate, shoe covers (overshoes) or dedicated shoes should be worn. Front-buttoned standard laboratory coats are unsuitable, as are sleeves that do not fully cover the forearms. Laboratory protective clothing must not be worn outside the laboratory and it must be decontaminated before it is laundered. Open manipulations of all potentially infectious material should always be conducted within a biological safety cabinet (class I or II). Respiratory protective equipment is necessary for some laboratory procedures[4].

1.2.2 The laboratory facility

There are several technical aspects to consider before a BSL2 or BSL3 facility is constructed. Planning should include the choice of equipment, ventilation system, technical maintenance and waste management. All technical aspects should be documented and strictly follow the relevant national rules and regulations. If no national rules have been established, international rules must be used. The choice between BSL2 and BSL3 or a combination of BSL2 and BSL3 zones in the same facility is based on European and national regulations that mainly refer to the type of manipulations carried out in the facility.

The laboratory must be separated from areas that offer unrestricted entrance to the building. If the laboratory cannot be located in a separate building, separation may be achieved by placing the laboratory at the blind end of a corridor, or constructing a partition and a door or access through an anteroom. The anteroom should have facilities for separating clean and dirty clothing, and a shower. The anteroom doors should be self-closing and

interlocking so that only one door can be open at a time. A break-through panel or emergency exit may be provided for emergency exit use.

As a rule, the following issues should be taken into consideration[4]:

- Ample space must be provided for the safe conduct of laboratory work around the equipment and for cleaning and maintenance.
- Walls, ceilings and floors should be smooth, easy to clean, impermeable to liquids, and resistant to the chemicals and disinfectants normally used in the laboratory. Floors should be slip resistant.
- Openings through these surfaces (e.g. for service pipes) should be sealed to facilitate decontamination of the room/s.
- Benchtops should be impervious to water and resistant to disinfectants, acids, alkalis, organic solvents, and moderate heat.
- Illumination should be adequate for all activities. Undesirable reflections and glare should be avoided.
- Laboratory furniture should be sturdy.
- Open spaces between and under benches, cabinets and equipment should be accessible for cleaning.
- Windows must be closed, sealed and break-resistant.
- Storage space must be adequate to hold supplies for immediate use and thus prevent clutter on benchtops and in aisles. Additional long-term storage space, conveniently located outside the laboratory working areas, should also be provided for storage of samples, cultures, records and for designated laboratory waste prior to autoclaving.
- A hand-washing station with hands-free controls should be provided near each exit door.
- To contain aerosols, a controlled ventilation system should continuously maintain the negative pressure. A visual monitoring device – preferable connected to an alarm – should be installed.
- The building ventilation system must also ensure that air from the containment laboratory is not re-circulated to other sections of the building. Air may be filtered by HEPA filters, reconditioned and re-circulated. When exhausted air from the laboratory is discharged to the outside of the building, it must be dispersed away from occupied buildings and air intakes. This air may be discharged through HEPA filters. A heating, ventilation and air-conditioning (HVAC) control system may be installed to prevent positive pressurisation of the laboratory. Consideration should be given to the installation of audible or clearly visible alarms to notify personnel of HVAC system failure. A system ensuring the proper work of the HEPA-filter units should be in place.
- If the facility is equipped with HEPA filters, they must be installed in a manner that permits gaseous decontamination and testing. The exhausted air from BSCs, which will have been passed through HEPA filters, must be discharged in such a way as to avoid interference with the air balance of the cabinet or the building exhaust system.
- BSCs should be situated away from walking areas and out of crosscurrents from doors and ventilation systems.
- There should be a programme in place for the regular testing and validation of BSCs.
- An autoclave for the decontamination of contaminated waste material should be available in the containment laboratory. If infectious waste has to be removed from the laboratory for decontamination and disposal, it must be transported in sealed, unbreakable and leakproof containers according to national or international regulations, as appropriate.
- Decontamination of wastewater from the laboratory (by hypochlorite or heat treatment) before introducing it to the public water system should be considered.
- Safety systems should be implemented to handle the risks of fire and electrical emergencies. Emergency shower and eyewash facilities should be installed.
- Suitably equipped and readily accessible first-aid areas or rooms should be available near the BSL3 laboratory.
- In the planning of new facilities, consideration should be given to the provision of:
  - a mechanical ventilation system that provides an inward flow of air without recirculation; and
  - a dependable supply of good quality water. There should be no-cross connections between sources of laboratory and drinking-water supplies. An anti-backflow device should be fitted to protect the public water system.
- There should be a reliable and adequate electricity supply and emergency lighting to permit safe exit. A back-up system is recommended, e.g. a stand-by generator for the support of alarm systems and other essential equipment such as BSCs, freezers, etc.

1.3 Procedures[1,4]

Based on local risk assessments, a set of detailed SOPs for the safe performance of all tasks carried out in the facility should be developed and implemented. As a rule, the following procedures should be adhered to:

- Pipetting by mouth must be strictly forbidden.

- All technical procedures should be performed in a way that minimises the formation of aerosols and droplets. The use of hypodermic needles and syringes should be avoided. They should never be used as substitutes for pipetting devices.
- All spills, accidents and overt or potential exposures to infectious materials must be reported to the laboratory supervisor. A written record of such accidents and incidents (including precautions that will be taken to avoid similar accidents in the future) should be maintained.
- There should be a procedure in place for emergency actions to be taken after exposure to infectious materials, including a medical surveillance programme.
- A written procedure for the clean-up of all spills must be developed; all staff members must be trained regularly and should have a good knowledge of, and a high level of compliance to all biosafety instructions.
- Potentially contaminated liquids must be decontaminated (autoclaved) before being discharged to the public sewer system.

1.4 Personal protective equipment (PPE)

The use of Personal protective equipment (PPE) should be regulated in local SOPs based on a local risk assessment as well as national and international regulations. It is crucial that the agreed level of PPE is known, respected and followed by all staff members and visitors to the laboratory.

The following table (Table 1.1), adapted from the WHO laboratory biosafety manual[4], lists examples of personal protection items for laboratory technicians.

Table 1.1. Examples of personal protective equipment

Personal protective equipment (examples)		
Equipment	**Hazard corrected**	**Safety features**
Laboratory coats, gowns, coveralls	Contamination of clothing	Back opening. Cover street clothing
Plastic aprons	Contamination of clothing	Waterproof
Footwear	Impact and splash	Closed-toe
Goggles	Impact and splash	Impact-resistant lenses (must be optically correct or worn over corrective eye glasses). Side shields
Safety spectacles	Impact	Impact-resistant lenses (must be optically correct). Side shields
Face shields	Impact and splash	Shield entire face. Easily removable in case of accident
Respirators	Inhalation of aerosols	Designs available include single-use disposable, full-face or half-face air purifying, full-face or hooded powered air purifying (PAPR) and supplied air respirators
Gloves	Direct contact with microorganisms; cuts	Disposable microbiologically approved latex, vinyl or nitrile; hand protection; mesh

Please refer to Chapter 5 for further details on laboratory safety levels and conditions when working with mycobacteria cultures.

1.5 The human resource component

Even the technically most advanced biosafety system will fail if the laboratory staff neglect the biosafety rules. It is recommended that experienced technical staff members remain involved throughout the planning and implementation of the biosafety programme[1,4].

A well-designed training programme for biosafety, offered yearly to all staff members, will raise the level of knowledge about the laboratory infection control plan. New staff members as well as students and/or visiting researchers should be informed about all relevant rules and recommendations before being allowed to work in the laboratory. It is recommended that all rules and regulations that govern the laboratory are communicated by the responsible person (often the laboratory supervisor) and a knowledge test is performed before the actual laboratory work with the pathogen. Procedures governing medical emergencies and accidental exposure to infectious material should be in place. All reported events should be investigated. The staff member responsible for laboratory infection control should discuss the cause of any incidence/accident with the involved persons. This should be done in an atmosphere of cooperation to avoid that staff members cover up details (or become less inclined to report incidences/accidents in the future). A written suggestion for improvement should be prepared, describing the preventive measures to be taken.

It is the responsibility of all staff members to achieve and maintain a safe working environment. Good biosafety laboratory practices should be known and embraced by all staff members, both to prevent accidents and to avoid that staff members claim ignorance of the safety guidelines after an accident.

1.6 Specific laboratory tasks related to hazards

As mentioned above, the risk of laboratory infections due to *M. tuberculosis* is related to the concentration of bacteria and the possible creation of infectious aerosols.

Biosafety measures must always be based on risk assessment. Here are some examples[4]:

- **Handling of containers with clinical specimens.** Even if this is unlikely to generate aerosols, exposure to the tubercle bacilli is possible. The outsides of containers used for the collection of clinical specimens are frequently contaminated by *M. tuberculosis* or other airborne pathogens. Specimen containers should be handled carefully and only opened in BSCs.
- **Centrifugation.** Fluid may spill from centrifuge tubes or tubes may break, releasing aerosols. Only closed rotors with appropriate and safe centrifugation tubes should be used. Centrifuge safety cups should be used and loaded/unloaded in the BSC.
- **Pipetting.** Pipettes and Pasteur pipettes in particular are likely to generate bubbles that burst and form aerosols. Pipetting should be performed only in BSCs and by using disposable pipettes. Pipetting by mouth should never be allowed.
- **Mechanical homogenising (vortexing, grinding, blending).** Appropriate procedures for avoiding the creation of aerosols should be applied. Mechanical homogenising should always be carried out in a BSC.
- **Sonication, heating or boiling of samples, e.g. for the extraction of nucleic acids.** Appropriate procedures should be applied to avoid splashing and the creation of aerosols.
- **Use of bacteriological loops.** Loops charged with infectious material should not be placed in a Bunsen burner due to the risk of splashing and the creation of aerosols. The use of disposable plastic loops is recommended.
- **Animal studies.** Major risks are self-inoculation and exposure to aerosols. The litter of infected animals can be contaminated and thus become a potential source of infection.
- **Transport and shipping of *M. tuberculosis* strains.** There are strict rules that must be followed when transporting clinical samples, and especially isolated *M. tuberculosis* bacteria. These rules depend on the actual transport situation, e.g. transport between countries or by airplanes. It is the responsibility of the shipping laboratory to identify the relevant regulations and follow them strictly.
- **Storage of collections of *M. tuberculosis* isolates and reference strains.** There should be local procedures with regard to biosafety for the storage of bacteria and clinical specimens containing bacteria. All freezers, fridges and other storage cabinets should be properly labelled, with the person in charge clearly marked.

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Appendix

The following checklist provides criteria for the planning or overhaul of BSL3 laboratories.

Table 1.2. Technical considerations for BSL3 laboratories

Checklist for BSL3 laboratories	
1.	The BSL3 laboratory should be housed in a solid building, ideally separated from other disciplines.
2	The BSL3 laboratory should be separated from other (BSL2) laboratories.
3.	The BSL3 laboratory can only be entered via an anteroom.
4.	Floors, walls, doors and working surfaces should be non-absorbing, resistant to acids, bases and decontaminants, easy to clean, and without sections that are hard to access. Seams, gaps and cracks should be completely caulked/grouted to seal them.
5.	The floor should be liquid-tight (higher skirting, welded seams).
6.	Doors should be self-closing.
7.	Windows should be sealed so they cannot be opened and are air-tight.
8.	The entrance door should have a window to monitor the BSL3 laboratory.
Airflow	
9.	Negative pressure must be kept in the anteroom and the laboratory (minimum pressure difference of 15 Pa, e.g. -15 Pa in the anteroom and -30 Pa in the laboratory).
10.	The negative pressure needs to be monitored constantly and displayed outside the laboratory, preferable next to the entrance.
11.	If the negative pressure gets out of range, an audio-visual alert in the anteroom and the laboratory should activate.
12.	Air from the laboratory and the anteroom should be exhausted via an independent air duct with a HEPA filter. The air intake duct does not need to contain a HEPA filter, but should have a one-way valve to prevent any backflow in case the negative pressure is not maintained.
13.	The ventilating system must have an emergency off-switch to prevent a build up of positive pressure in the laboratory if the exhaust fails.
14.	The ventilating system for a BSL3 laboratory should be completely independent and separated from other ventilation systems to prevent cross-contamination.
15.	There should be a minimum of six to 12 complete air exchanges per hour in a BSL3 laboratory.
16.	The air intake duct should be separated from the exhaust duct to prevent airflow contamination between the two ducts.
Entrance and access	
17.	The entrance of the BSL3 laboratory should be marked with a biohazard sign, information on the containment level, responsible staff members and biosafety office (including telephone numbers).
18.	Access to a BSL3 laboratory should be restricted to authorised staff members and controlled by key cards/electronic passes.
19.	An uninterruptible power supply for should provide emergency power.
20.	If staff technicians are allowed access, they should be use personal protective equipment and be supervised by regular laboratory staff. Work should be carried out in the early morning when no viable cultures are processed and the laboratory is relatively safe after the multiple air exchanges during the night and UV treatment. Equipment touched by staff technicians has to be disinfected with 80% ethanol. Regular maintenance personal should be subject to regular occupational health checks.
21.	Depending on the size of the working space, there should be a sealed emergency exit.
Anteroom	
22.	A door interlock system should be used to prevent the simultaneous opening doors, thus preventing leakage of potentially contaminated air from the BSL3 lab to the corridor. It should be possible to overrule this system.
23.	The anteroom is normally considered a part of the BSL3 area because anteroom and laboratory are part of the same ventilating system, when in fact the anteroom is a transition zone between uncontaminated and potentially contaminated area. It is therefore recommended that the anteroom is split in two parts: an unclean and a clean zone. The two zones should be clearly marked, e.g. by a laboratory bench.
24.	Some laboratories only have a small anteroom that is too small to be divided into two zones. If that is the case, laboratory coats should be left in the BSL laboratory.

25.	The anteroom should have a soap dispenser, an alcohol dispenser, a sink, and a disposable hand towel dispenser. The dispensers as well as the faucet/tap should be hands-free.
26.	The waste container and the container for worn lab coats should be sealable. Coats have to autoclaved before being laundered. Towels are considered relatively harmless and do not need to be autoclaved.
27.	An emergency eyewash facility should be installed near the sink.
Biological safety cabinets (BSCs)	
28.	Class I and II BSCs are acceptable. For maximum containment at the source, the installation of a Class III BSC can be considered, although this would pose ergonomic disadvantages.
29.	The BSC should be positioned in the laboratory so that airflow would not be disturbed by personnel or open doors. A Class III BSC is not affected by this.
30.	Exhaust air from a BSC can be discharged in three ways. 1. Air can be recirculated to the room, which is not advisable because of possible BSC filter leaks which then would bring contaminated air to the laboratory. This is not just a hypothetical risk, especially when BSCs are not well-maintained. 2. The BSC features a continuous airflow connection with a bypass for air treatment. 3. With a 'thimble' or 'canopy hood', exhaust can be recirculated to the room or discharged to the outside of the building via a dedicated duct or through the main exhaust system. When a BSC is switched on, it also contributes to the negative pressure in the laboratory. But only options two and three ensure that the negative pressure is still maintained when the BSC is off.
31.	BSCs have to be tested and certified at least once a year. Between maintenance intervals BSCs are decontaminated by fumigation with formaldehyde gas.
Digitalisation of information	
32.	Any paper-based communication between the BSL3 laboratory and the area outside the laboratory should be avoided. Instead, a computer-based laboratory management information system should be used.
Waste	
33.	Containers for BSL3 waste should be solid, unbreakable, closable, and autoclavable.
34.	A BSL3 laboratory should be equipped with an autoclave to decontaminate BSL3 waste. An autoclave with openings toward the laboratory *and* the hallway is ideal, as loading of BSL3 occurs directly from the BSL3 containment area. A stand-alone autoclave inside the BSL3 laboratory is also acceptable, provided there is an adequate solution to deal with the contaminated steam/condensate. If both options are not possible, an autoclave in the vicinity of the BSL3 laboratory (same building) is acceptable, but containers need to be leakproof and should only be moved under the supervision of the BSL3 laboratory, without any intermediate storage.
Wastewater	
35.	If the BSL3 laboratory has no sink, liquid waste has to be inactivated in an autoclave.
36.	If a sink is to be installed in a BSL3 laboratory, care should be taken that wastewater is not discharged in the public sewer system. Instead, wastewater should be collected in a dunk tank and inactivated (heat, chemicals) before it is discharged.
37.	Although the anteroom is officially considered part of the BSL3 laboratory, the risk of contamination by BSL3 microorganisms is considered so low that wastewater does not have to be decontaminated.

2. Quality assurance

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2.1 Background and principles

National tuberculosis programmes should be supported by laboratories that provide quality-assured and reliable results. Several European standards (ISO 17025, ISO 15189, ISO 9001) are dedicated to the management of quality assurance (QA)[5].

Quality assurance covers a number of distinct procedures that help to improve the quality of test procedures and therefore the confidence in national TB programmes. Every laboratory must establish and document quality assurance for tests used on patients' specimens/isolates. Obviously, quality can be equally good in laboratories with or without a formal QA system, but adherence to standards is helpful in identifying problems and the improvement of quality in a systematic manner.

For laboratories, the formal standards adopted by the majority of European countries are the European standards: ISO 17025 (General requirements for the competence of testing and calibration laboratories), ISO 15189 (Medical laboratories – particular requirements for quality and competence), and ISO 9001 (Quality management systems – Requirements). Accreditation by the two former standards addresses the quality system and provides a technical assessment, in contrast to certification by ISO 9001, which addresses the quality system only.

ISO 17025 and ISO 15189 are very similar in their specific requirements. ISO 17025 emphasises the needs of customers, whereas ISO 15189 emphasises the need of patients and clinicians. Accredited laboratories often only have ISO 17025 implemented, as ISO 15189 was only published in 2003. The decision to implement one or the other standard may rely on local and national requirements, the status of a health laboratory (commercial or public), proximity to clinical departments (diagnostic laboratory, reference laboratory), and the needs of other laboratories at the institute (due to accreditation fees).

Table 2.1. Overall scope of ISO 17025 and ISO 15189[5]

ISO 17025. General requirements for the competence of testing and calibration laboratories	ISO 15189. Medical laboratories. Particular requirements for quality and competence
'ISO/IEC 17025:2005 specifies the general requirements for the competence to carry out tests and/or calibrations, including sampling. It covers testing and calibration performed using standard methods, non-standard methods, and laboratory-developed methods. It is applicable to all organizations performing tests and/or calibrations. These include, for example, first-, second- and third-party laboratories, and laboratories where testing and/or calibration forms part of inspection and product certification. ISO/IEC 17025:2005 is applicable to all laboratories regardless of the number of personnel or the extent of the scope of testing and/or calibration activities. When a laboratory does not undertake one or more of the activities covered by ISO/IEC 17025:2005, such as sampling and the design/development of new methods, the requirements of those clauses do not apply. ISO/IEC 17025:2005 is for use by laboratories in developing their management system for quality, administrative and technical operations. Laboratory customers, regulatory authorities and accreditation bodies may also use it in confirming or recognizing the competence of laboratories. ISO/IEC 17025:2005 is not intended to be used as the basis for certification of laboratories.'[5]	'ISO 15189:2007 specifies requirements for quality and competence particular to medical laboratories. ISO 15189:2007 is for use by medical laboratories in developing their quality management systems and assessing their own competence, and for use by accreditation bodies in confirming or recognising the competence of medical laboratories.'[5]

Laboratory accreditation/certification (by a national body providing official recognition of laboratory quality) according to these standards recognises the professional competence of the laboratory and provides an official indicator of high performance standards. Central terms are given in Table 2.2 and further terms are defined in the 'International vocabulary of metrology – basic and general concepts and associated terms (VIM)'[1] and the 'IUPAC gold book'[2]. This chapter outlines the most important requirements from ISO/IEC 17025 and ISO 15189. The chapter takes into account the special requirements imposed by the medical environment and the contribution of medical laboratory services to patient care. It recognises that medical laboratories must provide not only testing of

patient samples, but also advisory, interpretative and educational services. The full text of these standards is available for purchase online[5].

Table 2.2. Central terms and abbreviations in quality assurance

Quality assurance (QA)	A system for continuously improving and monitoring the reliability, efficiency and clinical utilisation of laboratory tests. Quality control, quality improvement and method validation are integral components of quality assurance.
Quality control (QC)	A process of regular performance checks to ensure that a method is performing as expected. **Internal quality control (IQC)** includes controls tested in parallel with specimens/isolates. This evaluates the precision and accuracy of the test results, the performance of the test reagents, and how well laboratory staff perform when carrying out the test. **External quality control (EQC)** or proficiency panels are specimens/isolates received from an independent organisation in order to assess the performance of the participating laboratory. Inter-laboratory comparison is an alternative when proficiency panels are not available and includes the exchange of specimens/isolates with other laboratories (usually at least three) that perform the same tests.
Validation	Validation is the 'confirmation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled.' (ISO 17025)
Key indicators	For each test, key indicators should be identified and followed routinely in order to monitor trends (early detection of deviations).

2.2 Selection and implementation of tests

The laboratory should use testing methods that meet the needs of the customer and include procedures for sampling, handling, transport, and the storage and preparation of specimens/isolates. Methods from international, regional, or national standards by reputable technical organisations, from scientific journals, or as specified by a kit/equipment manufacturer should be used. Laboratory-developed methods, or methods adopted by the laboratory, may also be used if appropriate and validated. The laboratory should confirm that it can properly perform the methods before introducing the tests.

2.3 Validation

Non-standard methods, laboratory-developed methods, standard methods used outside their intended scope, modifications of standard methods, and changes in established methods should be validated. Validation is the 'confirmation by examination and the provision of objective evidence that the particular requirements for an intended use are fulfilled' (ISO 17025). Validation includes written requirements specification, determination of the assay characteristics, a check that the requirements are fulfilled by the assay, and a statement on the validity. For standardised methods it is only required to validate that the method is working locally as expected. Validation is a relative process: sensitivity and specificity of diagnosis is calculated in relation to a standard, verified procedure. Part of the validation may have been carried out by the manufacturer or by other laboratories. Since the '*in vitro* diagnostic directive' (IVD)[6] came into force in 2003, manufacturers are legally bound to carry out extensive validation. In this case, the validation can be a reduced equivalent, but the laboratory still has to verify that the method works locally as expected. In all cases, the laboratory should have access to documentation equivalent to a full validation and assess whether the validation is relevant to the use and whether the requirements are fulfilled.

It is not possible to recommend one validation method for all tests. Validation can be done using:

- a comparison of results achieved with other methods;
- inter-laboratory comparisons;
- a systematic assessment of factors influencing the result; and
- an assessment of the uncertainty of the results based on the scientific understanding of the theoretical principles of the method and practical experience.

An overview of validation principles can be found in Westgard (2008)[3] and Westgard (2010)[4]. Apart from that the following factors may be assessed:

- coefficients of variation;
- uncertainty of results;
- detection limit;
- sensitivity;
- specificity;
- linearity;
- limit of repeatability and/or reproducibility; and
- robustness.

In many cases, range and uncertainty of the values can only be given in a simplified way due to lack of information.

2.4 Controls and trend monitoring by key indicators

Appropriate internal quality controls (IQCs) should be selected and run in parallel with specimens/isolates in order to monitor routine performance regularly (e.g. control slides for microscopy). Reference strains can be purchased from different strain collections.

Key indicators can be used to monitor performance trends over time and allow early identification of deviations (e.g. proportion of contaminated cultures or proportion of inconclusive IGRA results over a certain time period). NRL from each country must provide guidelines for IQCs in accordance with the ISO standard requirements, the methods used, and local conditions (infrastructure, workload, equipment, and staff). Example of IQCs can be found in Chapters 3 to 7.

External quality control (EQC) programmes are essential parts of quality assurance. Programmes of relevance for mycobacteriology laboratories are available through INSTAND, UKNEQAS, CDC, LabQuality and the WHO supranational TB reference laboratories, to which all national TB reference laboratories have links. If EQC programmes for a test are not available, exchanges with other laboratories can be arranged. A minimum of three laboratories should participate; however, EQC programmes do not replace IQC.

2.5 Physical and environmental conditions

The basic set-up of a laboratory (energy supply, lighting, environmental conditions) should be conducive to its testing activities. Physical and environmental conditions that can negatively affect results should be monitored and documented. Areas with incompatible activities should be separated (e.g. primary specimens and mycobacterial isolates and pre- and post-amplifications areas) and precautions to prevent cross-contamination should be taken. Access to, and the use of, laboratories must be controlled if it affects the quality of tests. Measures should also be taken to ensure good housekeeping in the laboratory, thus improving biosafety (Chapter 1).

2.6 Equipment

Equipment and related firm- and software must be capable of achieving the required level of accuracy and should comply with specifications relevant to the tests. Procedures for handling, storage, use and maintenance should ensure proper functioning and prevent contamination or deterioration. Equipment with a measuring function must be calibrated initially and regularly thereafter. Procedures should be in place to ensure that equipment suspected of malfunction is not used until checked.

Records should be maintained for critical equipment: manufacturer's name; identification and serial number; compliance checks; location; manufacturer's instructions; results; reports; certificates of all calibrations/adjustments; acceptance criteria; date of next calibration; maintenance plan; registrations of damage, malfunction, modification or repair. The equipment itself should be labelled with the status of calibration, date of last calibration, and the next recalibration date.

2.7 Management and staff

Managerial and technical staff must be authorised to carry out the tests to the required standards. All necessary resources must be available to them. Management and staff should be free of any conflicts of interests, and key personnel should have appointed deputies. One person should be appointed quality officer. Management must document the competencies of the staff to ensure that equipment is properly handled, carry out testing, assess and approve results, and sign reporting forms. In some countries, formal authorisation may apply to some staff members. Staff members giving guidance or interpreting results should be knowledgeable about the applied technology and the significance of the results. Ideally, staff should be employed in permanent positions or have signed a contract. Job descriptions should be available for managerial, administrative, and technical staff involved in testing.

2.8 Documentation and registration

Laboratories document (in local language) their policies and procedures for testing and for equipment use to ensure the quality of results. All documents must be reviewed and approved by authorised staff before being issued, be read and understood by relevant staff and revised on a regular basis. The use of expired/changed documents must be prevented. All documents should be easily accessible. Handwritten corrections and annotations should quickly be incorporated into the official version of the documented. Handwritten corrections should be dated and signed by initials.

Procedures for registration of specimens should include specimen identification, access, archiving, maintenance, and disposal. Procedures should specify how long registration forms have to be kept on file. Registration forms

should be clearly legible, accessible, remain confidential to outsiders, and be kept in conditions that prevent damage and loss. Calculations and data transfers should be subject to appropriate and systematic checks. Registration documents should make it possible to replicate the entire testing process. When errors are identified, the correct information should be entered next to the error without overwriting the incorrect entry and then be signed or initialled and dated by the staff member who corrected the information.

Electronic data must be protected (entry; storage, including back-up, access, transmission, and processing). In addition, in-house developed computer software ought to be documented and validated.

2.9 Sampling guidance, review of requests, and service to customers

It is essential that the laboratory provides the customer with standardised operating procedures (SOPs) (based on existing guidelines) for sampling that are easily accessible at the sampling location, e.g. online. The sampling guidelines should address all factors that need to be controlled in order to ensure the validity of the test (e.g. specimen quality and volume, specimen number, transportation time and temperature). When specimens are suboptimal or unacceptable for testing, the result report should indicate this and/or this should be communicated to the appropriate staff members.

Procedures must be implemented for the review of requests, tenders and contracts for testing. For routine diagnostics, recommendations from the laboratory and the completed request form should be sufficient. The laboratory will identify customer needs, including guidance and analysis of results. In case of major deviations/delays, the customer should be informed.

2.10 Subcontracting, services and supplies

It is the responsibility of the laboratory to ensure that all works undertaken by subcontractors are completed to meet the required standards. Examples are the calibration of equipment or second-line drug susceptibility testing of *M. tuberculosis* at another laboratory. Policies and procedures should be in place for services and supplies that directly influence diagnostic quality: procedures should cover the purchase, receipt, and storage of reagents, as well as compliance of supplies with standards and regulations. The laboratory should assess suppliers of critical services and supplies. CE-marked reagents are produced according to the EU directive and are in compliance with national regulations.

2.11 Complaints, errors and corrective actions

Policies and procedures regarding complaints, deviations, and corrective actions should be established and registered. When errors are identified, the significance should be assessed and corrective actions implemented. The customer should be informed and the results corrected or withdrawn. It should then be assessed when the testing can be resumed. If the assessment indicates that the error could happen again (systematic error), corrective action must be taken immediately and its effect controlled. All steps regarding corrective actions have to be authorised first.

2.12 Improvement

Senior management should be committed to developing, implementing and improving the quality assurance system. The laboratory should improve the quality system regularly by establishing a quality policy and formulating quality objectives. Improvement methods include preventive actions, assessments of complaints and deviations, corrective actions (as above), observations from internal audits, data analyses, and management evaluations. Internal audits are carried out annually by trained, qualified staff not involved in the audited activities. Management reviews carried out annually assess the suitability of policies and procedures, staff reports, results from internal audit(s), corrective and preventive actions, assessments from external parties, results from external proficiency panels or inter-laboratory comparisons, changes in workload, customer enquiries, complaints, and other relevant factors.

2.13 Reporting the results

It is essential that the results of each test are reported accurately, clearly, unambiguously, and objectively. Each report includes a title, the name and address of the laboratory, the unique identification of the test report (all pages), an end-of-report message (or other indicator at the end of the report), the name and address of the customer, the used method, a description of specimens/isolate (including sampling date), patient identification, specimen type, the date of receipt of the specimen/isolate, the test result, and the person(s) authorising the report.

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3 The diagnosis of latent TB infection

Dimitrios Papaventsis and Vladyslav Nikolayevskyy

3.1 Background and principles

Most people who are initially infected with *Mycobacterium tuberculosis* do not develop active tuberculosis. This state (a person infected with the TB bacillus who does not develop active TB) is called latent TB infection (LTBI)[1]. This state is characterised by persistence and a low-rate multiplication of viable *Mycobacterium tuberculosis* bacilli within macrophages and evidence of an immune response against the bacterium, but without clinical manifestation and radiological and bacteriological evidence of active disease. One third of the world's population (almost two billion people worldwide) is estimated to be latently infected with tuberculosis – an enormous reservoir of potential tuberculosis cases[2]. Latency can be maintained for the lifetime of the infected person.

Primary infection leads to active disease in 10% of infected individuals, mostly within two years of infection[3]. When the host immune response weakens (e.g. through HIV infection, malnutrition, the use of steroids or other immunosuppressive medications, or advanced age), reactivation of latent infection occurs[4].

Being non-infectious, those latently infected with the TB do not pose an immediate risk of TB transmission. Detection of the LTBI, however, is an important means of global TB control and constitutes a major part of the WHO Global Plan to Stop TB[5]. Putting people with LTBI on chemoprophylaxis significantly reduces their risk of developing active TB. An ideal test for LTBI diagnosis should meet the following criteria:

- High sensitivity in all populations at risk.
- High specificity regardless of BCG vaccination and infection with environmental mycobacteria.
- Reliability and stability over time.
- Objective criteria for positive result, affordability and easy administration.
- Ability to distinguish recently infected individuals with increased risk of progression to active TB.

There are currently two groups of tests for LTBI diagnosis: tuberculin skin tests (TST) and interferon-γ release assays (IGRA).

3.1.1 Immune response to *M. tuberculosis*

The immune response to *M. tuberculosis* is multifaceted. Immunological mechanisms involved in maintaining a latent infection are complex, but are clearly necessary to prevent reactivation[1]. There are three potential outcomes of infection of the human host by *M. tuberculosis* (see also Figure 3.1):

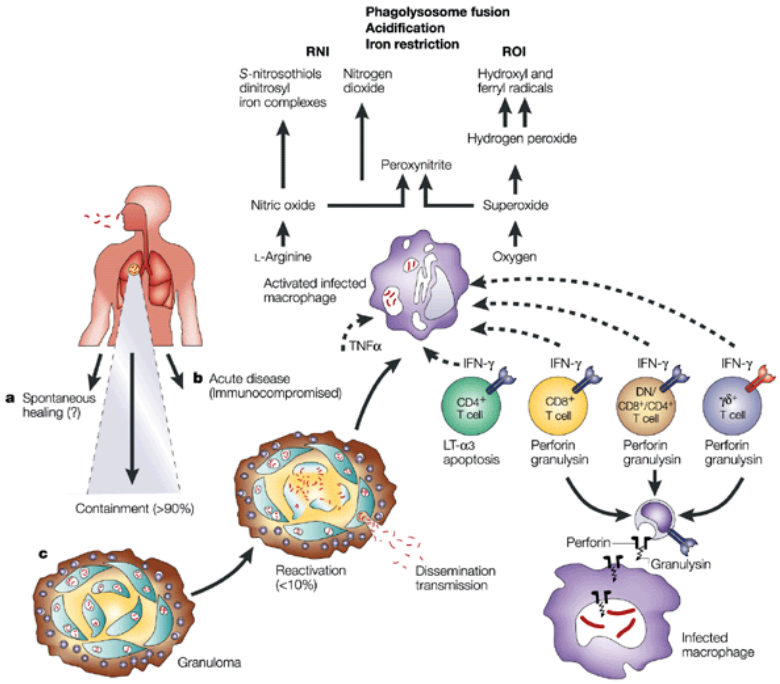
a) Spontaneous healing.

b) Latency. In most cases, mycobacteria are initially contained and disease develops later as a result of reactivation. The granuloma that forms in response to *M. tuberculosis* consists of macrophages, which can differentiate into epithelioid macrophages or multinucleate giant cells, CD4 and CD8 T-cells, and B cells. The T-cells produce interferon-γ, which activates macrophages. CD8 T-cells can lyse infected macrophages or kill intracellular bacteria. Tumour necrosis factor (TNF) is produced by macrophages and T-cells. Dendritic cells are also present, and a mature granuloma is surrounded by fibroblasts. *M. tuberculosis* is present within the macrophages and extracellularly.

c) Development of tuberculosis directly after infection in the immunocompromised host:

On depletion of CD4 T cells (e.g., during HIV infection), the granuloma does not function as well, production of interferon-γ may decrease, and macrophages are less activated. As a result, *M tuberculosis* begins to multiply and cause reactivation of infection. In the case of TNF neutralisation, the cells within the granuloma are no longer as tightly clustered, perhaps owing to chemokine or adhesion-molecule dysregulation. In addition, the macrophages are not as activated. These defects lead to a disorganised granuloma that is less able to control infection and greater immunopathology.[1]

Figure 3.1. Potential outcomes of infection of the human host by *M. tuberculosis*[17].



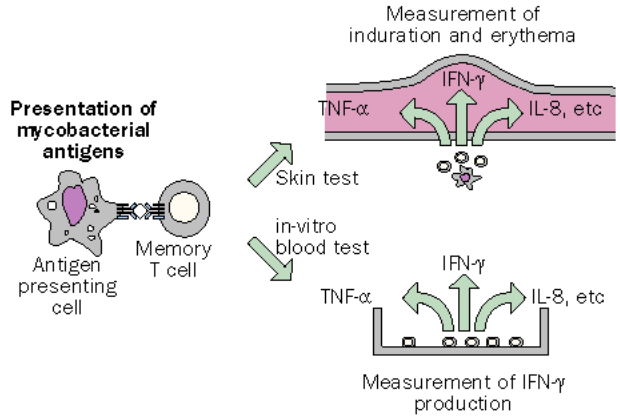
Although the host response is essential to control the infection, *M. tuberculosis* 'participates in the establishment of latency by using various strategies to evade elimination by the host'[1]. *M. tuberculosis* can subvert various antimycobacterial functions of macrophages. Once engulfed, *M. tuberculosis* ends up in a phagosome, the maturation of which is arrested at an early stage[17]. 'Within the phagosome, *M. tuberculosis* is subject to the antimycobacterial effect of reactive nitrogen intermediates (RNI)'[1]. *M. tuberculosis* inhibits phagosomal acidification and prevents fusion with lysosomal compartments. The bacilli can also inhibit the MHC class II-dependent antigen presentation pathway.

3.2 The tuberculin skin test (TST)

First introduced in 1890, the TST is an interdermal injection of purified protein derivative (PPD). The PPD is a crude antigenic mixture, shared among *M. tuberculosis*, *M. bovis*, and other non-tuberculous mycobacteria (NTM)[6]. The test measures *in vivo* a delayed-type hypersensitivity reaction based on immunological recognition of mycobacterial antigens in exposed individuals. Mycobacterial antigens are injected below the epidermal layer, causing infiltration of antigen-specific lymphocytes and the elaboration of inflammatory cytokines. This inflammatory reaction results in the characteristic indurated area at the site of injection (Figure 3.2). Until recently, the TST was the only tool for detecting LTBI. Limitations of the test include:

- a high proportion of false positive and false negative results;
- difficulty in separating true infection from the effects of BCG vaccination and NTM infection;
- technical problems in administration;
- immune response boosting after repeated TST;
- complicated and subjective interpretation; and
- a need for a second visit.

Figure 3.2. Inflammatory reaction from the tuberculin skin test (TST)[9]



3.3 Interferon-γ release assays (IGRAs)

3.3.1 Introduction

The *QuantiFERON-TB Gold IT* (QFT-G, by Cellestis Limited, Carnegie, Victoria, Australia) and the *T-SPOT* (by Oxford Immunotec Limited, Abingdon, UK) are two *in-vitro ex-vivo* tests for measuring cell-mediated immune responses (CMIR) to peptide antigens that simulate mycobacterial proteins. These antigens, ESAT-6, CFP-10 and TB7.7 (p4) (which is used only in QFT-G) are absent from all BCG strains and from most NTMs with the exception of *M. kansasii, M. szulgai* and *M. marinum*[7-9]. Individuals infected with *M. tuberculosis* complex organisms *(M. tuberculosis, M. bovis, M. africanum, M. microti, M. canetti)* have mononuclear cells in their blood that recognise these mycobacterial antigens. This recognition process leads *in vitro* to the stimulation and secretion of IFN-γ from sensitised T-cells. The detection and subsequent quantification of IFN-γ, measured by enzyme-linked immunoassay (Quantiferon) or enzyme-linked immunospot (T-SPOT), forms the basis of these tests[10] – as early as two weeks after infection with *M. tuberculosis*. Both tests are intended for use in conjunction with risk assessment, radiography, and other medical and diagnostic evaluations. Potential advantages of IGRAs over TST include: a) higher sensitivity, b) higher specificity (less influenced by BCG vaccination and NTM infection, c) less influence by technical problems in administration and interpretation, and d) the need for only one visit.

3.3.2 Current national guidelines and the clinical use of IGRAs

There is growing interest in the use of IGRAs, although most countries continue to recommend and use TST. More than 16 guidelines and statements have been published on IGRAs[11], which demonstrates considerable diversity in the approaches. Guidelines are predominantly available in high-income countries with established LTBI screening programmes. The two-step approach seems to be the most favoured strategy for IGRA use, especially in BCG-vaccinated contacts. There is also a trend towards using IGRAs alone prior to anti-TNF-α therapy. Some guidelines are still not proposing IGRA use in young children. Examples of current national guidelines on IGRA testing are shown in Table 3.1.

Table 3.1. Examples of current national guidelines on IGRA testing, adapted from Pai (2009)[10]

General testing approach	Countries
TST replacement by IGRA	Germany (anti-TNF-α), Swiss (anti-TNF-α), Denmark (anti-TNF-α, BCG-vaccinated contacts/adults)
Either TST or IGRA	USA, France, Australia (refugees), Japan (QFT preferred in all groups except in children < 5 years), Denmark (child contacts)
Two-step approach: TST first, followed by IGRA (either to improve sensitivity or specificity)	Canada, UK, Italy, Spain, Australia, Slovakia, Germany (contacts), Swiss (contacts), Netherlands (contacts, immigrants), Norway, Korea (contacts)

The clinical use of IGRAs in different groups has been recently reviewed[12]. The following paragraphs outline recommendations for IGRA testing in various groups of patients:

- IGRAs have been shown to be as good if not better than the TST in a contact investigation in low-burden regions.
- The Center for Disease Control (CDC) recommends that IGRAs may be used in all situations in which the TST is used but cautioned against its use in the setting of the severely immunocompromised and the very young[13].
- UK National Institute for Health and Clinical Excellence (NICE) 2006 guidelines recommend a two-step strategy for LTBI diagnosis: initial screen with TST; those who are positive (or in whom TST may be unreliable) should then be considered for IGRA testing, if available, to confirm positive TST results[14].

In summary:

- Persons thought to be at low risk of infection or progression or both:
  - CDC guidelines do not recommend TST or IGRA testing.
  - The excellent specificity of IGRAs would favour their use in cases/groups where the TST is currently performed (employee and school screening).
- Immunocompromised (individuals with HIV, chronic renal failure, solid organ transplant, fibrotic lesions, silicosis, cancer of the head and neck, malnutrition, diabetes, etc.):
  - IGRAs are at least as sensitive as TST.
  - Studies on routine testing for LTBI demonstrate significant discordance between TST and IGRA results, showing that the discordance is due to the failure of TST.
  - IGRAs are not a substitute for clinical judgment.
  - To maximise sensitivity in an immunocompromised individual with high risk of MTB infection, both an IGRA and the TST should be performed.
  - There is insufficient information to make a firm recommendation in the management of patients prior to the initiation of potent immunosuppressive therapy.
- School-age children :
  - IGRAs should be used as in adults.
- Children < 5 years old:
  - The number of studies of IGRA performance on children is growing, although evidence is still limited.
  - The results are broadly consistent with results of studies in adults (in terms of sensitivity and specificity).
  - IGRAs may be helpful in clinical cases in which TST performs poorly (congenital diseases, very young children with HIV infection or malnutrition or both, etc.).
  - Indeterminate results and test failure due to inadequate phlebotomy may be more common in young children.

Data regarding risk of disease progression are incomplete, but recent data suggest that a positive IGRA might predict future progression.

ECDC has recently published an evidence-based guidance document on the use of IGRAs in TB programmes. It presents the opinion of a panel of experts on the use of these assays for the diagnosis of latent TB infection as well as active TB disease.

3.4. Procedure 1: QuantiFERON-TB Gold IT[a]

(QFT-G, Cellestis Limited, Carnegie, Victoria, Australia)

3.4.1 General principles

The *QuantiFERON-TB Gold IT* system uses blood collection tubes that contain antigens representing specific *M. tuberculosis* proteins or controls. After blood collection (nil control, TB antigen and a mitogen tube), tube incubation at 37 °C ± 1 °C for 16 to 24 hours follows. When incubation is complete, the tubes are centrifuged, plasma is harvested, and the amount of IFN-γ produced is measured by ELISA. Results for test samples are reported in International Units (IU) relative to a standard curve prepared by testing dilutions of the secondary standard supplied by the manufacturer. The effect of heterophile antibodies is minimised by the addition of normal mouse serum to the green diluent and the use of $F(ab')_2$ monoclonal antibody fragments as the IFN-γ capture antibody coated to the microplate wells.

[a] Descriptions of laboratory procedures are based on the manufacturer's recommendations (Cellestis) and international safety, quality control and laboratory management regulations.

QuantiFERON-TB Gold package insert available from: http://www.cellestis.com/IRM/Content/pdf/QFTPI_English.pdf.

3.4.2 Baseline epidemiological data

Before performing the *QuantiFERON-TB Gold IT* test, baseline epidemiological data should be recorded: name, full address, contact info, gender, occupation, place of birth, time since immigration (if applicable), travel history, history of BCG vaccination and TST, clinical data (medication uptake, immunosuppression, weight loss, night sweats, fever, cough, abnormal CXR, previous TB treatment/chemoprophylaxis, etc.). Baseline data should be recorded on the patient data sheet that accompanies the specimen (see Chapter 3, Appendix 1).

3.4.3 Safety

Care should be taken when handling materials of human origin. All blood samples should be considered potentially infectious. Handling of blood samples and assay components, their use, storage and disposal should be in accordance with procedures defined in appropriate national, state or local biohazard and safety guidelines or regulations. Eye protection, gloves and normal laboratory protective clothing should be worn. Correct laboratory procedures should be adhered to at all times.

3.4.4 Materials provided by the manufacturer

Blood collection tubes

- Nil control (grey cap with white ring)
- TB antigen (red cap with white ring)
- Mitogen control (Purple cap with white ring)

Elisa components

- Microplate strips coated with murine anti-human IFN-γ monoclonal antibody
- Human IFN-γ Standard, lyophilised (8 IU/mL when reconstituted; 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, lyophilised (murine anti-human IFN-γ HRP, contains 0.01% w/v thimerosal)
- Wash buffer 20x concentrate (pH 7.2, contains 0.01 % w/v thimerosal)
- Enzyme substrate solution (contains H_2O_2, 3,3',5,5' tetramethylbenzidine)
- Enzyme stopping solution (contains 0.5M H_2SO_4)

3.4.6 Required materials (not provided)

- 37 °C ± 1 °C incubator (with or without CO_2).
- Calibrated variable-volume pipettes for delivery of 10 µl to 1000 µl with disposable tips.
- Calibrated multichannel pipette capable of delivering 50 µl and 100 µl with disposable tips.
- Centrifuge capable of centrifuging the blood tubes at least to 3,000 RCF (g).
- Microplate shaker capable of speeds between 500 and 1,000 rpm.
- Deionised or distilled water: 2L.
- Microplate washer (for safety reasons, an automated washer is recommended).
- Microplate reader fitted with 450nm filter and 620nm to 650nm reference filter.
- Variable speed vortex
- Timer
- Measuring cylinder: 1L or 2L
- Reagent reservoirs

3.4.7 Storage

- Blood collection tubes: store blood collection tubes at 4 °C to 25 °C (40 °F to 77 °F).
- ELISA kit reagents: store kit at 2 °C to 8 °C (36 °F to 46 °F). Always protect enzyme substrate solution from direct sunlight.
- Reconstituted and unused reagents: the reconstituted kit standard may be kept for up to three months if stored at 2 °C to 8 °C. Note the date the kit standard was reconstituted.
- The reconstituted 100x conjugate concentrate must be returned to storage at 2 °C to 8 °C and must also be used within three months. Note the date the 100x conjugate was reconstituted.
- Working strength conjugate must be used within six hours of preparation.
- Working strength wash buffer may be stored at room temperature for up to two weeks.

3.4.8 Methods

Sample collection and handling

Contents of the tubes should be thoroughly mixed with the blood. Incubation at 37 °C ± 1 °C should begin as soon as possible and within 16 hours of collection. For best results, the following procedures should be followed:

- For each subject collect 1mL of blood by venipuncture directly into each of the *QuantiFERON-TB Gold IT* blood collection tubes. If the level of blood in any tube is not close to the indicator line, it is recommended to obtain another blood sample. Under- or over-filling of the tubes outside of the 0.8 to 1.2 mL range may lead to erroneous results. High altitude (HA) tubes should be used at altitudes between 1000 and 2000 meters. Blood can also be collected using a syringe and 1mL transferred to each of the three tubes, ensuring appropriate safety procedures.
- Thorough mixing is required to ensure complete integration of the tube's contents into the blood. Mix the tubes by shaking vigorously for five seconds (ten times).
- Label the tubes appropriately.
- Prior to incubation, maintain tubes at room temperature (22 °C ± 5 °C). Do not refrigerate or freeze the blood samples.
- Perform specific tasks within set times.

Stage One: Incubation of blood and harvesting of plasma

- Re-mixing of the tubes is required if the blood is not incubated immediately after collection.
- Incubate the tubes upright at 37 °C ± 1 °C for 16 to 24 hours. No CO_2 or humidification incubator is required.
- Following incubation, blood collection tubes may be held between 2 °C and 27 °C for up to three days prior to centrifugation.
- After incubation, centrifuge tubes for 15 minutes at 2000 to 3000 RCF (g). If the cells are not separated from the plasma by the gel plug, the tubes should be re-centrifuged at a higher speed.
- Plasma samples can be loaded directly from blood collection tubes into the ELISA plate.
- Alternatively, plasma samples can be stored prior to ELISA, either in the centrifuged tubes or collected into plasma storage containers. Plasma samples can be stored for up to 28 days at 2 °C to 8 °C or below -20 °C (preferably less than -70 °C) for extended periods.

Stage Two: Human IFN-γ ELISA

1. Before use, plasma samples and reagents, except for conjugate 100x concentrate, must be brought to room temperature (22 °C ± 5 °C). Allow at least 60 minutes for equilibration.
2. Allow at least two strips for the standards and sufficient strips for the number of subjects being tested. Remove strips that are not required from the ELISA frame, reseal in the foil pouch, and return to the refrigerator for storage.
3. Reconstitute the human interferon-γ kit standard with the indicated volume of deionised or distilled water (see label; concentration of 8.0 IU/mL). Use the reconstituted kit standard to produce a dilution series of 4 IFN-γ concentrations (4.0, 1.0, 0.25, 0 IU/mL). Green diluent serves as the zero standard.
4. Reconstitute dried conjugate 100x concentrate with 0.3mL of deionised or distilled water. Mix gently to ensure complete solubilisation. Working strength conjugate is prepared by diluting the required amount of reconstituted conjugate 100x concentrate in green diluent as set out in package insert. Working strength conjugate should be used within six hours of preparation.
5. Prior to assay, mix plasmas thoroughly.
6. Add 50µL of freshly prepared working strength conjugate to each ELISA well.
7. Add 50µL of test plasma samples to appropriate wells. Add 50µL each of the standards 1 to 4. The standards should be assayed at least in duplicate (triplicate preferred).
8. Mix the conjugate and plasma samples/standards thoroughly using a microplate shaker for 1 minute at 500 to 1,000 rpm.
9. Cover each plate and incubate at room temperature (22 °C ± 5 °C) for 120 ± 5 minutes. Plates should not be exposed to direct sunlight during incubation. Deviation from specified temperature range can lead to erroneous results.
10. During the incubation, dilute one part wash buffer 20x concentrate with 19 parts deionised or distilled water and mix thoroughly. Wash wells with 400µL of working strength wash buffer. Perform the wash step at least six times. An automated plate washer is recommended for safety reasons when handling plasma samples.
    Thorough washing is very important to the performance of the assay. When an automated plate washer is used, standard laboratory disinfectant should be added to the effluent reservoir, and established decontamination procedures for potentially infectious material should be followed.
11. Tap plates face down on absorbent towel to remove residual wash buffer. Add 100µL of enzyme substrate solution to each well and mix for 1 minute at 500 to 1,000 rpm using a microplate shaker.

12. Cover each plate with a lid and incubate at room temperature (22 °C ± 5 °C) for 30 minutes. Plates should not be exposed to direct sunlight during incubation.
13. Following the 30 minute incubation, add 50µL of enzyme stopping solution to each well and mix thoroughly. Enzyme stopping solution should be added to wells in the same order and at approximately the same speed as the substrate in step 11.
14. Measure the optical density (OD) of each well within 5 minutes of stopping the reaction using a microplate reader fitted with a 450nm filter and with a 620nm to 650nm reference filter. OD values are used to calculate results.

3.4.9 Report interpretation

The predictive value of QFT-G results depends on the prevalence of *M. tuberculosis* infection in the tested population. Each QFT-G result and its interpretation should be considered in conjunction with other epidemiologic, historic, physical, and diagnostic findings. 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. Actual test data should not be reported and should be reported as follows:

- Positive result: A test is considered positive for an IFN-γ response to the TB antigen tube that is significantly above the nil IFN-γ IU/mL value. The nil sample adjusts for background, heterophile antibody effects, or non-specific IFN-γ in blood samples. The mitogen-stimulated plasma sample serves as an IFN-γ positive control for each specimen tested.
- Negative result: A test is considered negative for an IFN-γ response to the TB antigen tube that is not significantly above the nil IFN-γ IU/mL value.
- Indeterminate result: A low response to mitogen (<0.5 IU/mL) indicates an indeterminate result when a blood sample also has a negative response to the TB antigens. This pattern may occur with insufficient lymphocytes, reduced lymphocyte activity due to prolonged specimen transport or improper specimen handling, including filling/mixing of blood tubes, or inability of the patient's lymphocytes to generate IFN-γ.

QuantiFERON-TB Gold IT results are interpreted using the following criteria (Table 3.2).

Table 3.2. QuantiFERON-TB Gold IT results interpretation

TB antigen minus Nil (IU/mL)	Nil (IU/mL)	Mitogen minus Nil (IU/mL)	*QuantiFERON-TB Gold IT* Result	Report/interpretation
<0.35 OR ≥0.35 and <25% of Nil value	≤8.0	≥0.5	**Negative**	MTB infection NOT likely
≥0.35 and ≥ 25% of Nil value	≤8.0	Any	**Positive**	MTB infection likely
<0.35 OR ≥0.35 and <25% of Nil value	≤8.0	<0.5	**Indeterminate**	Results cannot be interpreted as a result of low mitogen response
Any	>8.0	Any	**Indeterminate**	Results cannot be interpreted as a result of high background response

3.4.10 Limitations

- Diagnosis of LTBI means tuberculosis disease must be excluded by medical evaluation.
- A negative result must be considered in conjunction with the individual's medical and historical data, particularly for individuals with impaired immune function.

There are technical factors related to indeterminate results:

- Longer than 16 hours from blood drawing to incubation at 37 °C ± 1 °C.
- Storage of filled blood collection tubes outside the recommended temperature range (22 °C ± 5 °C) prior to 37 °C ± 1 °C incubation.
- Insufficient mixing of blood collection tubes.
- Incomplete washing of the ELISA plate.

If technical issues are suspected with the collection or handling of blood samples, the entire QuantiFERON-TB Gold IT test should be repeated.

3.4.11 Quality control

Internal quality assessment (IQA)

Test accuracy depends on the generation of an accurate standard curve. Results derived from the standards must be examined before test sample results can be interpreted. Each laboratory should determine appropriate types of control materials and frequency of testing in accordance with local, regional, national, or other applicable accrediting organisations (e.g. positivity rate, indeterminate rate).

Quality control parameters:

- The mean OD value for standard 1 must be ≥ 0.600.
- The mean OD value for the zero standard (green diluent) should be≤ 0.150.
- The % coefficient of variation (%CV) between replicates for standards 1 and 2 must be ≤15%.
- Replicate OD values for standards 3 and 4 must not vary by more than 0.040 optical density units from their mean.
- 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.

External quality assessment (EQA)

Total quality management, including both internal and external quality assurance, is essential for the modern laboratory. External quality control schemes should be based on previous experience[15,16] and established as soon as possible.

3.5 Procedure 2: T-SPOT procedure[b]

3.5.1 General principles

T-SPOT (Oxford Immunotec, Abingdon, UK), unlike *QuantiFERON-TB Gold*, utilises enzyme-linked immunospot (ELISPOT) technique based on enumeration of activated specific T-cells responding to stimulation by specific antigens (ESAT-6 and CFP10) resulting in IFN-γ secretion. Stimulation by ESAT-6 and CFP10 antigens takes place in separate microtitre plate wells.

During the course of the procedure, peripheral blood mononuclear cells (PBMCs) are separated from a whole blood sample and counted so that a standardised cell number is used in the assay. The PBMCs are incubated with the antigens to allow stimulation of any sensitised T-cells present; secreted IFN-γ is captured by specific antibodies on the membrane on the base of the well. A second antibody, conjugated to alkaline phosphatase and directed to a different epitope on the (cytokine) IFN-γ molecule, is then added and binds to the cytokine captured on the membrane surface. Finally, a soluble substrate is added to each well; this is cleaved by bound enzyme to form a spot of insoluble precipitate at the site of the reaction. Each spot therefore represents the footprint of an individual cytokine-secreting T-cell, and evaluating the number of spots obtained provides a measurement of the abundance of *M. tuberculosis*-sensitive effector T-cells in the peripheral blood.

3.5.2 Baseline epidemiological data

As for the *QuantiFERON-TB Gold* assay, baseline epidemiological data are necessary for the correct clinical interpretation of the test results. Data should include name and surname, full address, contact information, gender, occupation, place of birth, time since immigration (if applicable), travel history, history of BCG vaccination and TB skin tests, relevant clinical data (medication uptake, immunosuppression, weight loss, night sweats, fever, cough, abnormal CXR, previous TB treatment/chemoprophylaxis, etc.). Baseline data should be recorded on the patient data sheet that accompanies the specimen (see Chapter 3, Appendix 1).

3.5.3 Safety

This diagnostic procedure involves handling of human blood samples and plasma, potentially infected with blood-born infections, including HIV, hepatitis B, and hepatitis C. Protective equipment (gloves, lab coats and goggles or shields) should be worn when handling blood/plasma specimens. Handling, storage and disposal of blood specimens should be in accordance with national, state or local biohazard and safety guidelines or regulations. Risk assessment should be performed prior to introduction of the procedures and SOPs should be developed and regularly updated.

[b] Descriptions of laboratory procedures are based on the manufacturer's recommendations (Oxford Immunotec) and international safety, quality control and laboratory management regulations.
Oxford Immunotec T-SPOT.TB96 package insert available from: http://www.oxfordimmunotec.com/96-UK.

Important notes:

- T-SPOT assay involves human PMBC cultivation. Therefore, observing aseptic technique is extremely important in order to avoid contamination of reagents, wells, cell cultures, and nourishing media;
- Blood should be collected and progressed into the assay within eight hours of blood collection. This time can be prolonged by using the T-cell *Xtend* reagent (also available from Oxford Immunotec). In this case the sample storage time before assay is increased to 32 hours. Only lithium-heparine tubes can be used in conjunction with T-cell *Xtend*.
- Calculations for the conjugate dilution, cell counting, etc. are provided on the CD-ROM supplied along with the kits.

3.5.4 Materials

Provided by the manufacturer with the kits (Table 3.3):

Table 3.3. Materials provided by the manufacturer

	T-SPOT TB 96 kit	T-SPOT TB 8 kit
1	1 microtitre plate: 96 wells coated with a mouse monoclonal antibody to IFN-γ.	1 microtitre plate: 96 wells, supplied as 12x8-well strips in a frame, coated with a mouse monoclonal antibody to IFN-γ
2	2 vials (0.7mL each) Panel A: contains ESAT-6 antigens	2 vials (0.8mL each) Panel A: contains ESAT-6 antigens
3	2 vials (0.7mL each) Panel B: contains CFP10 antigens	2 vials (0.8mL each) Panel B: contains CFP10 antigens
4	2 vials (0.7mL each) Positive control: contains phytohaemagglutinin (PHA)	2 vials (0.8mL each) Positive control: contains phytohaemagglutinin (PHA)
5	1 vial (50µL) 200x concentrated conjugate reagent: mouse monoclonal antibody to IFN-γ conjugated to alkaline phosphatise.	1 vial (50µL) 200x concentrated conjugate reagent: mouse monoclonal antibody to IFN-γ conjugated to alkaline phosphatase
6	1 bottle (25mL) substrate solution: ready to use BCIP/NBT (plus) solution.	1 bottle (25mL) substrate solution: ready to use BCIP/NBT (plus) solution.

All reagents except the conjugate are supplied ready to use. The conjugate should be diluted with PBS 1:200 immediately prior to use (50 µl working strength solution per well).

- Equipment and materials required but not provided with the kits
  - Class II microbiological cabinet (recommended to observe aseptic technique)
  - Centrifuge for preparation of PBMCs (capable of at least 1800xg and able to maintain the samples at room temperature (18-25 °C)
  - Haemocytometer
  - Inverted microscope (e.g. Wilovert S, Wetzlar, Germany)
  - A humidified incubator capable of 37 ± 1 °C with a 5% CO_2 supply
  - A microtitre plate washer or equipment to manually wash plates (e.g. multichannel pipette)
  - Pipettes and sterile pipette tips
  - Instruments for the plate reading: Microscope OR digital microscope OR magnifying glass OR Plate imager (e.g. ELR02, Autoimmun Diagnostika GmbH, Germany).
- Consumables:
  - Sterile pipette tips
  - Blood collection tubes with heparin or sodium citrate (such as Vacutainer CPT). EDTA tubes are NOT recommended.
- Reagents:
  - Ficoll-Paque* PLUS or alternative PBMC separation materials
  - Trypan blue dye (available from Sigma, catalogue number T8154)
  - Sterile PBS solution, available from Invitrogen as 'GIBCO Dulbecco's Phosphate-Buffered Saline (D-PBS) (1x)', catalogue number 14040-091). Do not use PBS containing Tween.
  - Distilled or deionised water.
  - Sterile serum-free cell culture medium such as 'GIBCO AIM V' (Invitrogen; catalogue number 31035-025) (for incubation)
  - Sterile cell culture medium RPMI 1640 (Invitrogen; catalogue number 21875-034) (for initial cell preparation and cell suspension dilution)

Sample collection

Blood should be collected as follows:

- Adults and children 10 years old and over: one 8mL or two 4mL CPT tubes or one 6mL lithium-heparin tube.
- Children 2 to 9 years old: one 4mL CPT or lithium heparin tube.
- Children up to 2 years old: one 2mL paediatric tube.

Blood should be stored at room temperature (no refrigeration or freezing) and assayed within eight hours. This time can be prolonged up to 32 hours if T-cell *Xtend* is used.

Sample preparation

Initial sample preparation steps depend on whether Vacutainer CPT or conventional lithium-heparin or sodium citrate tubes were used for the blood collection. Please note that T-cell *Xtend* reagent is not compatible with the CPT tubes. For details of the specimen preparation procedures involving T-cell *Xtend* please refer to the T-SPOT TB technical handbook available from: http://www.oxfordimmunotec.com/UK%20Technical%20Handbook.

CPT tubes (with gel plug)

- Centrifuge 8mL CPT tubes at 1600xg for 28 minutes or 4mL CPT tubes at 1800xg for 30 minutes at 18 °C where a refrigerated centrifuge is available. If a non-refrigerated centrifuge is used, ensure the temperature does not go above 25 °C.
- Collect the white, cloudy band of PBMCs using a pipette and transfer to a 15mL conical centrifuge tube. Make up the volume to 10mL with cell culture medium AIM V or RPMI 1640.

Lithium-heparin/sodium citrate tubes

- Dilute the blood with an equal volume of RPMI 1640 medium. Carefully layer the diluted blood (2–3 volumes) onto Ficoll-Paque PLUS (1 volume) and centrifuge at 1000xg for 22 minutes while maintaining the temperature between 18 and 25 °C.
- Collect the white, cloudy band of PBMCs using a pipette and transfer to a 15mL conical centrifuge tube. Make up the volume to 10mL with cell culture medium AIM V or RPMI 1640.
- Centrifuge at 600xg for 7min. Pour off the supernatant and resuspend the pellet in 1mL AIM V or RPMI medium.
- Make up the volume to 10 ml with fresh AIM V or RPMI medium and centrifuge at 350g for 7 minutes.
- Pour off the supernatant and resuspend the pellet in 0.7mL AIM V culture medium.

Cell counting and dilution

The T-SPOT.*TB* assay requires $2.5x10^5$ viable PBMCs per well. A total of four wells are required for each patient sample. The correct number of cells must be added to each well. Failure to do so may lead to an incorrect interpretation of the result. Care should be taken to ensure that the cell suspension is thoroughly mixed immediately prior to removal of aliquots for dilution or for counting.

For manual counting with a Neubauer haemocytometer, add 10µL of the final cell suspension to 40µL 0.4% (w/v) trypan blue solution. Place an appropriate aliquot onto the haemocytometer and count the cells in the grid. For other types of haemocytometers and for automated devices, follow the manufacturer's instructions.

Calculate the concentration of viable cells present in the stock cell suspension. The T-SPOT cell dilution calculator on the CD-ROM provided with each assay kit will facilitate this calculation.

Prepare 500µL of the final cell suspension at a concentration of $2.5x10^5$ cells/100µL. Ensure cells are thoroughly mixed before removing an aliquot for dilution.

Plate set up and incubation

The T-SPOT.TB assay requires four wells to be used for each patient sample. A nil control and a cell functionality positive control should be run with each individual sample. It is recommended that the samples are arranged vertically on the plate as illustrated below:

○ Nil control

○ Panel A

○ Panel B

○ Positive control

- Remove the pre-coated microtitre plate from the packaging and allow to equilibrate to room temperature. The microtitre plate is provided with a protective plastic base. This should not be removed at any stage of the procedure.
- Each patient sample requires the use of four individual wells as follows. (Do not allow the pipette tip to touch the membrane. Indentations in the membrane caused by pipette tips may cause artefacts in the wells).
  - Add 50µL AIM V culture medium to each nil control well.
  - Add 50µL Panel A solution to each well required.
  - Add 50µL Panel B solution to each well required.
  - Add 50µL positive control solution to each positive control well.
- To each of the four wells to be used for a patient sample, add 100µL of the patient's final cell suspension (containing 250,000 viable cells).
- Incubate the plate in a humidified incubator at 37 °C with 5% CO_2 for 16 to 20 hours.

Spot development and counting

1. Remove the plate from the incubator and discard the cell culture medium. Remove the substrate solution from the kit and allow to equilibrate to room temperature.
2. Add 200µL PBS solution to each well.
3. Discard the PBS solution. Repeat the well washing a further three times with fresh PBS solution for each wash. Discard all PBS from the final wash step by inverting the plate on absorbent paper before proceeding.
4. Dilute the concentrated conjugate reagent 1:200 in PBS to create the working strength solution.
5. Add 50µL working strength conjugate reagent solution to each well and incubate at 2–8 °C for 1 hour.
6. Discard the conjugate and perform four PBS washes as described in steps 2 and 3 above.
7. Add 50µL substrate solution to each well and incubate at room temperature for seven minutes.
8. Wash the plate thoroughly with distilled or deionised water to stop the detection reaction. Allow the plate to dry by standing it in a well-ventilated area or in an oven at up to 37 °C (spots become more visible as the plate dries). Allow four hours drying time at 37 °C or overnight at room temperature.
9. Count and record the number of distinct, dark blue spots on the membrane of each well. Use a magnifying glass, a suitable microscope, or an ELISPOT plate reader.
10. Apply the results interpretation and assay criteria (see below) to determine whether a patient sample is 'positive' or 'negative' to TB antigens.

Reading and results interpretation

T-SPOT.TB results are interpreted by subtracting the spot count in the nil control well from the spot count in each of the panels, according to the following algorithm:

- The test result is 'positive' if (Panel A minus nil control) and/or (Panel B minus nil control) ≥ 6 spots, AND a nil control count <10 spots.
- The test result is 'negative' if both (Panel A minus nil control) and (Panel B minus nil control) ≤ 5 spots (this includes values less than zero), AND a nil control count <10 spots AND a positive control count >20 spots (or show saturation).
- The test result is 'indeterminate' if:
  - a nil control count >10 spots regardless of spot counts in Panel A AND Panel B; or
  - a positive control count <20 spots if both (Panel A minus nil control) AND (Panel B minus nil control) ≤ 5 spots.

Due to potential biological and systematic variations, where the higher of (Panel A minus nil control) and (Panel B minus nil control) is 5, 6 or 7 spots, the result may be considered as borderline (equivocal). Borderline (equivocal) results, although valid, are less reliable than results where the spot count is further from the cut-off. Retesting of the patient, using a new sample, is therefore recommended. If the result is still borderline (equivocal) on retesting, then other diagnostic tests and/or epidemiologic information should be used to help determine TB infection status of the patient.

3.5.5 Reporting

The manufacturer recommends using the following wording in the laboratory reports:

- A 'positive' result indicates that the sample contains effector T-cells reactive to *M. tuberculosis.*
- A 'negative' result indicates that the sample probably does not contain effector T-cells reactive to *M. tuberculosis*

3.5.6 Quality control

Internal quality assessment (IQA)

Appropriate means of internal quality assurance and control should be determined, developed and implemented by each laboratory in accordance to local and governmental regulations. This should include:

- blind re-testing of specimens on a regular basis;
- keeping records on dates when kits are opened and finished, kit lot numbers;
- fridge and freezer temperature sheets.

External quality assessment (EQA)

Currently no formal EQA schemes exist for the IGRA tests. These should be established and implemented as soon as possible based on previous experience.

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Appendix: Patient data sheet

Sender's contact details:

Name _____

Laboratory/hospital _____

Postal address _____

_____

_____

Phone _____

Fax _____

For laboratory use

Patient's number _____

Date received _____

Time received _____

Date of test: _____

Please circle/mark/tick appropriate answer(s). Please do not leave any fields blank.

If answer requires further details, please specify.
Please remember that complete answers are essential for the correct interpretation of the test results.

Baseline epidemiological data

Patient's first name _____

Surname _____

Date of birth _____

Male ___ Female ___

Postcode _____

Occupation _____

Was the patient born abroad?
Yes ___.
No ___. Born in _____
If no, when did the patient come to _____ (country)? _____ (year)

Has patient lived, or spent more than two months travelling in another country?
Yes.
No.
Don't know.

History of BCG vaccination and TB skin tests

Has patient ever received the BCG vaccination? Yes ___ No ___ Don't know ___

If yes, please specify age: ___

BCG scar: Yes ___ No ___

Mantoux screening test done? Yes ___ No ___ Don't know ___. Reading _____ mm

Clinical data

Is patient taking any of the following medications?

___ Oral steroids
___ Cytotoxic drugs
___ Other immunosuppressive drugs (please specify)_____
___ None of the above.

Is the patient immunocompromised? Yes ___ No ___ Don't know ___

Is the patient HIV positive? Yes ___ No ___ Don't know ___

Does the patient have diabetes? Yes ___ No ___ Don't know ___

Does the patient have any of the following:
___ Fever
___ Night sweats
___ Loss of weight
___ Cough

Is the patient's CXR abnormal? Yes ___ No ___. If yes, please specify the location: _____

	R	L
Upper		
Middle		
Lower		

Cavities? Yes ___ No ___ Don't know ___

Consolidation? Yes ___ No ___ Don't know ___. Unilateral/bilateral? _____

Other relevant clinical data: _____
_____
_____
_____

4 Smear microscopy

Susana David, Vera Katalinic-Jankovic, Daniela Cirillo

4.1 Background and principles

Early laboratory diagnosis of tuberculosis still relies on the examination of stained smears. For universal application in resource-limited countries, microscopy of stained sputum smears is the best choice among diagnostic methods[7,12]. This technique is based on the fact that the cell wall of the *Mycobacterium spp*. genus is rich in complex lipids that prevent access to common aniline dyes, but when stained with carbol fuchsin or fluorochromes under special staining procedures, these are not easily decolorised even with alcohol-acid solutions. Because of this characteristic, all members of *Mycobacterium spp.*, not only *M. tuberculosis*, are referred to as acid-fast bacilli (AFB).

At present, two types of acid fast stains are used for the detection of mycobacteria in clinical specimens:

- Carbol-fuchsin staining (Ziehl-Neelsen [ZN] method and its modification performed without heating the dye [Kinyoun cold staining])
- Fluorochrome (auramine or auramine-rhodamine) staining.

Kinyoun staining is a modification of the classic ZN staining which excludes the heating step during the staining procedure and uses a higher concentration of carbol-fuchsin. Mycobacteria appropriately stained by ZN and Kinyoun appear as red rods. The performance of Kinyoun staining is lower than ZN, therefore this procedure is not recommended[6].

Methods which apply a fluorochrome have been used to stain acid fast bacteria for many years. Using this method, mycobacteria are detected as bright fluorescent rods against a darker background. Fluorochrome staining has an increased sensitivity and a shorter time is required for screening the slides when compared to Kinyoun or ZN staining[6,8] .

Smear microscopy is simple, inexpensive and efficient in detecting those cases of pulmonary tuberculosis that are most infectious. Since its yield is highly dependent on its execution, the quality of smear microscopy is crucial in the fight against TB in resource-limited settings[6,7].

A major limitation of smear microscopy is its low sensitivity (25–75% compared to culture), due to the high number of bacilli required for positivity (in the range of 5 x 10^3–10^4 bacilli per ml). Sensitivity of smear microscopy is influenced by numerous factors[7,12,13] such as the prevalence and severity of the disease, the type and quality of the specimen, the number of mycobacteria in the sample and the quality of the process of smear preparation, staining and reading. Smear microscopy does not allow for mycobacterial species identification nor does it give an indication of the viability of mycobacteria in the sample. HIV co-infected TB patients may have disseminated paucibacillary disease with fewer AFB. Smear microscopy is often negative or may require more scrutiny in screening to identify these lower numbers of AFB.

4.2 Procedure 1: Ziehl-Neelsen (ZN) [7,14,15]

Each batch of prepared reagent should be recorded in a reagent preparation workbook which includes: the signature of the technician who prepared it, the date of preparation and the results of quality control testing[2,6].

4.2.1 Ziehl-Neelsen (ZN) reagent preparation

Good staining reagents, made with high-quality carbol fuchsin dye are essential to detect AFBs[6]. Contamination of reagents by environmental mycobacteria should be prevented by using freshly distilled water.

Standard reagents:

- Basic fuchsin powder
- Phenol crystals (the crystals should be almost colourless)
- Alcohol (denaturated 95% ethanol)
- Water (distilled or purified)

Decolourising solution:

- Concentrated sulphuric acid (≥95%)
- Water (distilled or purified)

or

- Hydrochloric acid (37%)

- Alcohol (denaturated 95% ethanol)

The counterstain solution:

- Methylene blue powder
- Water (distilled or purified)

A. Carbol fuchsin (CF) reagent

The quality of basic fuchsin varies among different manufacturers with regard to its purity and solubility. The content of basic fuchsin should represent 85%–88% in weight. If carbol fuchsin purity is known, it should be used to calculate the final stain concentration of 0.3%. To calculate the required amount of basic fuchsin, divide the actual amount by the dye content. For instance, if the dye content is 75%, you must divide the amounts by 0.75. So 3 g/0.75 = 4 grams will be weighed for the 0.3% stain. If the powder with a dye content of ≥85% is used, there is no need to calculate the correction factor. If the dye purity is unknown or if the basic fuchsin dissolves poorly or precipitates are still visible after filtration, it may be wise to use a higher concentration (1%) when preparing the staining reagent.

0,3% Carbol fuchsin	
Basic fuchsin	3.0 g
95 % ethanol	100 mL
Phenol crystals	50 g
Distilled water	900 mL

- Weigh 3.0 g of basic fuchsin powder and 50 g of phenol crystals separately.
- Add 100 mL of alcohol (denatured ethanol) to a one-litre conical flask.
- Add the 50 g of phenol and swirl the flask until it is dissolved.
- Add the 3.0 g of basic fuchsin powder and continue to mix well until the fuchsin powder completely dissolves.

Check for remaining powder or crystals on the bottom. If there are any, continue swirling with slight heating. Only after the fuchsin is completely dissolved, add 850 mL of water and mix by further swirling.

If precipitates are visible, the carbol fuchsin staining reagent should be filtered. Filter the carbol fuchsin again during the staining process, using a funnel with filter paper (or by placing a piece of filter paper directly on the slide). Other staining reagents do not need to be filtered. If any particles are detected in the carbol fuchsin solution, the solution must be refiltered.

B. Decolourising solution

25% sulphuric acid	
Concentrated sulphuric acid	250 mL
Distilled water	750 mL

- Add 750 mL of distilled water to a two-liter conical flask.
- Measure 250 mL of concentrated sulphuric acid in a cylinder.
- Pour it slowly into the flask containing the water, directing the flow of acid gently along the inner side of the flask. Always add the acid slowly to the water, not vice versa.
- Mix well by swirling the flask.

3% HCl ethanol	
Concentrated hydrochloric acid	30 mL
95% ethanol	970 mL

- Add 970 mL of 95% ethanol to a two liter conical flask.
- Measure 30 mL of concentrated hydrochloric acid in a cylinder.
- Pour it slowly into the flask containing alcohol, directing the flow of acid gently along the inner side of the flask with constant swirling. Always add the acid slowly to the alcohol, not vice versa.
- Mix well by swirling.

C. Counterstain

Methylene blue chloride	3.0 g
Distilled water	1000 mL

- Weigh 3 g of methylene blue powder.

- Add the powder to 0.5 L of pure water in a conical flask.
- Swirl the contents of the flask to dissolve the dye.
- Add 0.5 L of water and mix again.

4.2.2 Storage of reagents

The flasks with freshly prepared reagents should be covered until quality control procedures have been performed and the results have been evaluated[7]. Solutions should be stored in clean brown bottles and clearly labelled. The label should indicate the reagent name, concentration and the preparation date. Reagents preserved in tightly closed bottles can be used for up to one year. Bottles should be kept out of direct sunlight. If clear bottles are used, stocks of reagents should be stored in a closed cabinet.

4.2.3 Quality control of freshly prepared staining reagents

After preparing staining reagents, quality control checks should be performed on every batch[7,14]. Quality control is essential to ensure the effectiveness of staining reagents and the complete absence of AFB contamination.

Quality control results should be recorded in a logbook. In the logbook, every batch should be clearly identified by the name of the reagent and the preparation date found on the bottle labels. Perform QC by using one or more freshly prepared staining reagents, carrying out the usual staining procedure as described for positive controls. Test the performance of carbol fuchsin by staining and examining two scanty smears stained once, and two negative smears stained three times[7].

4.3 Procedure 2: Fluorochrome staining[2,14,15]

4.3.1 Fluorochrome reagents preparation

A. Fluorochrome reagents

Auramine O (solution 1)	
Auramine	0.1 g
95% ethanol	10 mL

Phenol (solution 2)	
Phenol crystals	3.0 g
Distilled water	87 mL

Prepare solutions 1 and 2 by dissolving the auramine in ethanol (solution 1) and phenol crystals in water (solution 2).

Mix solutions 1 and 2 and store in a tightly stoppered dark coloured bottle away from heat and light. Label bottles with the name of the reagent, the date of preparation and expiry date. Store at room temperature for three months. Turbidity may develop on standing but this does not affect the staining reaction.

B. Decolourising solution

0,5% Acid alcohol	
Concentrated hydrochloric acid	0.5 mL
70% ethanol	100 mL

Carefully add concentrated hydrochloric acid to the ethanol. Always add acid slowly to the alcohol, not vice versa. Store in a dark-coloured bottle. Label bottles with the name of the reagent, the date of preparation and expiry date. Store at room temperature for three months. For each volume of stain, 2 to 3 volumes of decolourising solution is needed.

C. Counterstains

Either potassium permanganate or acridine orange may be used as counterstains.

Potassium permanganate	
Potassium permanganate ($KMnO_4$)	0.5g
Distilled water	100ml

Dissolve potassium permanganate using distilled water in a tightly stoppered dark-coloured bottle. Label bottles with the name of the reagent, the date of preparation and expiry date. Store at room temperature for up to three months.

Acridine orange	
Anhydrous dibasic sodium phosphate (Na_2HPO_4)	0.01 g
Distilled water	100 mL
Acridine orange	0.01 g

Dissolve sodium phosphate in distilled water. Add acridine orange and mix until dissolved. Store in a tightly stoppered dark-coloured bottle away from heat and light. Label bottles with the name of the reagent, the date of preparation and expiry date. Store at room temperature for up to three months.

4.3.2 Safety measures

Never add water to acid! To reduce exposure to toxic phenolic fumes, preparation of reagents and staining with solution containing phenol should be performed in a well-ventilated area or under a chemical hood. Always wear protective laboratory coats, gloves and safety glasses when handling a strong acid. In case of an accident with acid, rinse the affected body part immediately with plenty of water.

4.4 Sample collection

Smear microscopy for *Mycobacterium spp.* detection can be used for a wide variety of biological samples. For the diagnosis of respiratory tuberculosis, sputum is the most commonly used sample. To ensure optimal recovery of TB bacilli from sputum, two to three specimens should be collected and processed for mycobacterial microscopy and culture[7,15].

Country guidelines should be consulted for the number of recommended samples. Early morning specimens have the highest yield of AFB; however, it is now proven that good diagnostic specimens can be collected at any time. It is not recommended to perform smear microscopy from blood or very bloody samples due to the low sensitivity of the procedure. It is also not recommended to routinely perform smear microscopy from urine samples due to the frequent detection of saprophytic mycobacteria colonising the urogenital tract.

Samples should be collected in clean, wide-mouthed and leakproof specimen containers[2,6]. Single use disposable plastic containers (50 ml capacity) are preferred in order to avoid transferring the specimens from one container to another. Alternatively, 50 ml disposable sterile conical tubes can be used.

Patients should receive clear written instructions on the proper collection of the sputum specimen for TB diagnosis. For patients on treatment, specimens should be collected at intervals specified by the country's guidelines[7]. Sputum collection should never be performed in the laboratory. It is a procedure generating infectious aerosols and should only be performed at a distance from other people or in rooms with negative pressure and adequate air changing[7,14].

A good specimen should be approximately 3–5 ml in volume[5]. Sputum specimens should appear thick and mucoid or clear but with purulent grains[7]. The colour varies from opaque white to green. Bloody specimens will appear reddish or brown. Note: clear saliva or nasal discharge is not suitable as a TB specimen, although saliva should not automatically be rejected: induced and follow-up sputa resemble saliva [2,15].

4.4.1 Specimen handling

For optimum patient management, process the specimen as soon as possible (i.e. < 24 hours). For microscopic examination, the interval between collection and staining is not critical. Acceptable results can be obtained even if specimen delivery has been delayed.

4.4.2 Criteria of acceptability

Upon arrival in the laboratory, the quality of sputum samples should be assessed and reported in the referral form[7]. TB-positive sputa can vary in colour and aspect. If the sample is liquid and is clear as water, without particles or streaks of mucous material, process the sample but ensure that the poor quality of the sample is reported on the result form. When possible, encourage the patient/physician to submit a new specimen; however, even saliva can yield positive results. All specimens should be processed, except for broken or leaking containers which should be discarded and another specimen requested.

Accept very small quantities if the patient has difficulty producing sputum and if the aspect is correct. Blood-streaked sputum is suitable, but pure blood should not be examined[1,2].

4.5 Smear preparation[7]

Although smear preparation for AFB detection[7] is a relatively safe procedure in terms of infected aerosol production, it is recommended to prepare the slides in a BSC class I or IIB[14,15]. If the smear is prepared after centrifugation of the sample (concentrated smear), the centrifuge holder must be opened within a BSC.

- Smears should be prepared using new, clean, grease-free and unscratched slides. Using a pencil/marking pen, record the laboratory register serial number and order number of the specimen on the frosted end of the slide. If plain unfrosted slides are used, label them using a diamond pencil.
- Using the end of an applicator stick or wire/disposable loop, select and pick up the yellowish purulent particles of sputum.
- Prepare the smear in an oval shape in the centre of the slide. The smear size should be 2–3 cm in length and 1–2 cm wide, which will allow 100–150 fields to be counted in one length.
- For good spreading of the sputum, firmly press the stick perpendicular to the slide and move in small concentric circles or coil-like patterns.
- Place the used stick into a discard container.
- Use a separate stick for each specimen.
- Thorough spreading of the sample is very important, especially in the case of thick or purulent material; it should be neither too thick nor too thin. Prior to staining, hold the smear about 4–5 cm over a piece of printed paper. If letters cannot be read, it is too thick.
- For concentrated samples (after centrifugation at 3000x g for 20 minutes, see sample preparation for TB culture) 1 or 2 drops of sediment should be smeared on the slide.
- Allow the smear to air-dry completely at room temperature within the BSC.
- Do not dry smears in direct sunlight or over a flame.
- Pass the slide over a flame 2–3 times for about 2–3 seconds each time. Do not heat the slide for too long or keep it stationary over the flame or else the slide will be scorched.
- Alternatively, slides can be fixed for two hours on hot plates (65–75 °C), within the BSC.

Table 4.1 lists the equipment needed for direct (unconcentrated) smear microscopy.

Table 4.1. Equipment required for smear preparation and staining

Equipment required for smear preparation and staining
Container to store specimen
Wire/disposable loop with an inner diameter of 3 mm to spread sputum on the slide
Microscope slide (grease-free and unscratched)
Pencil/marking pen to put the identification number on the microscopy slide
Forceps to hold smear slide
Bunsen burner to fix the smear slide and to flame the smear during staining
Staining rack to hold the smear slide
Slide rack to place stained smear slide to dry in the air

4.6 Staining procedures [2,14,15]

4.6.1 Ziehl-Neelsen staining method

- Cover the entire surface of each heat-fixed slide with carbol fuchsin.
- Using a Bunsen burner, gently heat the slides until vapor rises. Do not allow them to boil.
- Allow the stain to remain on the slide for ten minutes. Adequate time is required for the carbol fuchsin to penetrate and stain the cell well.
- Gently wash the stain from each slide with a stream of cold water until all the free stain has washed away.
- Cover each slide with acid alcohol; wait three minutes.
- Rinse slides again carefully with water and tilt each slide to remove excess water.
- Flood the slide with the counterstain methylene blue for one minute.
- Rinse slides again carefully with water, drain and air dry.

4.6.2 Fluorochrome staining method

- Prepare and heat fix smears.
- Place the numbered smears on a staining rack in batches (maximum 12).
- Flood the slides with auramine O stain and allow them to stain for 15 minutes.
- Be sure that the stain stays on the smear. Do not heat and do not use paper strips.

- Rinse the slide with water. Aim the flow of water at the edge of the slide and slowly peel the stain from the slide.
- Flood the slides with 0.5% acid alcohol and allow them to decolourise for three minutes.
- Ensure that the slides are flooded thoroughly with acid alcohol.
- Rinse off the 0.5% acid alcohol with water, drain the excess water from the slide.
- Flood each slide with potassium permanganate and allow quenching for two minutes.
  Note: It is critical that the potassium permanganate remains on the slides for no longer than two minutes as over-quenching of fluorescence can occur.
- Wash off the potassium permanganate. Drain the excess water from the slide.
- Allow smears to air dry. Do not blot. Read as soon as possible after staining.

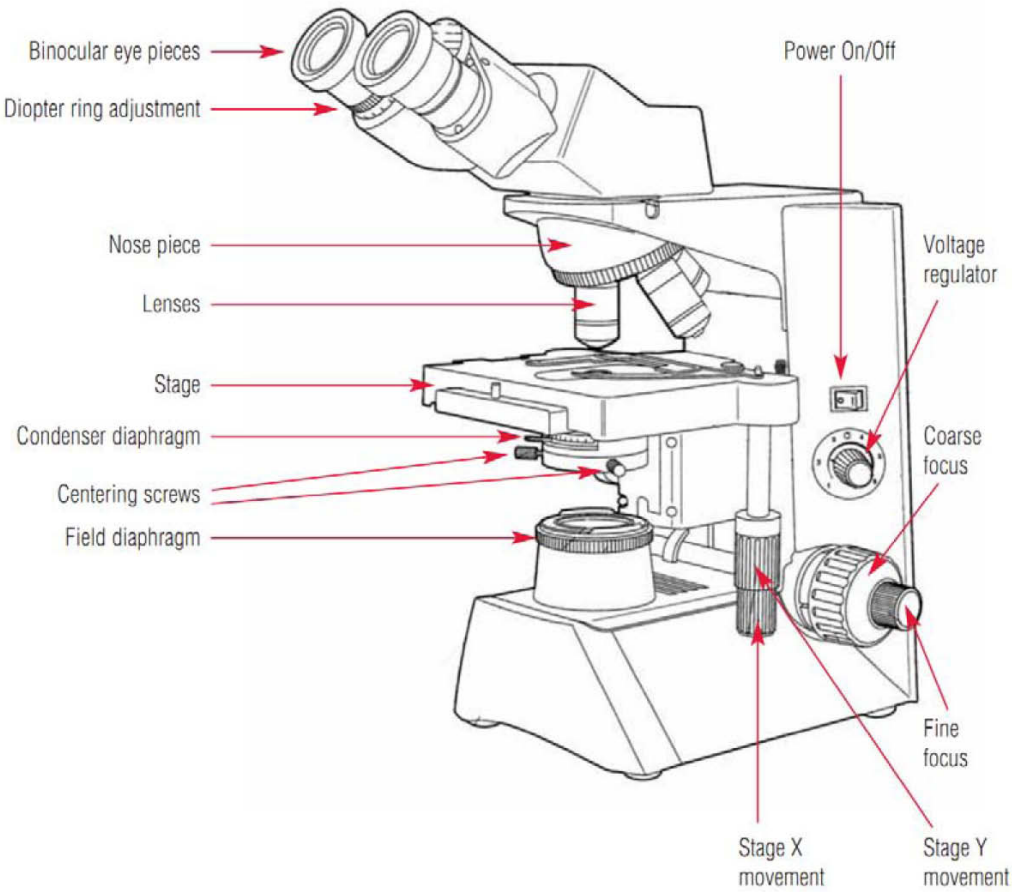
4.6.3 Automated staining

Automated stainers that can process a large number of samples are commercially available. The machines require dedicated reagents and are able to perform both ZN or fluorocrome staining. Accurate and appropriate maintenance after each staining session is required to maintain consistent, high-quality staining.

4.7 Microscopy

Light microscope components are shown in Figure 4.1.[7,16]

Figure 4.1. Microscope components[17]



Source: Lumb R, Bastian I. Laboratory diagnosis of tuberculosis by sputum microscopy. Adelaide: Institute of Medical and Veterinary Science; 2005. p. 38.[17]

4.7.1 Maintenance[3,4,9]

Install the microscope on a rigid, flat, level surface, away from direct sunlight, dust, vibration (e.g. from centrifuges), water (sink, spray from a tap), chemical reagents or humidity.

The modern light microscope needs no particular daily maintenance, but considerable care is required in its use. For further information, please refer to microscope manual for care and maintenance information.

4.7.2 Fluorescence microscopy

The identification of mycobacteria with the fluorescent dye auramine O is based on the affinity of the fluorochrome to the mycolic acids in the cell wall. Auramine O is excited by blue light and emits in the region of ~500 nm to ~650 nm.

Fluorescence microscopy displays some important advantages:

- High contrast fluorescence images allow for easier detection of AFB.
- The use of low- to medium-power lenses (typically 10x, 20x and 40x) permits a larger field of view than conventional microscopy, where typically a 100x lens is used.
- The fluorochrome staining method is simpler than the ZN method.

A binocular microscope equipped with a fluorescent light source and suitable filter set is used for auramine-stained smears. Fluorescent light is provided by a vapour lamp (such as mercury or xenon lamps). The mercury vapour lamp provides the strongest light but it has a limited lifespan of about 100 to 200 hours, which must be monitored with a timer. Also, these lamps are very expensive and fragile.

4.7.3 Light emitting diode (LED) microscopy

There is a compelling base of evidence promoting ultra-bright LED microscopy as a substitute for both conventional fluorescence microscopy and direct ZN microscopy[11]. LED-based microscopy facilitates identification of acid-fast bacilli in comparison with ZN, can be used with auramine staining, is cost-effective (lifespan of the lamp is more than 10 000 hours), has low power requirements, and can be easily introduced in microscopy centres, including peripheral facilities. In addition, light intensity can be easily regulated.

Since LED-based microscopy has been acknowledged as an important development in direct fluorescence microscopy, the WHO has recommended that it replace conventional fluorescence microscopy and that it be phased in as an alternative to conventional ZN microscopy in both high- and low volume laboratories. During the implementation of LED microscopy, the following issues should be addressed: training requirements, validation during the introductory phase, monitoring of trends in case detection and treatment outcomes. Adapted systems should then be introduced for internal quality control as well as external quality assurance.

4.8 Recording and reporting

Recording and reporting of results[2,6,7] is summarised in Table 4.2.

Table 4.2. Reporting of microscopy smears

IUATLD/WHO scale (1000x field = HPF) Result	Microscopy system		
	Bright field (1000x magnification: 1 length = 2 cm = 100 HPF)	Fluorescence (200–250x magnification: 1 length = 30 fields = 300 HPF)	Fluorescence (400x magnification: 1 length = 40 fields = 200 HPF)
Negative	Zero AFB/1 length	Zero AFB/1 length	Zero AFB/1 length
Scanty	1–9 AFB/1 length or 100 HPF	1–29 AFB/1 length	1–19 AFB/1 length
1+	10–99 AFB/1 length or 100 HPF	30–299 AFB/1 length	20–199 AFB/1 length
2+	1–10 AFB/1 HPF on average	10–100 AFB/1 field on average	5–50 AFB/1 field on average
3+	>10 AFB/1 HPF on average	>100 AFB/1 field on average	>50 AFB/1 field on average

4.8.1 ZN smear examination

Examine one length of the smear (2 cm) or 100 fields with light microscope, using 1000x magnification. If less than 10 AFB are found in 100 fields, the number of AFB should be counted. For high positives, examination of only 20 to 30 fields is sufficient.

4.8.2 Auramine (fluorochrome) smear examination

Examine one length of an auramine slide with a fluorescent microscope, using 200–250x magnification, to cover 30 fields in one length, equivalent to 300 fields at 1000x magnification. Alternatively, 400x magnification can be used, covering 40 fields at this magnification.

Negative report: Negative for acid-fast bacilli where no organisms observed in 100 oil immersion fields.

Positive report: Positive for acid-fast bacilli; provide AFB quantification.

The results should be recorded in the TB laboratory register, and recorded on the sample examination request form as well as forwarded to the person requesting the sample examination[10].

Figures 4.2 and 4.3 give examples of smear microscopy using auramine and ZN staining.

Figure 4.2. AFB smear microscopy of biological specimens: (a) auramine stain; (b) Ziehl-Neelsen stain

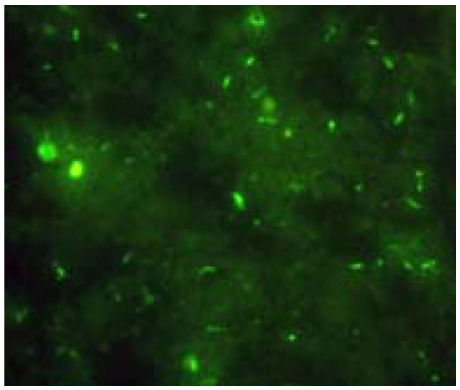
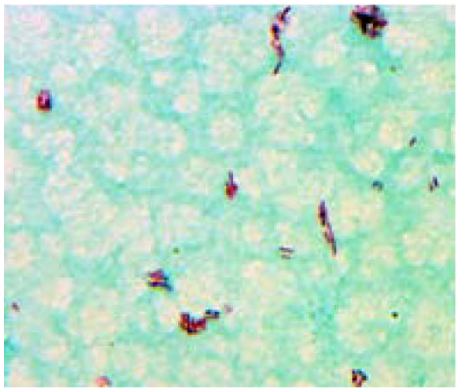
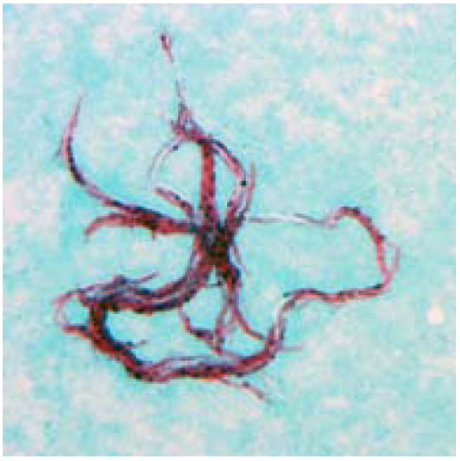
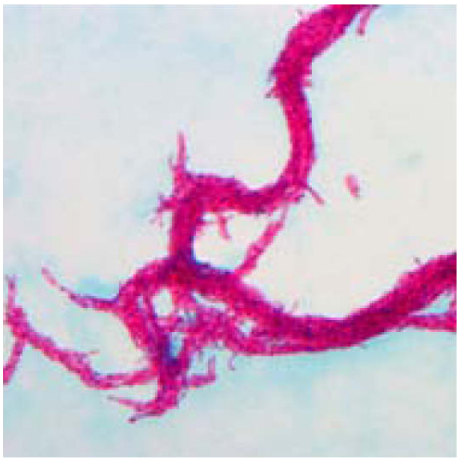
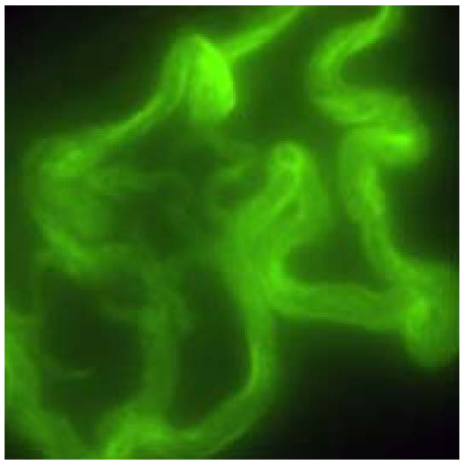
a.  b. 

Figure 4.3. AFB smear microscopy of *M. tuberculosis* cultures. Images show cords: (c, d) Ziehl-Neelsen stain; (e) auramine stain

c.  d. 

e. 

Pictures in Figures 4.2 and 4.3 were kindly provided by Professor Zofia Zwolska, Head of the Microbiology Department, National Tuberculosis Reference Laboratory, National Tuberculosis and Lung Diseases Research Research Institute, Warsaw Poland Institute, Warsaw Poland.

4.9. Quality control

4.9.1 Quality control parameters

Quality control in microscopy is a process of internal monitoring of the performance of bench work in the laboratory. It should be an effective and systematic process, ensuring that laboratory work is accurate, reliable and reproducible. This is done by assessing the quality of specimens; monitoring the performance of microscopy procedures, reagents and equipment; reviewing microscopy results; and by documenting the validity of microscopy methods.

A positive and a negative control slide should be included in each run of stains, verifying the correct performance of the procedure as well as the staining intensity of the acid-fast organisms[14,15].

Table 4.3 shows the most common causes of errors in smear microscopy. Control slides should be assessed prior to reading the patient smears to confirm the correctness of staining. If QC slides are acceptable, patient smears should be read and reported. If the control slide(s) are unacceptable, review procedures and reagent preparations. After identifying and correcting the problem, all patient slides should be repeated with a new set of controls. The results of the quality control of reagents should be reported in the reagent preparation workbook.

Table 4.3. Common causes of errors in smear microscopy	**Cause**	**Action to be taken**
False negative	Smear too thick, detaching during staining	Improve homogenisation, reduce the material deposited.
	Smear too thin	Increase the amount.
	Poor staining	Check QC of reagents, prepare new reagents, check dilution.
False positive	Cross-contamination	Avoid contact between slides during staining procedure. Clean objective lens after reading each slide. Check water/solutions for environmental contamination.
	Red precipitates	Prepare new solution. Filter before use.

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5 Culture tests for *Mycobacterium tuberculosis* complex

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5.1 Background and principles

Bacteriological cultures can provide a definitive diagnosis of tuberculosis. The primary advantage of culture tests over sputum microscopy is its higher sensitivity, allowing for the detection of very low numbers of bacilli (approximately 10 bacilli/ml of sputum compared with at least 5000 bacilli/ml of sputum for microscopy). The use of cultures increases the potential of diagnosing TB at early stages of the disease. Culture tests are also used for the detection of treatment failures and in extrapulmonary TB. The use of culture tests increases the number of TB cases found by 30–50%. Moreover, cultures are used for species identification and drug susceptibility testing (DST)[1,2].

European standards for tuberculosis care call for the use of cultures, in liquid or on solid media, on at least the first sample of every new suspected TB case[1,2]. If this is not feasible, culture tests should at least be performed for the:

- diagnosis of cases with clinical and radiological signs of pulmonary TB where smears are repeatedly negative;
- diagnosis of extrapulmonary TB;
- diagnosis of childhood TB;
- diagnosis of TB among HIV-positive adults and children; and
- diagnosis and monitoring of MDR- and XDR-TB.

Tuberculosis, although mainly a pulmonary disease, can affect any organ of the body. The isolation of the aetiological agent for effective microbiological diagnosis is dependent on:

- the selection of the correct type of specimen;
- the quality of the sample; and
- the adequate use of storage and transportation procedures.

Processing of inappropriate clinical specimens for mycobacterial cultures is a waste of both financial and human resources[6]. Clinical staff should be properly trained and accept only suitable specimens.

Because mycobacteria are usually slow growing and require a long incubation time, a variety of other microorganisms can overgrow the cultures of specimens obtained from non-sterile sites. Appropriate pre-treatment and processing of samples, as well as the use of selective culture media is critical for eliminating contaminants as much as possible while not seriously affecting the viability of mycobacteria[10].

5.2. Biohazards and biosafety in the TB laboratory

Good microbiological techniques (GMT) – working methods designed to eliminate or minimise exposure to pathogens via, for example aerosols, splashes, accidental inoculation – are essential for minimising biohazards[13]. Nosocomial transmission of *M. tuberculosis* from specimens is a major concern to laboratory workers. All specimens suspected to contain *M. tuberculosis* should be handled with appropriate precaution at all times and opened only within an appropriate biosafety cabinet. Infectious aerosols are produced in the TB laboratory whenever a liquid suspension containing tubercle bacilli is handled. Biosafety measures in the laboratory are essential to protect workers against exposure to infectious aerosols. Please refer to Chapter 1 for more details on procedures and laboratory safety practices.

Because of their viscosity, sputa are a minimal source of infectious aerosols. By contrast, aerosols produced during processing (especially during centrifugation steps) of homogenised sputa and during culture handling, must be minimised and therefore processed and contained in a BSC.

Classification of laboratory practices used for *M. tuberculosis* diagnosis should be based on a risk assessment (number and type of tests, prevalence of tuberculosis and of MDR-TB). Specimen processing for mycobacterial culture should be performed in a BSC in at least a biosafety level 2 (BSL2) laboratory, whereas procedures involving manipulation of *M. tuberculosis* cultures (identification, sub-culturing and DST) must be performed in a BSC in laboratories complying with BSL3 standards. All aerosol-generating procedures should be performed in a class I or II BSC[9,11].

The health of laboratory workers should be regularly monitored by the employer. They should be educated about the symptoms of TB and be provided with readily accessible free medical care if symptoms arise. Confidential HIV

counselling and testing should be offered to laboratory workers. Options for the reassignment of HIV-positive workers away from the high-risk areas should also be considered.

5.2.1. Minimum WHO recommendations for TB culture/DST facilities

The WHO recommends that all specimen processing procedures are carried out in a laboratory built and equipped for BSL2. The minimum requirements for a BSL2 tuberculosis laboratory are: restricted access to the laboratory, the presence of a fully functional and maintained BSC, and an autoclave or other means of decontamination available in the same building. More information on biosafety is given in Chapter 1[9-13].

Identification, subculturing, and DST should be performed in a BSL3 containment room with an anteroom and directional airflow from functionally clean to dirty areas, with at least 6 to 12 air exchanges per hour. The containment room may be the blind end of a corridor, or be formed by constructing a partition and door so that access to the laboratory is through an anteroom (e.g. double-door entry) or through the basic BSL2 laboratory. The autoclave should be in the vicinity of the laboratory so that the movement of contaminated materials is minimised. BSCs have to be ducted to the outside or vented through a thimble. Recirculation of air from BSCs into the laboratory room and recirculation to other areas within the building are not permitted. Please refer to Chapter 1 for further detail on laboratory safety levels and conditions.

5.2.2 Personal protective equipment (PPE)

The decision to use additional PPE should be based on risk assessments. Risk assessments should be reviewed routinely and revised when necessary.

Masks and respirators

One of the most common misconceptions is that a standard surgical mask can provide protection against *M. tuberculosis* infectious aerosols. Surgical masks made from poorly fitting porous material, leave large gaps between the face and mask and therefore only help in the prevention the spread of microorganisms *from the wearer* to others by capturing the large wet particles in the exhaled air.

Although BSCs and airflow in the laboratory are the main means of protection against exposure to contaminated aerosols generated during culture and DST activities, the need for additional personal protection must be assessed and considered in certain settings, such as when MDR-TB and/or HIV are prevalent. Staff may be HIV-infected and highly susceptible to contaminated aerosols.

Protection from inhalation of infectious aerosols can be provided by respirators, which are devices with the capacity to filter particles of 0.3–0.4 µm diameter and fit closely to the face to prevent leakage around the edges. The N95 (FFP2) respirator is a lightweight, disposable nose and mouth respirator; it effectively filters out more than 95% of particles of diameter 0.3 µm and above. The FFP3 respirator removes more than 98% of such particles. Each user should be instructed about the proper use of the respirator and informed about its limitations. Respirators should be correctly fitted to the face to prevent leakage from around the face seal. This is done by placing the mask over the nose and mouth with the top elastic band over the crown of the head and the bottom elastic band over the back of the neck. The metal strip covering the nose should be firmly moulded over the bridge of the nose. Facial hair between the wearer's skin and the sealing surfaces of the respirator will prevent a good seal. Respirators should also be worn during emergency cleaning of spillages that involve the release of viable organisms in the work area. Respirators should be stored in a convenient, clean and sanitary location and should be discarded after eight hours (cumulative) of use and not be kept for more than one week[7].

Gloves

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

Gloves must be changed after every session that requires their use and after every interruption of the activity. Never wear gloves outside the laboratory. Every time hands are removed from the BSC, gloves must be pulled off and discarded in a waste container in the BSC[7].

Disposable latex, latex-free vinyl (clear) or nitrile gloves can be used and the correct size (small, medium or large) should be available for all individuals. Hypoallergenic gloves should be provided in case of allergy to latex proteins and/or to the cornstarch used for powder. Re-using single-use gloves is not advised. Used gloves should be discarded as contaminated material. Following the safe removal of gloves, wash hands immediately with water and liquid soap. Proper hand-washing with soap and adequate care in the handling of contaminated materials are critical elements of safe laboratory practice.

Gowns

Always wear a gown inside the laboratory (never outside) and change at least weekly. Long-sleeved with narrow cuffs, back-opening gowns or overalls give better protection than laboratory coats and are preferred in microbiology laboratories. When worn, laboratory coats should be fully buttoned. An area of the laboratory must be designated for storage of used and new clothing. Laboratory gowns must be stored away from personnel clothing. Laundering services should be provided at/near the facility. Considerations should be given to having extra clothing suitable for visitors, maintenance and emergency response personnel[7].

To remove personal protective equipment, always remove in the following order:

- disposable gloves;
- gown/coat/suit/overalls;
- respirator/mask.

5.3 Specimen collection, storage and transport

5.3.1 Sample collection

Proper specimen collection procedures and containers, adequate specimen volumes and appropriate transport conditions can all affect TB culture results. Correct labelling of specimens is critical. This includes patient and sample identification, sample type, and date of collection.

As a general rule, it is preferable that specimens are collected before starting specific treatments. Specimens should always be collected with care to avoid contamination by host or environmental microorganisms and submitted in sterile, leakproof, disposable, appropriately labelled, laboratory-approved containers without any fixatives.

If centrifugation is used for culture tests, the use of collection containers suitable for centrifugation should be considered. Decontamination and centrifugation in the collection container avoids having to transfer samples to another container.

Sputum samples

Most specimens received by the laboratory are sputum samples. Patients should be clearly instructed on how to collect the sputum specimen; written instructions must be provided.

A systematic review of 37 eligible studies[6] clearly showed that most TB cases (average 85.8%) were detected with the first sputum specimen. With the second sputum specimen, the average incremental yield was 11.9%; with the third specimen (when the first two were negative) the incremental yield was 3.1%. It is expected that microscopic analysis of two sputum smear samples will improve case-finding, reduce time to diagnosis, accelerate initiation of treatment, and decrease the number of patients dropping out of the diagnostic pathway. Based on this evidence, WHO has recommended that two sputum samples in a single day be used to diagnose pulmonary TB in settings where a well-functioning EQA system is in place, the workload is high, and human resources are limited.

A good sputum specimen should be approximately 3–5 ml of recently-discharged material from the bronchial tree. It is usually thick and mucoid. It may be fluid and contain pieces of purulent material. The colour may vary from opaque white to green. Bloody specimens will appear reddish or brown. Clear saliva or nasal discharge is not suitable as a TB specimen, although saliva should not automatically be rejected: induced and follow-up sputa resemble saliva. To avoid contamination or dilution of a good sample, specimens should not be pooled.

Other specimens

Body fluids (spinal, pleural, pericardial, synovial, ascitic, blood, pus, and bone marrow) should be aseptically collected in sterile containers using aspiration techniques or surgical procedures. Pleural effusion is a suboptimal specimen: tubercle bacilli are mainly in the pleural wall and not in the fluid. The minimum volume for pleural effusion is 20–50 ml. A pleural biopsy specimen is ideal.

For fluids that may clot, sterile potassium oxalate (0.01–0.02 ml of 10% neutral oxalate per ml fluid), heparin (0.2 mg/ml), or sodium citrate (two drops of 20% sodium citrate for every 10 ml of fluid) should be added as an anticoagulant to the culture.

Aseptically collected tissues should be placed in sterile containers without fixatives or preservatives and transported quickly to the laboratory. For prolonged transportation, dehydration should be prevented by adding sterile saline and maintaining a temperature of 4–15 °C.

Urine is expected to be contaminated. To minimise excessive contamination of urine specimens, external genitalia should be washed before specimen collection. Once received in the laboratory, a urine sample must either be processed immediately or centrifuged and the pellet refrigerated. As excretion of tubercle bacilli is intermittent, three consecutive early-morning midstream specimens must be collected.

Other respiratory specimens that can be submitted to the laboratory for mycobacterial culture are bronchial secretion (minimum volume 2–5 ml) and bronchial alveolar lavage (BAL) samples (minimum volume 20–50 ml). Transbronchial and other biopsies taken under sterile conditions should be kept wet during transportation by adding 0.5–1 ml sterile 0.9% saline.

In children who produce little, if any sputum, aspiration of the early-morning gastric juice can be used for TB diagnosis. The gastric aspirate should be transported immediately to the laboratory and neutralised by adding 100 mg of sodium bicarbonate.

5.3.2 Storage of specimens

Specimens should be correctly collected and delivered as quickly as possible to the laboratory. Every effort must be made to organise and expedite specimen transportation and processing. Although TB bacilli can survive in sputum for one week in the absence of preservatives, the probability of successfully culturing the bacilli decreases with time and this is especially critical for paucibacillary specimens. If specimens cannot be transported to the laboratory within one hour, it is recommended to store them at 4 °C. This does not apply to whole blood specimens, which are not to be refrigerated. On arrival at the laboratory, specimens should again be refrigerated until they can be processed. The delay between collection and inoculation should not exceed seven days.

5.3.3 Transportation of specimens

Packaging of infected specimens that are to be sent by surface or air mail must be carried out according to national biosafety guidelines or international rules. For international transfer of infectious substances, the International Air Transport Association (IATA, www.iata.org) should be contacted.

Specimens and cultures should be packaged in a three component packaging consisting of:

- a leakproof primary receptacle(s);
- a leakproof secondary packaging; and
- an outer packaging of adequate strength for its capacity, mass and intended use.

For the purposes of transport, infectious substances are defined as substances which are known or are reasonably expected to contain pathogens[8]. Category A corresponds to an infectious substance which is transported in a form that, when exposure to it occurs, is capable of causing permanent disability, and/or life-threatening or fatal disease in otherwise healthy humans or animals. All other infectious substances belong to Category B.

Cultures of *M. tuberculosis* belong to Category A. However, for surface transport, when *M. tuberculosis* cultures are intended for diagnostic or clinical purposes, they may be classified as category B. For surface transport there is no maximum quantity per package.

For air transport:

- no primary receptacle should exceed 1 litre (for liquids) or the outer packaging mass limit (for solids); and
- the volume shipped per package should not exceed 4 litres or 4 kg.

These quantities exclude ice, dry ice, or liquid nitrogen when used to keep specimens cold.

5.4 Homogenisation and decontamination of specimens

Most (but not all) specimens are considered contaminated. Pulmonary specimens including sputum, bronchial secretions, bronchoalveolar lavage, bronchial aspirates and brushings are usually contaminated by normal host microbiota. Extrapulmonary specimens may be divided into two main groups according to the extent of contamination:

- Aseptically collected specimens, usually free from other microorganisms (sterile).
- Specimens contaminated by normal flora or specimens not collected aseptically (not sterile).

Normally, contaminated extrapulmonary specimens are gastric lavage, laryngeal aspyrates, urine, skin, autoptic materials, and uterine mucosa. Sterile specimens include pus from cold abscess, CSF, synovial or other cavity body fluids, as well as surgical biopsies.

Contaminated specimens must be subjected to rigorous decontamination procedures that liquefy the organic debris and eliminate the unwanted normal flora. Normal flora would rapidly overgrow the entire surface of the medium and consume it before the TB bacilli started to grow. Specimens must be homogenised to free the bacilli from the mucus, cells or tissue in which they may be embedded.

Digesting/decontaminating agents are to some extent toxic to tubercle bacilli; to minimise the number of dead mycobacteria, the digestion/decontamination procedure must therefore be followed precisely. A proportion of cultures will be contaminated by other organisms: a contamination rate of 3–5% is acceptable on solid media.

Cultures in liquid media may show higher contamination rates (5–10%). Furthermore, if specimens (especially sputum) take several days to reach the laboratory, the contamination rate may be higher. A contamination rate that approaches 0 indicates that the decontamination procedure was too harsh.

5.4.1 Digestion and decontamination of sputum samples

Digestion and decontamination using the sodium hydroxide (modified Petroff) method

Sodium hydroxide is toxic, both for contaminants and for tubercle bacilli; strict adherence to the indicated timings is therefore essential. This decontamination procedure can only be used for samples which will then be inoculated on solid media.

Reagents:

- Sodium hydroxide (NaOH) solution, 4%
- Phosphate buffer 0.067 mol/litre, pH 6.8

Sodium hydroxide (NaOH) solution, 4%:

- Sodium hydroxide pellets (analytical grade): 4 g
- Distilled water: 100 ml

Dissolve NaOH in the distilled water. Aliquot in 2 ml quantities. Sterilise by autoclaving at 121 ºC for 20 minutes.

Phosphate buffer, 0.067 mol/litre, pH 6.8:

- Stock solution A: disodium phosphate, 0.067 mol/litre

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

- Stock solution B: monopotassium phosphate, 0.067 mol/litre

Dissolve 9.07 g of KH_2PO_4 in 1 litre of distilled water.

Mix 50 ml of solution A and 50 ml of solution B. Use a pH meter to confirm that the correct pH for the buffer is reached. Adjust as necessary, using 10% phosphoric acid or 10% sodium hydroxide.

Aliquot in volumes required for adding to a single centrifugation tube (e.g. 50 ml amounts if 50 ml centrifuge tubes are used), discarding the extra volume. Sterilise by autoclaving at 121 ºC for 20 minutes. Leftover volumes of buffer can then be pooled and sterilised again for further use.

Procedure:

- If sputum has not already been collected in centrifuge tubes, select sterile plastic screw-top centrifuge tubes (one for each specimen).
- With a permanent marker, write the number of the specimen on the wall of the tube (not on the cap).
- Write the number of each specimen and the inoculation date on two tubes of media.
- Mark the volume of sputum on the centrifuge tube (at least 2 ml, not more than 5 ml). Add an equal volume of 4% NaOH and tighten the screw-cap.
- Vortex to digest.
- Allow to stand for 15 minutes at room temperature.
- Fill the tube to within 2 cm of the top (e.g. to the 50 ml mark on the tube) with phosphate buffer.
- Centrifuge at 3000g for 15 minutes.
- Carefully pour off the supernatant through a funnel into a discard can containing 5% phenol or another mycobacterial disinfectant.
- Resuspend the deposit in approximately 0.3 ml phosphate buffer.
- Inoculate the deposit on two slopes of egg-based medium labelled with the ID number. Use a pipette to inoculate each slope with 3–4 drops (approximately 0.1–0.15 ml).
- Smear one drop on a slide, marked with the ID number, for microscopic examination.

Digestion and decontamination using the N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method

The most widely used method for the digestion and decontamination of contaminated specimens is the N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method.

Decontamination using NALC-NaOH is based on the mucolytic properties of N-acetyl-L-cysteine (NALC) which enable the decontaminating agent, sodium hydroxide, to be used effectively at a low final concentration. Consequently, the NALC method results in more positive cultures than other methods, as it kills only about 30% of the tubercle bacilli in clinical specimens. The time needed for processing a single specimen is approximately 40 minutes, while 20 specimens would take approximately 60 minutes.

This method is suitable for cultures on both solid and liquid media. However, the disadvantages of the method are that NALC loses activity and must therefore be made fresh every day. Commercially prepared solutions are available, but expensive.

After exposure to the decontaminant and subsequent centrifugation, it is essential the sediment be resuspended in a 1:10 dilution of buffer (or water) to reduce the concentration of any toxic components that may inhibit the growth of TB bacilli.

As a measure of precaution, an aliquot of the sediments should be kept for one week in the refrigerator and re-decontaminated if the inoculated cultures show signs of contamination. Optionally the sediment can be frozen (-20 °C) in a screw-cap sterile 1.5-2 ml vial that is properly labelled.

Reagents N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method:

- Sodium hydroxide-citrate solution

Solution A: Sodium hydroxide 4%	
Sodium hydroxide pellets (analytical grade)	40g
Distilled water	1000 ml

  - Dissolve NaOH in the distilled water.

Solution B: Trisodium citrate $3H_2O$ 2.94%	
Trisodium citrate $3H_2O$	29.4g
Distilled water	1000 ml

  - Dissolve trisodium citrate $3H_2O$ in the distilled water.
  - Mix solutions A and B, aliquot in 100 ml quantities, and sterilise by autoclaving at 121 °C for 15 minutes. Store at 4 °C in refrigerator.

- N-acetyl-L-cysteine (NALC)
  - NALC-NaOH solution should be freshly prepared for daily use only.
  - Prepare by adding 0.5g NALC to 100 ml of the sodium hydroxide-citrate solution just before use: aliquot in 4 ml amounts.

- Phosphate buffer, 0.067 mol/litre, pH 6.8
  - See above for preparation.

Sputum processing

Sputum specimens should not be pooled because of the risk of cross-contamination. Always digest/decontaminate the whole specimen – do not attempt to select portions of the specimen as is done for direct microscopy. Gently decant from the specimen container into the centrifuge tube. If the specimen is too viscous to pour, an equal volume of digestant/decontaminant can be added to the sputum in the specimen container before the mixture is poured carefully into an appropriate screw-top centrifuge tube.

Sputa should not be processed in batches of more than 6–8 as the method is strictly time dependent.

Procedure:

- If sputum has not already been collected in centrifuge tubes, select sterile plastic screw-top centrifuge tubes (one for each specimen).
- With a permanent marker, write the number of the specimen on the wall of the tube (not the cap).
- Write the number of each specimen and the inoculation date on two tubes of media.
- Transfer the sputum (at least 2 ml, but no more than 5 ml) into a centrifuge tube. Add an equal volume of NALC-NaOH solution.
- Tighten the cap of the tube and shake or vortex. Mix for no more than 20 seconds.
- Keep at 20–25 °C for 15 minutes for decontamination.
- Fill the tube to within 2 cm of the top (e.g. the 50 ml mark on the tube) with 0.067 mol/litre phosphate buffer (pH 6.8) or distilled water. Vortex mix.
- Centrifuge at 3000g for 15 minutes.
- Carefully pour off the supernatant into a discard bottle containing the appropriate disinfectant.
- Resuspend the deposit and inoculate onto two slopes of LJ medium (and one slope of LJ with pyruvate if needed) or into liquid medium. Using a pipette (not a loop), inoculate each slope with 3–4 drops (0.2–0.4 ml). Smear one drop on a slide (marked with the ID number) for microscopic examination.

5.4.2 Digestion and decontamination of specimens other than sputum

Laryngeal swabs

Smear examination is not done for laryngeal swabs. Swabs yield little material: as much of the material as possible must be collected and not wasted.

- Swabs must be cultured on the day that they are received using sterile precautions.
- Use sterile forceps to transfer the swab to a sterile centrifuge tube.
- Add 2 ml of sterile distilled water.
- Decontaminate according to NaOH-NALC method (see above, Section 5.4.1).
- Before adding the phosphate buffer solution, remove the swab from the tube using sterile forceps.
- Fill the tube to within 2 cm of the top (e.g. the 50 ml mark on the tube) with phosphate buffer, 0.067 mol/litre, pH 6.8. and mix the contents by inversion.
- Centrifuge at 3000g for 15 minutes.
- Carefully pour off the supernatant into a discard bottle containing an appropriate disinfectant.
- Inoculate the deposit on two slopes of LJ medium (and one slope of LJ with pyruvate, if needed) or in liquid medium. Using a pipette (not a loop), inoculate each slope with 3–4 drops.

Gastric lavages

Gastric lavage specimens should be processed as soon as possible after collection; acidity can kill mycobacteria in the specimen so gastric lavage specimens must be processed within four hours. The gastric aspirate should be collected in a tube containing 100 mg of sodium bicarbonate for neutralisation and should be transported immediately to the laboratory. Proceed as for sputum.

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

Mucopurulent materials

Handle as for sputum when the volume is 10 ml or less.

Handle as for mucoid gastric lavage when the volume is more than 10 ml.

Fluid materials

If the specimen has been collected aseptically, centrifuge and inoculate the sediment directly onto culture media, preferably liquid medium.

Materials that should not be decontaminated are:

- spinal, synovial or other cavitary body fluids;
- bone marrow;
- pus from cold abscesses;
- surgically resected specimens (excluding autopsy material); and
- material obtained from pleural, liver and lymph nodes as well as biopsies (if not fistulised).

To maximise the recovery rate, the entire CSF volume (or other small volume of aseptically collected fluid) should be cultured, preferably in liquid medium.

If the specimen was not aseptically collected:

- Handle as for sputum when the volume is 10 ml or less.
- Handle as for fluid gastric lavage when the volume exceeds 10 ml.

Tissue

If a biopsy needs to be processed for smear and culture, it is necessary to homogenise the biopsy in a sterile porcelain mortar or preferably in a small non-reusable tissue grinder with 2–5 ml of sterile saline.

Mortars, pestles and tissue grinders must be cleaned and sterilised thoroughly to prevent false-positive results or contamination due to organisms left over from previous specimens. Lymph nodes, biopsies and other surgically resected tissue should be cut into small pieces with a sterile scalpel or scissors. Homogenise the specimen in a sterile porcelain mortar or tissue grinder using 5 ml sterile saline and a small quantity of sterilised sand. Inoculate the suspension onto culture media.

5.5 Culture media: principles

As *M. tuberculosis* grows slowly, with a generation time of 18–24 hours (other bacteria reproduce within minutes), usual bacteriology techniques are not applicable to mycobacterial cultures. Moreover, growth requirements are such that *M. tuberculosis* will not grow on primary isolation on simple chemically defined media. The only media that allows for abundant growth are egg-enriched media containing glycerol and asparagine, and agar or liquid

media supplemented with serum or bovine albumin. Many different media have been developed for *M. tuberculosis* growth and are generally classified into two main groups: solid media (egg- and agar-based) and liquid media. Antibiotics can be added to culture media in order to prevent the growth of non-specific flora.

Both solid and liquid media are recommended for *M. tuberculosis* isolation from biological samples. An advantage of solid over liquid media is that colonies of mixed cultures and contaminants can be observed.

The choice of media depends primarily on the type of specimen. Non-selective media are recommended for use with samples from normally sterile sites (bone marrow, tissue biopsy samples, cerebrospinal fluid and other body fluids etc.), while selective media, that contain antimicrobial agents to prevent growth by contaminating bacteria and fungi, are recommended for use with contaminated specimens (sputum, abscess contents, bronchial washings, gastric lavage fluid, urine, etc)[1,2].

The most commonly used nonselective media are:

- egg-based media: Löwenstein-Jensen (LJ) medium and Ogawa medium;
- agar-based media: Middlebrook 7H10 and Middlebrook 7H11; and
- liquid media: Middlebrook 7H9 broth.

Commonly used selective media that are available in some countries are:

- egg-based media: Gruft modification of LJ (containing malachite green, penicillin and nalidixic acid as selective agents), and Mycobactosel LJ (containing malachite green, cycloheximide, lincomycin and nalidixic acid as selective agents);
- agar-based media: selective 7H11 (Mitchison's medium), containing carbenicillin, amphotericin B, polimixin B and trimethoprim as selective agents;
- liquid media: in general they contain a modified Middlebrook 7H9 broth plus a mixture of antimicrobial agents. Several automated systems have been commercially developed for rapid detection of mycobacteria in liquid medium:
  - BACTEC MGIT 960 system (BD [Becton, Dickinson and Company] Diagnostic Systems);
  - ESP Culture System II (Trek Diagnostic Systems);
  - MB/BacT (bioMérieux).

5.5.1 Solid media

Egg-based media

LJ medium, which contains malachite green as an inhibitor of non-mycobacterial organism, is the most commonly used egg-based medium, especially for sputum culture. LJ is user-prepared or commercially prepared in slant tubes. LJ containing glycerol favours *M. tuberculosis* growth, while LJ without glycerol but containing sodium pyruvate enhances *M. bovis* growth; both media should be used in geographic regions where patients may be infected with either organisms[3]. Ogawa medium is LJ without asparagin. Non-selective eggs-based media can be stored in the refrigerator for several months provided that the tube caps are tightly closed to minimise drying by evaporation.

A disadvantage of egg-based media it that when contamination does occur it may involve the entire slant surface, so the culture is generally lost. If specimens contain few bacilli it may take three to eight weeks before cultures become positive.

Agar-based media

These media are prepared in slant tubes or plates and are less likely than egg-based media to become contaminated. Middlebrook 7H10 and 7H11 media are usually prepared in the laboratory from commercially available agar-powdered bases with addition of Middlebrook oleic acid-albumin-dextrose-catalase (OADC) enrichment. Because of the transparency of 7H10 and 7H11 plates, *M. tuberculosis* microcolonies with typical cord formation can be detected and counted using a microscope as early as one week after incubation. Also, visibility of colonial morphology on agar plates is better than on egg-containing slants, aiding the identification of mycobacteria. Middlebrook 7H11 should be preferred to 7H10 because it contains 0.1% casein hydrolysate, a substance favouring the recovery of isoniazid-resistant mycobacteria. Furthermore, 7H11 is also better for growing multi-drug resistant (MDR) strains as these may not grow at all on 7H10 agar plates.

A disadvantage of Middlebrook media is that the surface dries more rapidly than egg-based media. It is important to know that daylight, heating and storage at 4 °C for more than four weeks may cause the release of formaldehyde in a sufficient concentration to inhibit the growth of mycobacteria.

5.5.2 Liquid media

Liquid media offer a considerable time advantage over solid media for the detection of *M. tuberculosis* growth: 7–14 days in Middlebrook 7H9 liquid medium, compared with 18–28 days in Middlebrook 7H11 agar, or 21–42 days in LJ medium[5].

One of the most widely used automated systems for rapid detection of mycobacteria in liquid medium is the BACTEC MGIT 960 system[2]. The system's culture tubes consist of modified Middlebrook 7H9 broth, a growth supplement, and an antimicrobial agent mixture. A similar principle is used in the ESP Culture System II and the MB/BacT system. In the BACTEC 960 system and ESP Culture System II, *M. tuberculosis* growth is detected by the rate of oxygen consumption within the headspace of the cultures; in the MB/BacT system, a colourimetric sensor detects the production of CO_2 dissolved in the culture medium.

5.5.3 Quality control of media

Quality control (QC) of the media is needed to ensure that the strain isolated from a specimen is from the patient and not a contaminant present in the ingredients of the medium. The description below holds mainly for quality control of solid media, as it is on such media that colonies are visible to the eye and species identification thus possible.

Commercially prepared media should not be needed to be quality controlled for sterility, growth and selectivity, provided that the documentation of the QC manufacturer's procedures are obtained[12]; the information should include the preparation date, the lot number, the expiration date, the test organisms used, the date of testing and the result. In all other cases (user-prepared media and when documentation of sterility, growth and selectivity is not provided), the media must be checked for:

- medium conditions: colour, dehydration, contamination, bubbles, etc.
- sterility: by incubating from 1 to 3% of each batch at 35 to 37 °C in 5 to 10% CO_2 for up to 21 days
- performance: by testing growth of positive and negative control strains

The organisms used as positive controls are *M. tuberculosis* H37Ra (ATCC 25177), *M. kansasii* ATCC 12478, *M. scrofulaceum* ATCC 19981, *M. intracellulare* ATCC 13950, *M. fortuitum* ATCC 2841. *Escherichia coli* ATCC 25922 is used to demonstrate partial inhibition by non-selective media and complete inhibition by selective media.

Procedure[1,2]:

- Prepare a 0.5 McFarland suspension of the organisms in 7H9 broth.
- Inoculate media with 10µl of the control suspension using a pipette or a calibrated loop. For testing selective properties of the media, inoculate them with 10µl of 1:10 suspension in sterile 0.85% NaCl. Incubate all media at 35 to 37 °C in 5 to 10% CO_2 for up to 21 days.
- Expected results are as follows:

Positive controls	**Result**
M. tuberculosis ATCC 25177	Growth on all media
M. kansasii ATCC 12478	Growth on all media
M. scrofulaceum ATCC 19981	Growth on all media
M. fortuitum ATCC 2841	Growth on all media
M. intracellulare ATCC 13950	Growth on all media (not included when testing selective media containing penicillin or carbenicillin)
Negative control	**Result**
Escherichia coli ATCC 25922	Partial inhibition in nonselective media, total inhibition in selective media

5.6 Culture tube inoculation

5.6.1 Solid media

In media purchased ready-to-use, condensed moisture is frequently observed on the culture slants and should be removed before use. Each slant should be inoculated with 0.2–0.4ml (2–4 drops or 4 loopfuls) of the centrifuged sediment. The use of sterile disposable Pasteur pipettes is highly recommended. The inoculum should be distributed over the entire surface of the slant.

At least two slopes of LJ medium per specimen should be inoculated with 0.2 ml of each sediment. In areas where *M. bovis* is isolated, an additional slope containing pyruvate is recommended. A too-small inoculum is a common cause of false-negative results. In the upper part of the slant the medium is thin and dehydrates readily; if mycobacteria are seeded only on this upper section, they might not grow, again leading to false-negative results.

5.6.2 Liquid media

Inoculation on liquid media should be performed under rigorous sterile conditions to avoid the risk of contamination. Liquid media is more susceptible to contamination than solid media and therefore needs to be

supplemented with a mixture of specific antibiotics to kill the contaminants. These antibiotic mixtures are available from commercial companies selling culture media for automated culture systems.

Each properly labelled liquid culture tube should be inoculated with 0.5 ml of sediment and the sediment must be deposited under the surface of the medium keeping the tube tilted at an angle of 45 °. The tube is then returned to a vertical position, leaving the inoculum below the surface of the liquid.

5.7 Culture incubation[7]

All cultures should be incubated at 35-37 °C. Always check the temperature indicator before incubating the cultures. The cultures should be incubated until growth is observed or discarded as negative after 8 to 12 weeks (6 weeks if liquid media is used).

Inoculated solid cultures should be incubated with caps loosened in a slanted position for at least one week to ensure an even distribution of the inoculum. Thereafter, caps should be tightened to prevent desiccation of the media and if space is needed in the incubator the tubes can be placed upright. Tops should be tightened to minimise evaporation which can result in dry out of the media.

5.8 Culture examination[7]

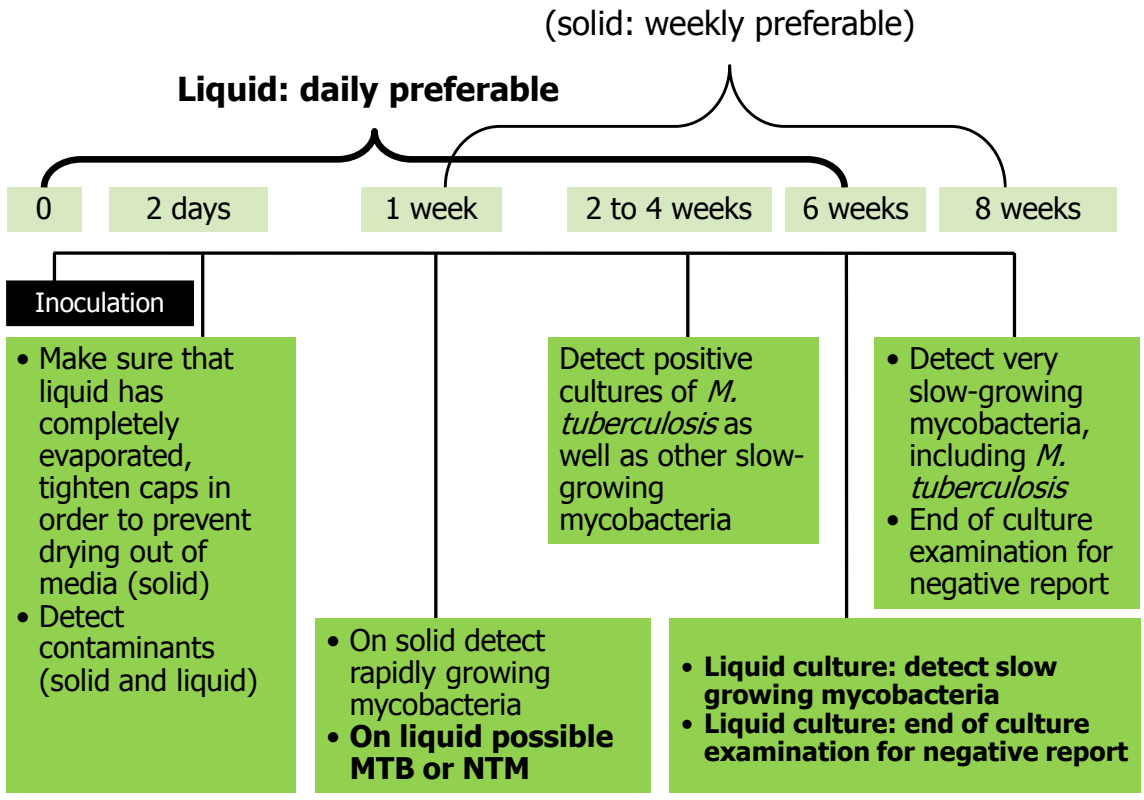
All cultures should be examined 48 hours after inoculation in order to:

- check absorption of liquid inoculated;
- tighten caps to prevent drying out of media; and
- detect early contaminants.

Thereafter, cultures should be examined weekly or if this is not feasible, at least three times during the eight-week incubation period (see Figure 5.1).

- 7 day check: To detect rapidly growing mycobacteria.
- 3–4 week check to detect positive cultures of *M. tuberculosis* as well as other slow-growing mycobacteria.
- End of culture check (after eight weeks) to detect very slow-growing mycobacteria, including *M. tuberculosis*, before discarding and reporting the culture as negative.

Figure 5.1. Minimal examination schedule for solid cultures



Source: Culture, DST and quality assurance package, WHO[14]

Liquid cultures should be examined daily and reported negative after six weeks of incubation in the absence of growth unless incubated in the automated systems.

5.9 Presumptive identification of *M. tuberculosis*[7]

With experience, a mycobacteriologist can presumptively identify *M. tuberculosis* colonies, which are typically rough, crumbly, waxy, non-pigmented (cream, buff-coloured) and slow-growing. A clear morphology is apparent after two to four weeks of incubation.

M. bovis is a slow-grower – colonies appear white, small and round with a wrinkled surface and irregular thin margins. This microorganism is infrequent and is usually isolated from people in contact with cows. In high-burden TB settings and if only solid media is in use, environmental mycobacteria are also infrequent when compared to *M. tuberculosis* isolation and generally represent contamination or colonisation.

Suspicious colonies should be confirmed by Ziehl-Neelsen (ZN) staining. Processing a positive culture causes an increased biological risk and biosafety measures must be strictly enforced; i.e. work performed within a BSC. To minimise cross-contamination risks, positive cultures should be processed after the specimen processing is finished. To do this, a very small quantity of bacteria is removed from the culture using a loop and gently rubbed into one drop of sterile saline or water on a slide. The ease with which the organisms emulsify in the liquid should be noted: unlike environmental mycobacteria, tubercle bacilli do not form smooth suspensions. The smear is allowed to dry, fixed by heat and stained by the ZN method. Observed by microscopy, TB bacilli are frequently arranged in serpentine cords of varying length or show linear clumping. Individual cells are 3–4 µm in length.

If liquid media is used for culture, a positive TB culture may be recognised from the following characteristics:

- signs of growth are observed at least one week after inoculation;
- there is growth in the form of visible floccules in the medium; and
- floccules tend to remain isolated after shaking.

For ZN staining of liquid cultures, again following all biosafety regulations, deposit a drop of medium on a glass slide (albumin-coated smears are recommended for liquid culture microscopy), let it dry in the BSC, fix, and proceed with the staining protocol. *M. tuberculosis* bacteria grown in broth tend to form large side-to-side aggregates called 'cords' from the Latin *corda* meaning "rope". Under certain conditions, some non tuberculous mycobacteria (NTM), such as *M. kansasii, M. marinum*, *M. fallax* and *M. chelone* may also form cords. *M. avium* does not present cording. Identification of TB complex should always be performed before proceeding with drug susceptibility testing (DST).

5.10 Contaminations

Some bacteria and moulds can survive the decontamination procedure and contaminate the culture, with the most common early contaminants of solid media being moulds. Any culture showing the presence of mould contamination should be discarded.

Contaminated cultures are recognizable from various characteristics. The surface of contaminated culture media may be completely covered by the growth of non-mycobacteria. Some bacterial species can liquefy or discolour the solid medium: either the slant collapses to the bottom of the tube or there is a very clear change of colour of Löwenstein-Jensen (LJ) medium (from very dark blue-green to cream). In such cases, cultures should be sterilised and discarded. Certain contaminating organisms produce acid from constituents of the medium and the lowering of pH unbinds some of the malachite green from the egg that is the basis of the medium (indicated by the medium changing to dark green). Tubercle bacilli will not grow under these conditions and cultures should be discarded.

If the contamination is present only in part of the slant and the medium maintains its characteristics, the cultures can be retained until the eighth week. The appearance of late contamination does not exclude the presence of *M. tuberculosis* if colonies with suspicious morphology are visible; a smear should therefore be prepared from these colonies or from the surface of the medium. The smear should be ZN stained. If microscopy indicates the presence of acid-fast bacilli, an attempt can be made to collect bacteria from the slant's surface, followed by re-decontamination and re-inoculation of the culture.

If liquid media are used, contamination should be suspected if homogeneous turbidity is seen. ZN staining should be performed by depositing a drop of medium on a glass slide, letting it dry in the BSC, fixing and proceeding with the staining protocol.

The different kinds of contaminants that should be considered are: non-tuberculous mycobacteria, fungi, bacteria, and yeasts.

After ZN staining, the culture should be handled according to the results:

- Presence of only AFBs in the deposit, with no non-AFBs, indicates pure growth of mycobacteria – the deposit should be processed for identification and DST (inoculation of a non-selective agar plate, such as blood agar, can be used for purity check).
- Presence of AFBs with non-AFBs in the deposit indicates contamination of possible growth of mycobacteria – the deposit should be processed for decontamination and culture on solid media.
- No AFBs and only non-AFBs in the deposit indicate growth of contaminants – the deposit should be discarded.
- Any presence of contaminants should be recorded in the laboratory register and if the culture is discarded, it should be reported as a 'contaminated culture'.

Evaluation of the contamination rate should be performed every six months for quality assurance purposes. A contamination rate of 3–5% is considered a good balance between the need to kill contaminating bacteria and the need to keep the majority of tubercular mycobacteria present in the sample. A contamination rate of 0–1% may indicate too strong a decontamination process. The contamination rate should refer to the number of contaminated tubes, not to the number of registered specimens.

Common contaminants are detailed below[4]:

Non-tuberculous mycobacteria (NTM):

- Fast- or slow-growers
- Acid-fast bacilli
- Not usually arranged in cords

Fungi:

- Usually slow-growers
- Non-acid-fast
- Hyphae are thicker than mycobacteria

Bacteria:

- Usually non-acid-fast except for some closely related genera (*Gordonia*, *Tsukamurella*, *Nocardia*, *Rhodococcus*, *Dietzia*) and *Legionella micdadei*

Yeast:

- Usually non-acid-fast

Oocystis:

- Usually non-acid-fast except *for Cryptosporidium, Isospora, Cyclospora*

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6 Identification of *Mycobacterium tuberculosis* and drug resistance in cultures and sputum using molecular assays and immunoassays

Doris Hillemann, Sarah Mitchell, Francis Drobniewski

6.1 Background and principles

Historically, the direct detection of mycobacteria in patient specimens is by staining and subsequent microscopy. This is a rapid and cheap method; however, it requires a high bacterial count in the specimen for a reliable result. In addition to the low sensitivity, differentiation of the microorganisms is rarely possible using microscopy. Hence, the need for culture of mycobacteria in special liquid and solid media is still the gold standard for detecting mycobacteria in different specimens (Chapter 5). However, the culture of mycobacteria can take between six and eight weeks as TB bacilli and many other mycobacteria grow very slowly. Therefore, early and specific treatment of the patient is delayed with standard biochemical and growth-based differentiation tests.

Molecular biology is now becoming more important in the diagnosis of mycobacteria. It supports culture either by serving as a rapid direct test on specimens or by enabling a rapid and unequivocal species differentiation from culture material. Nucleic-acid-based methods have largely displaced the classical methods.

Molecular genetic tests offer considerable time advantages in the identification of mycobacteria, enabling a more rapid initiation of resistance tests and specific treatment. They are useful tools for the detection and differentiation of mycobacteria from cultures and can have a high specificity and sensitivity. It should be noted however, that they do not/should not replace the currently endorsed standard methods of detecting mycobacteria and determining drug-susceptibility patterns, but rather be used as a support to the diagnostic work-up. Test results should always be confirmed using the standard methods.

This chapter includes methods for the identification of mycobacteria as well as drug susceptibility testing from culture and from specimens.

6.2 Procedure 1: Chromatographic immunoassay for the qualitative detection of *Mycobacterium tuberculosis* complex from cultures

Capilia TB-neo, TAUNS Laboratories Co, Numazu, Japan, and BD MGIT TBc Identification Test, BD (Becton, Dickinson and Company) Diagnostic Systems, Sparks, MD, USA.

6.2.1 Introduction

Definite diagnosis of TB can be made by identification of *M. tuberculosis* complex organisms from a clinical sample after growth in solid or liquid media. Since *M. tuberculosis* complex strains (with the exception of some substrains of *M. bovis* BCG) but not non-tuberculous mycobacteria (NTM) secrete specifically and predominantly the MPB64 protein (mycobacterial protein fraction from BCG of Rm 0.64), this can be used for the discrimination between MTBC and NTM. Immunochromatographic assays based on the reaction of monoclonal antibodies against MPB64 have been developed and evaluated[1-3].

6.2.2 Materials

No special equipment is required for the test; it is sufficient to have the test plates provided by the producer, a 100 µl pipette, and a timer. The test plates consist of a placing area, a test area containing the anti-MPB64 antibodies, and a control area where anti-species immunoglobulin antibodies are fixed.

6.2.3 Methods

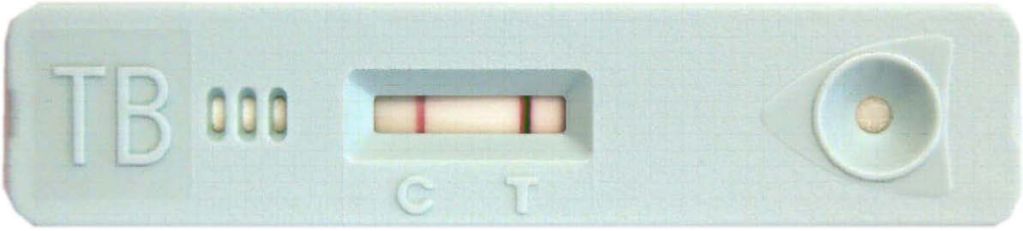
The testing method is based on immunochromatographic principles, in which antibodies labelled with colloidal particles (such as colloidal gold) react with target antigens to form a migrating antigen-antibody complex, which is captured by a second fixed antibody. A colour reaction takes place where the labelled particles are fixed.

The tests can be used with positive liquid media tubes or visible colonies grown on solid media. In the case of liquid cultures, a 100 µl volume is dropped on to the test device. For solid cultures, 1 µl bacteria (=1mm loop) or 1 AFB+ colony (at least 1mm) are resuspended in the respective buffer, vortexed and a 100 µl volume of these suspensions is used. Reading of the results should occur after 15 minutes but within 60 minutes of contact.

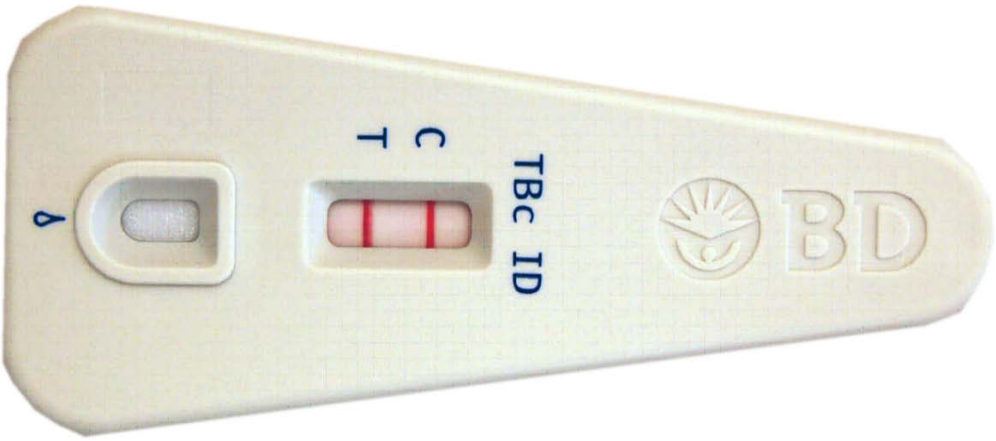
6.2.4 Results/interpretation

For a specific MPB64 antigen-antibody reaction, a red-purple colour band becomes visible within 15 minutes. The culture is interpreted as positive for MTBC if the colour reaction takes place in the test and control area. The intensities of the bands may vary. The specimen is interpreted as negative if a colour reaction takes place only in the control area. The test is invalid if no band is visible in the control area or if the background colour inhibits the test interpretation.

Figure 6.1. Examples of chromatographic immunoassays for the qualitative detection of *Mycobacterium tuberculosis* complex. Developed with 100 µl of a AFB+ smear-positive liquid culture after 15 minutes incubation time



a. Capilia TB-neo



b. BD MGIT TBc identification test

Although most MTBC strains may be correctly identified with the tests exhibiting a high sensitivity (92.4%–99.2%) and specificity (100%), some test-negative strains have been isolated. The reason for the failure of the test is the absence of MPB64 antigens in the culture medium, proven by the detection of a mutation in the MPB64 encoding *mpb64* gene[3]. Figure 6.1 gives examples of chromatographic immunoassays.

6.2.5 Biosafety

Appropriate biosafety precautions for handling mycobacteria must be used. The dropping procedure, development, and reading of the test should be carried out in an appropriate BSC in a BSL 3 laboratory. As used test devices may contain viable mycobacteria, they should be discarded safely according to institutional guidelines for handling BSL-3 material (see Chapter 1).

6.3 Procedure 2: Line probe assay for the identification of the genus *Mycobacterium* and 16 different mycobacterial species including *M. tuberculosis* complex from culture

INNO-LiPA MYCOBACTERIA v2, Innogenetics, Ghent, Belgium

6.3.1 Introduction

INNO-LiPA MYCOBACTERIA v2 is a line-probe assay for the simultaneous detection and identification of the genus *Mycobacterium* and 16 different mycobacterial species. The test is based on nucleotide differences in the 16S–23S rRNA spacer region and can be performed on either liquid or solid cultures[7,8].

6.3.2 Materials

A DNA thermal cycler, microtube centrifuge, water baths or heating blocks, a blotting device including a tray for the strips, a vortex, a rack, adjustable micropipettes, disposable pipette tips, 1.5 ml screw-top microcentrifuge tubes, gloves, thermostable DNA polymerase, autoclaved distilled water, TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and reagents from the kit (amplification buffer, primer mix, $MgCl_2$ solution, reverse line blots, hybridisation solution, stringent wash solution, denaturation solution, conjugate solution, conjugate buffer, substrate solution, rinse buffer).

6.3.3 Methods

The test comprises three major parts: DNA extraction, DNA amplification and hybridisation.

For DNA extraction from solid cultures, a loopful of bacteria is transferred and resuspended in 0.5 ml TE buffer in a screw-top microcentrifuge tube. For liquid cultures, 500 µl of the liquid culture containing some bacterial clumps with as much bacteria as possible (e.g. taken from the bottom) are transferred directly to a screw-top microcentrifuge tube. All samples are boiled for ten minutes at a temperature of at least 95 °C. After centrifugation at 17 900g for five minutes, 2 µl of the supernatant can be used for amplification.

For amplification, the following mixture should be prepared:

- 14.8 µl autoclaved distilled water
- 10 µl amplification buffer
- 10 µl primer mix
- 10 µl $MgCl_2$ solution
- 0.2 µl *Taq* polymerase (1 U, 5U/ µl)
- 5 µl extracted DNA

A negative control containing water instead of the extracted DNA must be prepared with every run of the test.

The amplification profile is as follows:

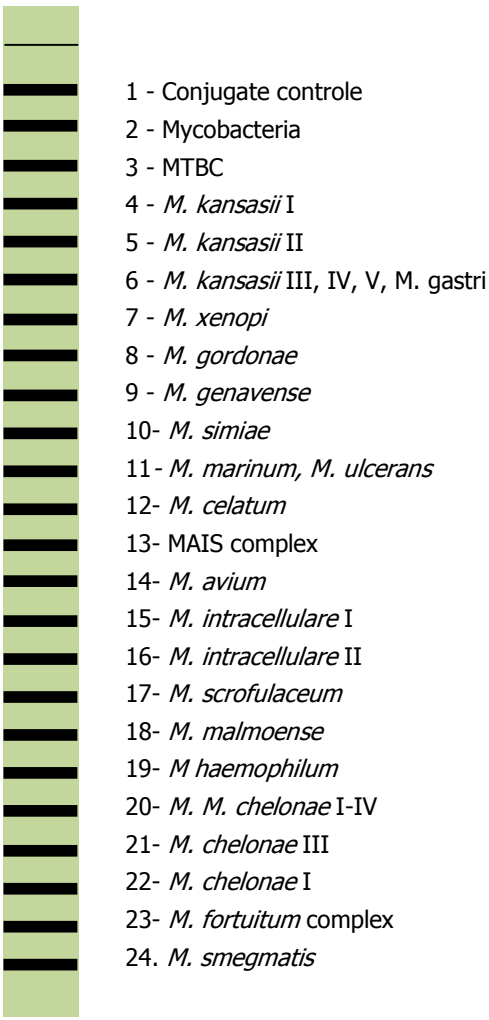
4 min	95 °C
30s	95 °C
30s	62 °C, repeat steps 35 times
30s	72 °C
End 4 °C	

If no automatic blotting system is used, hybridisation can be performed manually. For this purpose, 10 µl of the amplification product is added and carefully mixed with the denaturation solution in a disposable trough together with the test strip. Incubation time is five minutes at room temperature. After the addition of 2 ml prewarmed hybridisation solution, the trough is shaken gently in a water bath at 62 °C for 30 minutes. After removal of the hybridisation solution, the strips are washed with stringent wash solution twice at room temperature, and once at 62 °C for 10 minutes. The next two washing steps with rinse solution for 1 minute each are also at room temperature. The strips are then shaken for 30 minutes in the conjugate solution (streptavidin labelled with alkaline phosphatase). Washing of the test strip twice for one minute using rinse solution is followed by one washing step with substrate buffer. The test strip is then incubated in substrate solution for 30 minutes or until clearly visible bands develop. Colour development can be stopped by washing twice for three minutes with distilled water. After drying the strips on absorbent paper they can be interpreted.

6.3.4 Results/interpretation

The following *Mycobacterium* species can be detected simultaneously according to the interpretation scheme: *M. tuberculosis* complex, *M. kansasii*, *M. xenopi*, *M. gordonae*, *M. genavense*, *M. simiae*, *M. marinum* and *M. ulcerans*, *M. celatum*, MAIS, *M. avium*, *M. intracellulare*, *M. scrofulaceum*, *M. malmoense*, *M. haemophilum*, *M. chelonae* complex, *M. fortuitum* complex, and *M. smegmatis* (Figure 6.2).

Figure 6.2. Interpretation scheme for INNO-LiPA



In one study[8], the overall specificity and sensitivity of the assay was estimated at 100% for the genus-specific probe. For the other probes, the sensitivity was 100%, while the total specificity was 92.2% or 94.4% depending on whether two specimens of very rare species (one *M. thermoresistibile* and one *M. agri*) were excluded from the study or not. Another group reported excellent sensitivity and specificity for all species and complexes for which the test is licensed[7].

6.3.5 Biosafety

Appropriate biosafety precautions for handling mycobacteria have to be used. Screw-top Eppendorf tubes should be used throughout when heating mycobacteria. The transfer of bacteria in the tubes prior to and during boiling should be carried out in an appropriate BSC in a BSL 3 laboratory. After heating, further steps can be performed outside the BSC.

6.4 Procedure 3: Line probe assay for the identification of the *M. tuberculosis* complex and the detection of rifampicin resistance in *M. tuberculosis*[1]

[1] INNO-LiPA Rif.TB kit, Innogenetics, Ghent, Belgium

6.4.1 Introduction

The LiPA rpoB PCR can be performed on all respiratory specimens and other specimens where the detection of rifampicin resistance in *M. tuberculosis* is the primary purpose of the investigation[6,11]. The PCR described is for use with all non-cerebral spinal fluid (CSF) specimens unless otherwise specified.

6.4.2 Materials

INNO-LiPA Rif.TB kit – Innogenetics K1044.
A full explanation of the principle, methodology and interpretation of the LiPA can be found in the instruction booklet included in each kit.

Gloves and guarded tips should be used throughout the procedure.

6.4.3 Methods

Each PCR run should include:

- each sample in duplicate;
- an inhibition control for each sample;
- the decontaminated extracted negative control;
- five extracted negative controls (water);
- the decontaminated, extracted, positive control; and
- a positive control with a low amount of DNA.

DNA extraction

See Section 6.4.3. Ensure DNA extraction is carried out in a BSL3 laboratory.

Amplification

A one step amplification is performed on DNA from culture though a nested PCR (higher sensitivity) is recommended on DNA extracted from specimens.

To be carried out in a PCR clean room. A clean room is any laboratory area without mycobacterial DNA and PCR amplification products. Never bring PCR amplification products back into a clean area; this includes clothing of the laboratory scientist, reagents, equipment, etc. unless thorough decontamination to remove DNA/RNA has been carried out.

While samples are in the 80 °C heating-block (see DNA extraction), prepare the PCR tubes.

- Remove enough 2X buffer (mix) and outer primers (LiPAOP1/2 2 pmol/µl) from the freezer and allow to thaw.
- Prepare the bulk PCR mix in a screw-cap microfuge tube. For each reaction required add:

20µl 2X buffer
10µl primer mix
0.2µl Taq DNA polymerase

- Mix by inverting the tube 10 times, then briefly centrifuge before distributing 30µl aliquots into labelled 0.2ml thin-wall PCR tubes.
- Close lids gently and transfer to the specimen preparation room.

In the specimen preparation room:

- Place the PCR tubes in a PCR rack and place in a Class-1 cabinet.
- Using a clean guarded tip for each tube, add 10µl of DNA extract or control to the appropriately labelled PCR tube. Once all extracts and controls have been transferred, add 1µl of inhibition control to the inhibition control tubes.
- Close tubes tightly and transfer to the PCR product lab. Note: Remove coat and carry tubes in gloved hand. Do not take anything in to the PCR product lab.
- Place tubes in the thermal cycler and close lid. The PCR cycles should be:

a.		95 °C	15 min
b.	30 cycles	95 °C	60 s
		58 °C	30 s
		72 °C	90 s
c.		70 °C	10 min
		4 °C	∞

The reaction should take about 2.5 hours (it is convenient to run this overnight). Shortly before the cycling is complete, prepare the second round reactions.

Nesting the PCR reactions

Preparing the second-round reactions.

In the PCR clean room:

- Remove enough 2X buffer (mix) and inner primers (LiPAIP1/2 2 pmol/µl) from the freezer and allow to thaw.
- Prepare the bulk PCR mix in a screw-cap microfuge tube. For each reaction required add:

10µl purified water
20µl 2X buffer
10µl primer mix
0.2µl Taq DNA polymerase

- Mix by inverting the tube 10 times and distribute 40µl aliquots into labelled 0.2ml thin-wall PCR tubes.
- Close lids gently and transfer to the PCR product lab.

Nesting procedure

- When the first-round cycling is complete remove the tubes from the thermal cycler and place in the rack labelled 'nesting'. Centrifuge the tubes in the picofuge for one minute.
- Assemble all the required equipment in the Template-Tamer workstation – the nesting pipette, the nesting tips, and the second-round reaction tubes.
- Change gloves directly before nesting. Add 1µl of first-round reaction directly to the liquid in the appropriate second-round tube. Be careful not to touch the underside of the microfuge lid.
- Spin the tubes briefly in the picofuge and load onto the thermal cycler before closing the lid.
- Place tubes in the thermal cycler and close lid. The PCR cycles should be:

a.		95 °C	15 min
b.	30 cycles	95 °C	30 s
		65 °C	30 s
		72 °C	30 s
c.		70 °C	10 min
		4 °C	∞

Hybridisation

In PCR product lab:

- Remove INNO-LiPA Rif.TB box from fridge. Preheat the hybridisation solution (HS) and stringent wash (SW) solution to 62°C. Allow other contents to warm up to room temperature while bath heats to 62°C. Check with thermometer and adjust accordingly. Temperature must be ± 0.5 °C for accurate results.
- Place required number of hybridisation trays in plastic rack.
- Mix the denaturation solution (DS). Using a plugged 20µl pipette tip and a P20 pipette, place 10µl of DS into all trays to be used in the assay.
- Using a new 20µl plugged pipette tip for each sample, add 10µl of PCR product and mix (by pipetting up and down six times) with the DS in the appropriate tray.
- Allow the PCR product to denature for five minutes (important: DNA must be single-stranded for the hybridisation to work properly).
- Using forceps, remove the appropriate number of test strips and place on clean paper. Label the strips by marking them with a pencil above the red marker line. Avoid touching the strip below the red marker line as this can damage and remove the probes.
- Swirl the HS to ensure all contents are dissolved. Using a P1000 pipette and a plugged pipette tip, place 1 ml of HS in each tray. Mix briefly by rocking. Place a test strip in each tray and ensure that they are fully submerged.
- Place tray in the shaking bath and leave for 30 minutes.

- After 30 minutes remove the tray from the bath and place on absorbent paper. Remove the HS using the tap vacuum to discard into the sink and add 1 ml of SW solution. Agitate gently for 1 minute. Repeat this wash step once. Remove solution and add 1 ml of SW solution.
- Place back in the shaking water bath for another ten minutes.
- While stringent wash is in progress make up the follow solutions:
  - Rinse solution: dilute 1 in 5 with sterile distilled water. For each test make 5 ml of the working strength solution.
  - Conjugate: dilute 1 in 100 in conjugate diluent. Make up 1 ml for each test (if performing multiple tests consider making up one extra volume to give excess volume if required). To do this, for each test to be performed add 10μl of conjugate concentrate and 990μl of conjugate diluent to a plastic universal and mix.
- After 10 minutes of stringent wash remove the tray from the water bath and place on absorbent paper. Remove SW solution as above and add 1ml of rinse solution and agitate for 1 minute. Repeat this wash step. Remove all the rinse solution and add 1ml of conjugate solution.
- Leave agitating on the small orbital platform shaker at room temperature (20–25 °C) for 30 minutes.
- Just before the end of this incubation step, prepare the colour substrate. To do this, dilute the substrate concentrate 1 in 100 with substrate buffer, i.e. for each test to be performed add 10μl of substrate concentrate and 990μl of substrate buffer into a plastic universal and mix.
- Wash strips twice for a minute each in rinse solution as above. Wash strip for a further minute in 1 ml of substrate buffer.
- Add 1 ml of diluted substrate to each test and agitate at room temperature for 30 minutes (can be left longer if colour development is faint).
- Transfer strips to a tray containing 50ml distilled water and leave for five minutes with occasional agitation.
- Remove strips and place on clean white paper; allow strips to dry before interpretation.
- Seal the dried strips under plastic tape on the appropriate request form for storage.

6.4.4 Results/interpretation

Products of c260bp in any of the negative controls must be analysed by LiPA. A LiPA-positive for MTBC in any of the negative controls invalidates the assay.

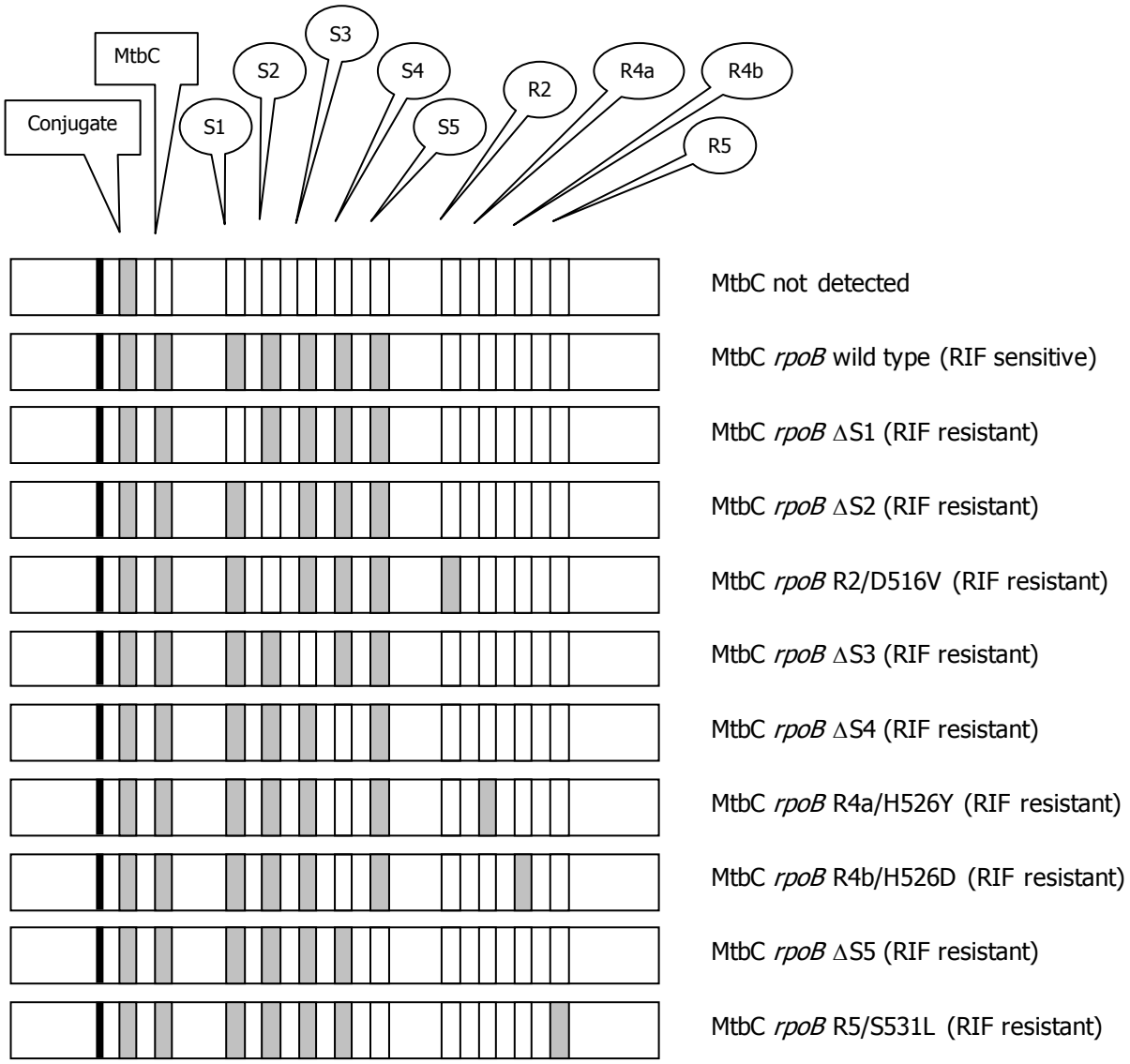
No product in the low-positive control but positive-inhibition controls indicates a reduced sensitivity for the assay.

Record the test as negative for MTBC if the duplicate PCRs are negative and the inhibition control is positive. The report should state:

'The molecular amplification test was negative for *Mycobacterium tuberculosis* complex. This does not exclude the diagnosis of tuberculosis.'

If both duplicates are PCR-positive (c260bp), one duplicate is analysed by LiPA. A guide to the interpretation of the LiPA is given in Figure 6.3.

Figure 6.3. Interpretation of LiPA



A wild-type genotype contains no mutations. Genotypes other than wild type are mutant genotypes.

If the LiPA is negative for MTBC, the test is negative for MTBC and the report should state:

'The molecular amplification test was negative for *Mycobacterium tuberculosis* complex. This does not exclude the diagnosis of tuberculosis.'

If the LiPA is positive for MTBC and has a wild-type genotype, the MTBC is likely to be rifampicin sensitive and the report should read:

'The performed test indicated:

- the presence of *Mycobacterium tuberculosis* complex; and
- the region coding for the B-subunit of the RNA polymerase contained no mutations associated with rifampicin resistance.

Note: This test is unable to detect true rifampicin resistance in a small percentage of isolates.'

If the LiPA is positive for MTBC and has a mutant genotype, the MTBC is likely to be rifampicin resistant and the report should read:

'The performed test indicated:

- the presence of *Mycobacterium tuberculosis* complex; and
- there were mutations in the rpoB gene which would confer rifampicin resistance.'

If one duplicate is PCR-positive, this should be analysed by the LiPA. If the LiPA is negative for MTBC, record the test as negative for MTBC (see above). If positive for MTBC, the test is inconclusive and the PCR must be repeated in duplicate from the extracts. If the repeat results are still discordant then the report should read:

'The PCR was performed in duplicate on two separate occasions. On each occasion only one duplicate was positive for *Mycobacterium tuberculosis* complex. This sample is likely to be positive for *Mycobacterium tuberculosis* complex with no mutations for rifampicin resistance. If possible, please send a repeat sample for confirmation.'

If the repeat results are both negative, then the report should read:

'Unfortunately, the PCR was equivocal. If possible, please send another sample for testing.
Note: It will not be possible to repeat the PCR on CSF samples and the result on these samples should be recorded as inconclusive.'

If the inhibition control is PCR-negative but the sample is PCR-positive in duplicate, then analyse the product by LiPA and record the result as above.

If the inhibition control and the duplicate tests are PCR negative, the PCR is repeated in duplicate from the extract. It will not be possible to repeat the PCR on CSF samples. If the inhibition control is still PCR negative, then record the test as inhibited. The report should read:

'The PCR was negative although the results suggest the presence of PCR inhibitors.'

The following note should be added to reports on the analysis of paraffin-wax-embedded blocks:

'The sensitivity of this assay may be severely reduced when performed on paraffin-wax-embedded tissue. A more appropriate specimen for the detection of *Mycobacterium tuberculosis* would be unfixed material.'

6.5 Procedure 4: Line probe assay for the identification of species from the genus *Mycobacterium*[1] and the detection of potential multidrug and extensively multidrug-resistant tuberculosis (MDR- and XDR-TB) from cultures and smear-positive specimens[2]

[1] Hain Lifescience GenoType CM, AS and MTBC tests

[2] Hain Lifescience GenoType MTBDRplus and MTBDRsl

6.5.1 Introduction

The GenoType series is based on DNA strip technology and allows for the genetic differentiation of species belonging to the genus *Mycobacteria*. The Hain CM test is used to identify common mycobacteria and the AS test to further differentiates additional species. The GenoType MTBC test differentiates between the species of the *M. tuberculosis* complex (MTBC). The CM, AS and MTBC strips detect and analyse positive mycobacterial cultures and must not be used to detect mycobacteria directly from patient material.

The GenoType MTBDR*plus* test allows for the detection of MTBC and simultaneously its resistance to rifampicin and/or isoniazid by mutations in the *rpo*B and *kat*G/*inh*A (high/low isoniazid resistance) genes, respectively[9]. The GenoType MTBDR*sl* detects MTBC and simultaneously its resistance to fluoroquinolones (e.g. ofloxacin and moxifloxacin) and/or aminoglycosides/cyclic peptides (injectable antibiotics as capreomycin, viomycin/kanamycin, amikacin) and/or ethambutol. The MTBDR*plus* and MTBDR*sl* are validated for DNA extracted from both positive cultures and smear-positive pulmonary specimens. Note: these tests should not be used to detect mycobacteria directly from smear-negative materials unless the laboratory independently validates their use. They are licensed for smear-positive sputum only.

All procedures are identical and are divided into three steps: DNA extraction, a multiplex amplification using biotinylated primers, and reverse hybridisation.

6.5.2 Materials

The GenoType series all use the same reagents except for the primer/nucleotide/dye mix (PNM), which is specific to each test kit.

Materials required and provided by the manufacturer

Ready to use:

- PNM (primer/nucleotide/dye mix). Note: different PNM for each test kit

- Denaturing solution (contains <2% NaOH, irritating to eyes and skin)
- Hybridisation solution (contains 8–10% anionic)
- Stringent wash solution (contains >25% quaternary ammonium compound, <1% anionic)
- Rinse solution (contains buffer, <1% NaCl, <1% anionic)
- Nitrocellulose membranes

Prepare for use:

- Conjugate (contains streptavidin-conjugated alkaline phosphatase): Calculate how many tests are being performed (+3) and add concentrate (CON-C) to diluent (CON-D) in the proportions of 10µl CON-C to 1 ml CON-D per test. Note: this must be made fresh each time.
- Substrate (contains dimethyl sulphoxide (irritant): Add the contents of the substrate concentrate (SUB-C) to the contents of the substrate diluent (SUB-D). Once made, the solution can be kept for four weeks at room temperature, provided it is protected from light – it must be stored in the brown SUB-D bottle.

Materials required not provided by the manufacturer

- Absorbent paper
- Adjustable pipettes and disposable tips
- DNA extraction reagents
- PCR tubes (DNase and RNase free)
- Shaking water bath/TwinCubator
- Shaking platform/TwinCubator
- Thermal cycler
- Thermostable DNA polymerase with buffer (hot-start enzyme recommended)
- Thin-walled PCR tubes
- Tweezers
- Ultrasonic bath
- Water (molecular biology grade)

6.5.3 Methods

DNA extraction

DNA extraction must be carried out in a BSL3 laboratory in a BSC.

- DNA extraction from solid cultures: Using a 1µl loop, transfer a small amount of growth to a 1.5ml screw-top Eppendorf tube containing 100µl molecular-grade water.
- DNA extraction from liquid cultures and primary specimens: For very large volumes of culture (over 2ml) centrifuge at 3000g for 20 minutes and discard the supernatant, leaving about 1 ml. Resuspend the deposit in the remaining fluid and add 100µl to a 1.5ml screw-top Eppendorf tube. Note: Primary specimens should not be used with AS and CM strips. For smear-positive sputum specimens, centrifuge 500µl of decontaminated sample (see NALC-NaOH decontamination – see Section 5.4.1) as above and resuspend in 100µl molecular-grade water in a 1.5ml screw-top centrifuge tube. Add 100µl to a 1.5ml screw-top centrifuge tube; if there is a very small volume, make up to 0.25ml with sterile Middlebrook.
- Treatment of blood and bone marrow cultures or cultures containing pus or blood prior to Hain genotyping is not validated by Hain and therefore only an alternative method. Excessive cellular matter can have an inhibitory action on polymerase chain reactions (PCRs); therefore, it is necessary to treat those samples prior to DNA extraction:
  - Pipette 0.5 ml of the culture into a plastic universal
  - Add 0.25ml Triton X and 0.25 ml 1N NaOH
  - Vortex
  - Add 9ml sterile distilled water and vortex again
  - Centrifuge at 3000g for 20 minutes
  - In the cabinet, pour off all the supernatant into another universal and discard
  - Resuspend the pellet by adding 0.25ml sterile Middlebrook and vortexing
  - Add 100µl of this deposit to a 1.5ml screw-top centrifuge tube

For the negative control: Add 100µl of molecular water to a 1.5ml centrifuge tube.

All tubes:

- Add 100µl chloroform and vortex.
- Place in a covered water bath at 80 °C for at least 30 minutes.

At this stage the *Mycobacterium spp.* are inactivated by heating or chloroform treatment, can be considered safe, and processing can continue outside the BSL3 laboratory.

- Place the microcentrifuge tube at –20 °C for 10 minutes.

- After 10 minutes take samples out of freezer and allow to thaw whilst preparing PCR mix.

PCR amplification

To be prepared in a clean room. A clean room is any laboratory area without DNA and PCR amplification products produced later using a molecular amplification system. Never bring PCR amplification products back into a clean area; this includes clothing of the laboratory scientist, reagents, equipment etc. unless thorough decontamination to remove DNA/RNA has been carried out.

PCR reaction mix

Make up PCR reaction mix:

- 35µl PNM mix (remember to use the correct kit PNM for the test performed)
- 5µl PCR buffer
- 2µl magnesium chloride final concentration 2.5 mM
- 2.8µl molecular water (Sigma)
- 0.2µl (1U) Hot-start Taq
  Total 45µl

Pipette 45µl into a 0.2ml x 8-strip thin-wall tube (+ individual caps) as required. It may be helpful to use a different-coloured tube for each day of the week to help minimise contamination of the products.

Amplification

- This procedure should be conducted in the DNA-specimen preparation room.
- Centrifuge thawed samples (DNA preparation) at 13000g for 3 minutes.
- Label 0.2ml x 8-strip thin-wall tubes with the corresponding worksheet number (1 to N).
- After centrifugation carefully pipette 5µl of the top aqueous solution to the appropriate tube with PCR mix and seal the cap. Carry on until all have been pipetted.
- Place the 0.2ml x 8-strip thin-wall tube (+ individual caps) into a diagnostic thermocycler, close the lid and screw tight.
- Run the amplification cycle:

a.		95 °C	15 min
b.	10 cycles	95 °C	30 s
		58 °C	2 min
c.	20 cycles	95 °C	25 s
		53 °C	40 s
		70 °C	40 s
d.		70 °C	8 min
		4 °C	∞

The reaction time is approximately 1 hour and 50 minutes.

Hybridisation of PCR amplicon onto the Hain strips[c]

Hydridisation can either be done automatically, for example with a Bee Blot machine, or manually, using a TwinCubator machine. Always wear gloves when handling the strips.

Preparation:

- Prewarm shaking water bath/TwinCubator to 45 °C. Prewarm solutions HYB and STR to 37–45 °C before use.
- The reagents must be free from precipitates (note, however, that solution CON-D is opaque). Mix if necessary.
- Warm the remaining reagents (with the exception of CON-C and SUB-C) to room temperature. Using a suitable tube, dilute conjugate concentrate (CON-C, orange) and substrate concentrate (SUB-C, yellow) 1:100 with the respective buffer (CON-C with CON-D, SUB-C with SUB-D) in the amounts needed. Mix well and bring to room temperature. For each strip, add 10µl concentrate to 1 ml of the respective buffer. Dilute CON-C before each use. Diluted SUB-C is stable for four weeks if stored at room temperature and protected from light.
- Dispense 20 µl of denaturation solution (DEN, blue) in a corner of each of the wells used.
- Add 20 µl of amplified sample to the solution, pipette up and down to mix well and incubate at room temperature for five minutes.

[c] Descriptions of laboratory procedures are based on the manufacturer's instructions (Hain Lifescience). The original text is available from: http://www.tbevidence.org/documents/rescentre/sop/MTBDRsl.pdf.

- Meanwhile, take the strips out of the container using tweezers and mark them with a pencil underneath the marker line. Always wear gloves when handling strips.
- Carefully add 1 ml of prewarmed hybridisation buffer (HYB, green) to each well. Gently shake the tray until the solution has a homogenous colour.
- Take care not to spill the solution into the neighbouring wells.
- Place a strip in each well.
- The strips must be completely covered by the solution and the coated side (identifiable by the marker line near the lower end) must face upward. Using pipette tips, turn over strips which may have turned when immersed in the solution. Carefully clean tweezers under warm running water and dry after each use to avoid contamination. (These measures also apply to all subsequent steps.)
- Alternatively, place tray in TwinCubator and incubate for 30 minutes at 45 °C.
- Adjust the shaking frequency of the water bath to achieve a constant and thorough mixing of the solution. To allow adequate heat transfer, the tray must be dipped into the water to at least one third of its height.
- Completely aspirate the hybridisation buffer using, for example, a Pasteur pipette connected to a vacuum pump.
- Add 1 ml of stringent wash solution (STR, red) to each strip and incubate for 15 minutes at 45 °C in shaking water bath/TwinCubator.
- Work at room temperature from this step forward.
- Completely remove the stringent wash solution.
- Pour out wash solution in a waste container and remove all remaining fluid by turning the tray upside down and gently tapping it on absorbent blotting paper. (This also applies to all other wash steps.)
- Wash each strip once with 1 ml of rinse solution (RIN) for one minute on shaking platform/TwinCubator (pour out RIN after incubation).
- Add 1 ml of diluted conjugate (see above) to each strip and incubate for 30 minutes on a shaking platform/TwinCubator (pour out RIN after incubation).
- Remove the solution and wash each strip twice for one minute with 1 ml of rinse solution (RIN) and once for 1 minute with approximately 1 ml of distilled water (e. g. use wash bottle) on a shaking platform/TwinCubator (pour out solution each time).
- Ensure all traces of water are removed after the last wash.
- Add 1 ml of diluted substrate (see above) to each strip and incubate (protected from light) without shaking for ten minutes.
- Depending on the test conditions (e. g. room temperature), the substrate incubation time can vary between three and 20 minutes. Extended substrate incubation times can lead to increased background staining and might impair interpretation of the results.
- Stop the reaction by briefly rinsing twice with distilled water.
- Using tweezers remove the strips from the tray and dry them between two layers of absorbent blotting paper.

6.5.4 Results/interpretation

Templates are provided with the manufacturer's insert.

Figure 6.4. Interpretation sheet for GenoType Mycobacterium CM

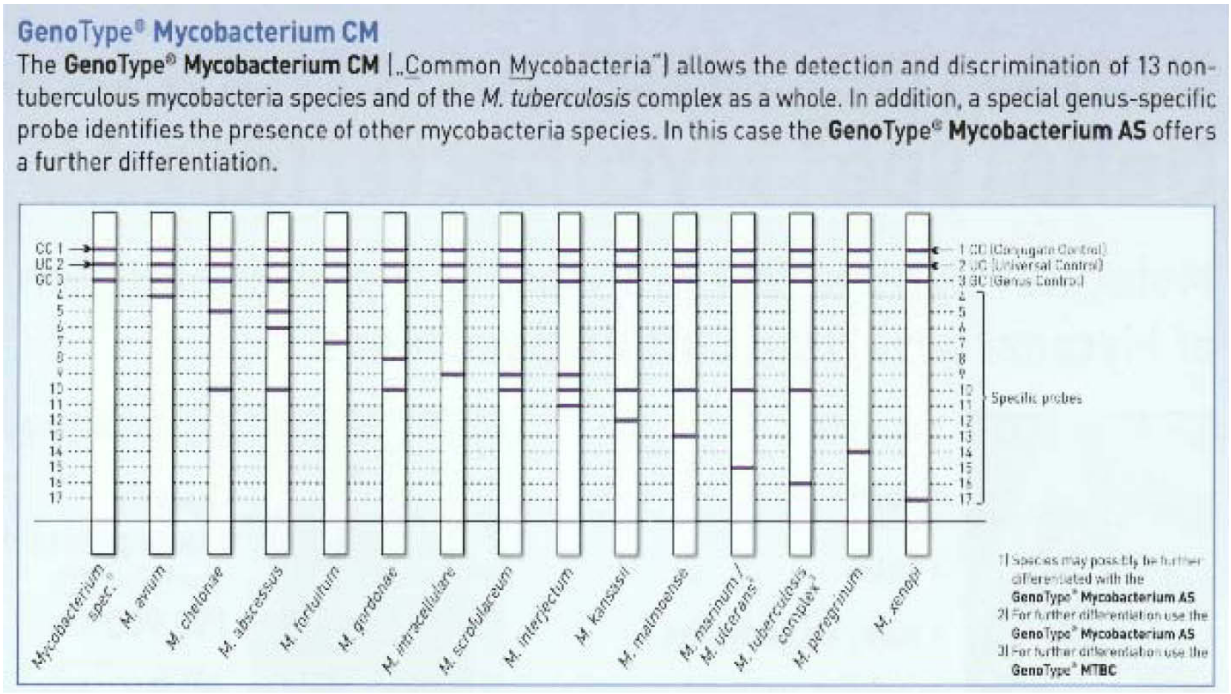


Figure 6.5. Interpretation sheet for GenoType Mycobacterium AS

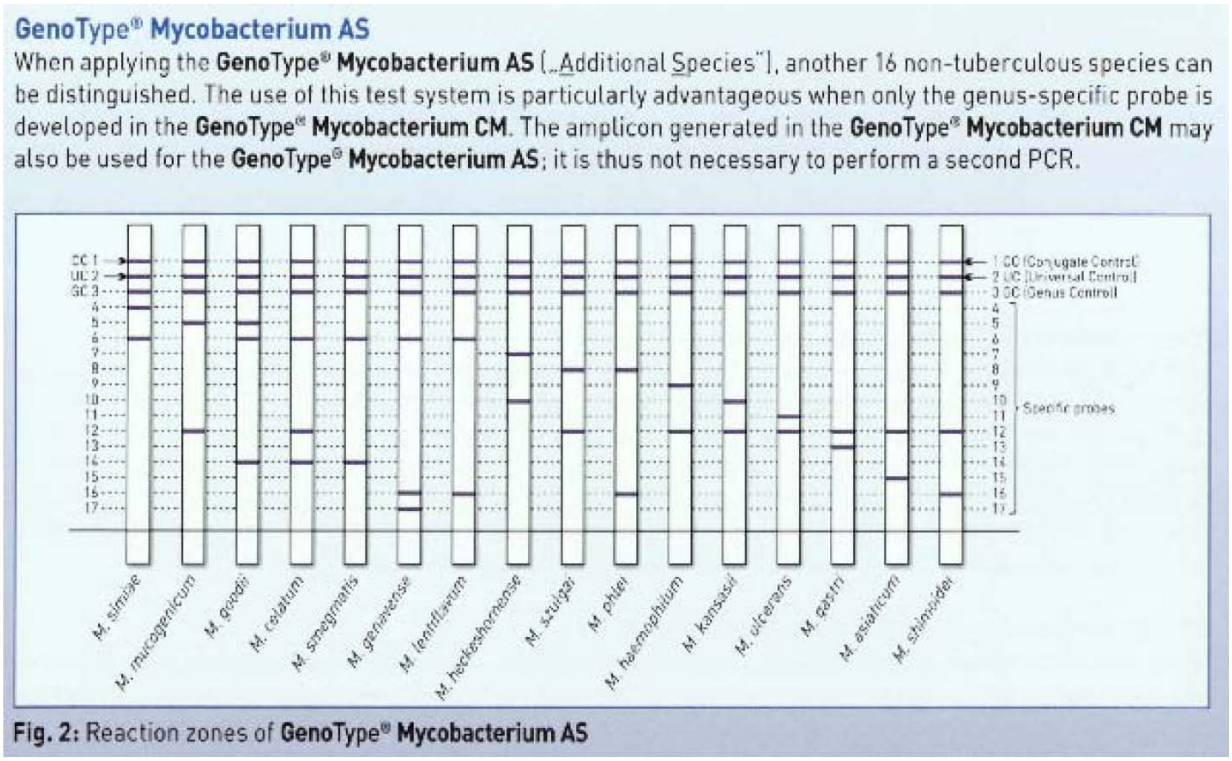


Figure 6.6. Interpretation sheet for GenoType Mycobacterium MTBC

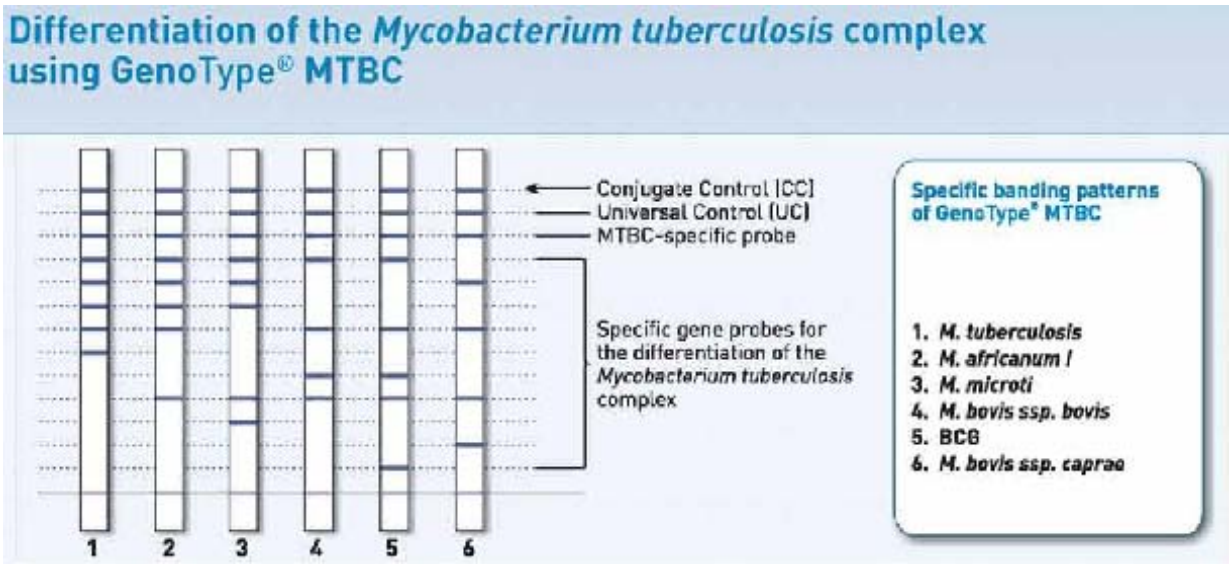


Figure 6.7. Interpretation sheet for GenoType Mycobacterium MTBDRPlus

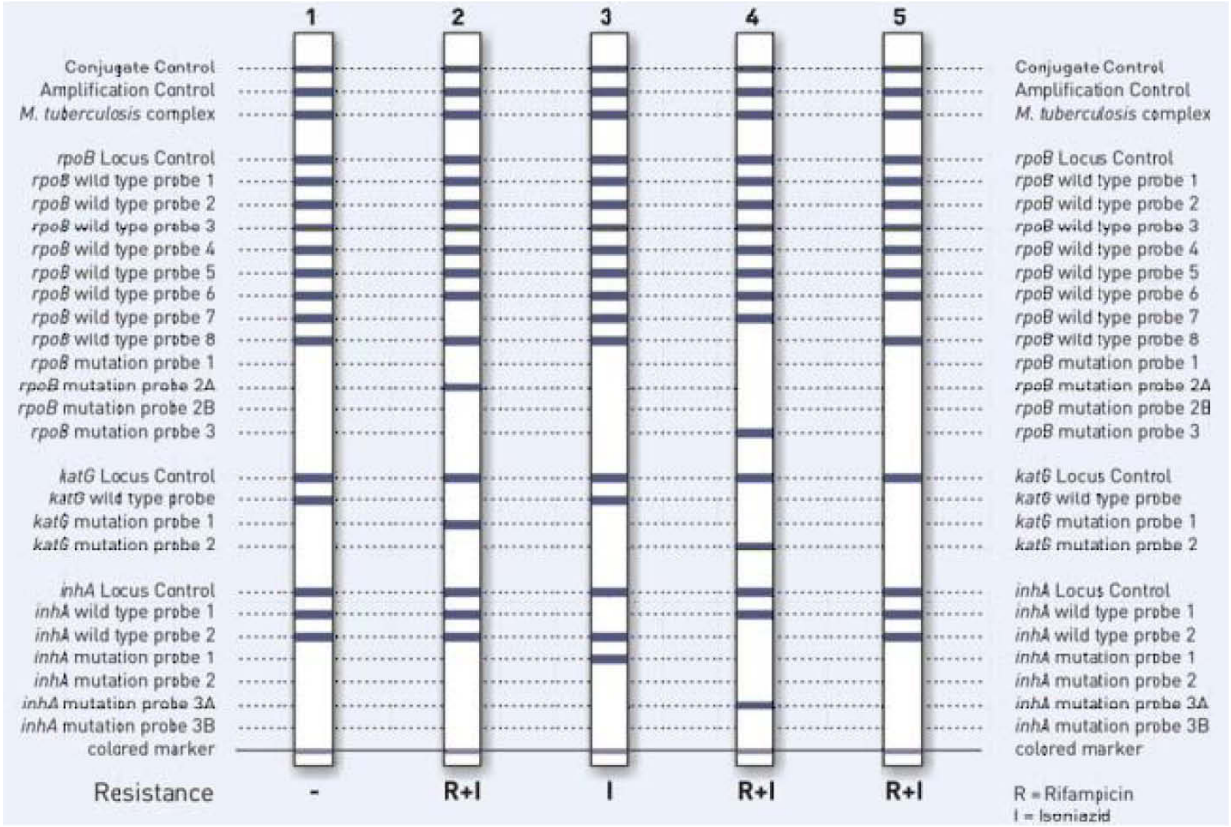
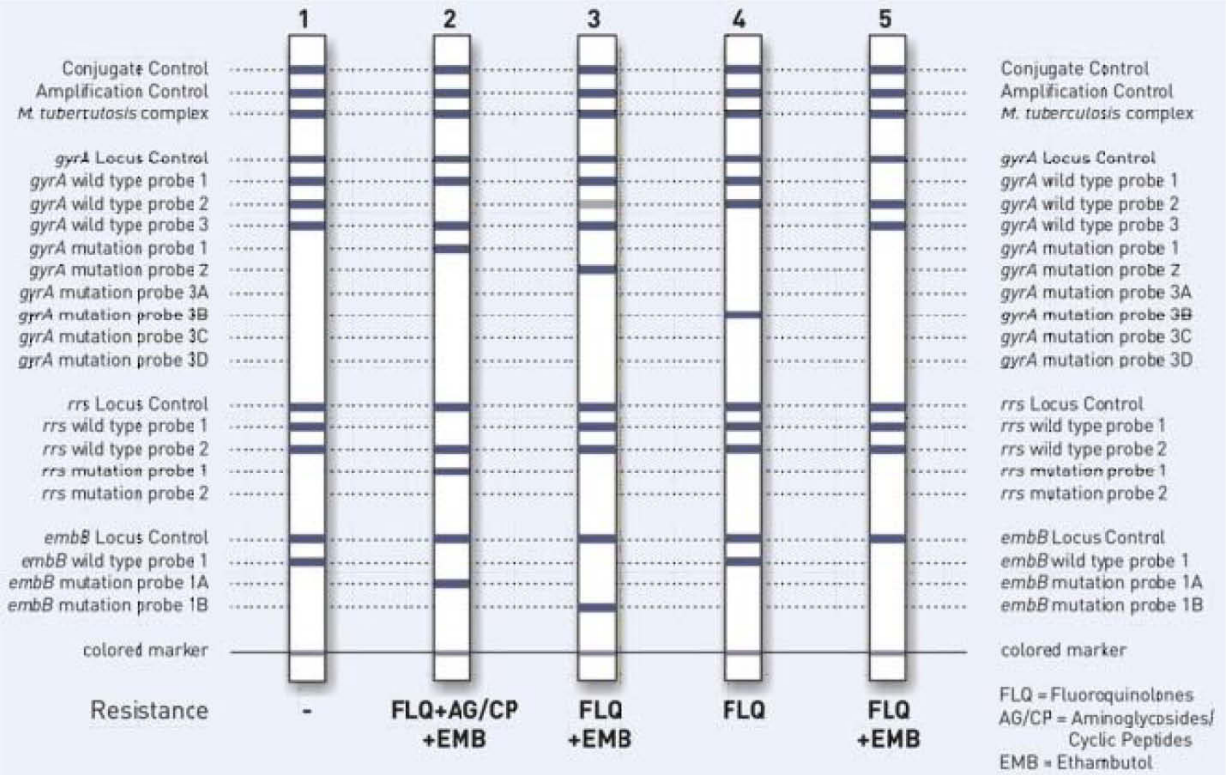


Figure 6.8. Interpretation sheet for GenoType Mycobacterium MTBDRsl



To validate a run in any of these systems, all negative controls must be negative, and positive controls must give the wild-type hybridisation pattern shown on all strips (Figures 6.3 to 6.7). Thus, a line must develop in the conjugate control (CC) zone and in the universal control (UC) zone, which detects all known mycobacteria and members of the group of gram-positive bacteria with a high G+C content. Furthermore, banding in the MTBC/TUB region indicates amplicons are generated from MTBC members.

No, or poor, hybridisation with a low positive control but a positive amplification control indicates a reduced sensitivity for the assay.

6.5.5 Examples of reporting for GenoType Mycobacterium MTBDRplus

Record the test as negative for MTBC if only the CC and AC are positive. The report should state:

- The PCR test was negative for *Mycobacterium tuberculosis* complex.
- This does not exclude the diagnosis of tuberculosis.

If the MTBDRplus strip is positive for MTBC and has a wild type rpoB, katG and inhA, as in strip 1 (Figure 6.7), the MTBC is likely to be rifampicin sensitive, and the report should read:

The performed test indicated:

- the presence of *Mycobacterium tuberculosis* complex; and
- the region coding for the β-subunit of the RNA polymerase contained no mutations associated with rifampicin resistance.

Note: Detection of non rpoB mutation does not rule out rifampicin resistance as the test is unable to detect true rifampicin resistance in a small percentage of isolates (not all rifampicin-resistance-associated mutations are covered).

If the MTBDR*plus* strip is positive for MTBC and has a mutant *rpoB*, but wild type *katG* and *inhA* the MTBC is likely to be rifampicin resistant, and the report should read:

The performed test indicated:

- the presence of *Mycobacterium tuberculosis* complex; and
- there were mutations in the rpoB gene which would confer rifampicin resistance.

If the MTBDRplus strip is positive for MTBC and has a wild type rpoB, but mutant katG and/or inhA (e.g. strip 3, Figure 6.7) the MTBC is likely to be isoniazid resistant and the report should read:

The performed test indicated:

- the presence of *Mycobacterium tuberculosis* complex; and
- the region coding for the β-subunit of the RNA polymerase contained no mutations associated with rifampicin resistance.
- There was a mutation in katG/inhA that is usually seen in isoniazid resistant isolates.

Note: This test is unable to detect true rifampicin resistance in a small percentage of isolates.

If the MTBC probe is negative but multiple locus control probes are visible or the MTBC probe is positive alone, report as equivocal. The report should read:

- 'The PCR test gave an equivocal result.'

If the amplification control and the test is negative, the PCR is repeated from the extract at 1/10 dilution in water. If the inhibition control is still negative then record the test as inhibited. The report should read:

- 'The PCR was negative but the results suggest the presence of PCR inhibitors which could give a false negative result.'

6.5.6 Biosafety

Appropriate biosafety precautions must be taken when handling mycobacteria. The transfer of bacteria to tubes and the addition of chloroform and boiling during DNA preparation should be carried out in an appropriate safety cabinet in a BSL3 laboratory. After microorganisms are inactivated by heating or chloroform treatment, the process can be continued outside the BSL3.

6.6 Procedure 5: Nucleic acid hybridisation test for the identification of *M. tuberculosis* complex[1]

[1] AccuProbe *Mycobacterium tuberculosis* Complex Culture Identification Test Kit, Gen-Probe Incorporated, San Diego, CA, USA.

6.6.1 Introduction

Fast species identification of a clinical isolate is important for rapid diagnosis. With a nucleic acid hybridisation test[1], members of the MTB complex can be identified within less than an hour[4,5]. The test does not react to NTMs and does not differentiate between members of the MTB complex, but the result of MTB complex is sufficient for most clinical laboratories since *M. tuberculosis* is the most prevalent of the MTB complex organisms.

6.6.2 Materials

An illuminometer, sonicator, water bath or heating block, rack, adjustable micropipettes, gloves, vortex, and reagents from the kit are required[1].

6.6.3 Methods

The test uses a single-stranded DNA probe with a chemiluminescent label that is complementary to the ribosomal RNA of the target. After the ribosomal RNA of the bacteria is released, the labelled DNA probe combines with the target RNA to form a stable DNA:RNA hybrid, which can be measured in a illuminometer. The decision to test a culture with an MTB complex probe may also depend on the appearance under the microscope (in liquid medium) and the pigmentation of the bacteria or the pellet.

For solid cultures, 100µl of reagent 1 (lysis reagent) and 100µl of reagent 2 (hybridisation buffer) are transferred into a reagent tube. For liquid cultures, only 100µl of reagent 2 is needed. Samples (a loopful of bacteria or 100µl of a mixed culture suspension) are added and mixed well by vortexing.

The tubes are incubated for 15 minutes in the sonication bath and subsequently for ten minutes at 95 °C. A 100µl volume of the lysed specimens are transferred into probe containing tubes, which are incubated for 15 minutes at 59.5–61 °C (exact time and temperature is critical).

For the visualisation of the hybridisation product, 300µl of the selection reagent are added, mixed well by vortexing, and again incubated for ten minutes at 59.5–61 °C (exact time and temperature is critical). The samples can be analysed in an illuminometer after having cooled off to room temperature (one hour). The whole procedure can be completed within one hour.

6.6.4 Results/interpretation

The results of the tests are based on cut-off values. Samples producing signals greater than or equal to these cut-off values are considered positive, results below these cut-off values are considered negative; intermediate results should be repeated. The cut-off values are device-specific.

The result is only valid if the included negative (e.g. *M. avium* ATCC 25291) and positive (e.g. *M. tuberculosis* ATCC 25177) controls are within a specified range of values. If the initial test gives a negative result, additional probes have to be performed.

The nonradioactive DNA probes for the identification of *M. tuberculosis* complex have been evaluated with 134 clinical isolates, which included 36 MTB complex isolates. Sensitivity and specificity was 100%[4]. Another study investigated 359 liquid vials positive for acid-fast bacilli, including 224 MTB complex isolates, and found an initial sensitivity of 87.2% for MTB complex[5].

A principle limitation of the probe technology is the inability to identify mixed cultures. If a mixed culture is suspected, additional probes might yield a result. However, only a limited number of species are covered by these additional probes.

6.6.5 Biosafety

Appropriate biosafety precautions for handling mycobacteria have to be used. The transfer of bacteria to the tubes for sonication and heating should be carried out in a BSL3 laboratory in an appropriate biosafety cabinet. After heating, the next steps can be performed outside the biosafety cabinet.

6.7 Procedure 6: Single-use sample-processing cartridge system with integrated multicolour real-time PCR capacity for the detection, identification of *M. tuberculosis* complex bacteria and the detection of rifampicin resistance[1]

[1] The Xpert MTB/RIF assay with the Cepheid GeneXpert System, Cepheid, Sunnyvale, CA

6.7.1 Introduction

The culture of mycobacteria and/or the methods for molecular detection require a specific infrastructure and trained staff, which may limit the availability of these technologies to specialised laboratories. A recently developed fully automated molecular test for the detection of TB bacteria can overcome this problem[10]. It can be used directly on patient sputum samples or decontaminated sputum samples.

6.7.2 Materials

The test requires a specific hardware platform, as well as the test kits, disposable pipettes, and gloves.

The platform integrates sample processing, PCR, and the analysis of the PCR fragment.

6.7.3 Methods

The test is based on a disposable plastic cartridge containing all reagents required for bacterial lysis, nucleic acid extraction, amplification, and amplicon detection. The only manual step is the addition of a bactericidal lysis buffer to sputum or decontaminated sputum.

The lysis buffer is added in a 2:1 ratio (v/v) to untreated sputum of > 1 ml volume and in a 3:1 ratio to decontaminated sputum pellets > 0.5 ml volume. The additional buffer in decontaminated pellets is necessary in order to meet the assay minimum sample volume requirement. The closed sputum cup or centrifuge tube is incubated at room temperature for 15 minutes, during which time the samples must be manually agitated twice. Following this, 2ml of the inactivated sample mixture are transferred to the cartridge (equivalent to 0.7 ml untreated sputum or 0.5 ml decontaminated pellet). Cartridges are then inserted into the platform and the programme is started. The extraction, PCR and detection process takes 90 minutes and the results are automatically generated by the GeneXpert system unit.

6.7.4 Results/interpretation

Results in the detection of TB bacteria can be positive, negative or indeterminate.

6.7.5 Biosafety

Appropriate biosafety precautions for handling mycobacteria have to be used. The transfer of the samples in the cartridges should be carried out in an appropriate biosafety cabinet in a BSL3 laboratory. After addition of the lysis buffer, a marked reduction of viable bacteria occurs. The filled cartridges should not be opened again or manipulated. They should be discarded according to the institutional guidelines.

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7 First- and second-line drug susceptibility testing for *Mycobacterium tuberculosis* complex

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7.1 Background and principles

This chapter describes the susceptibility testing for *Mycobacterium tuberculosis* and other members of the *M. tuberculosis* complex (MTBC; *M. africanum*, and *M. bovis* are the most common) to antituberculous drugs[13].

Drug susceptibility testing (DST) in tuberculosis has two main objectives: (i) individual treatment management of a tuberculosis case, and (ii) anti-tuberculosis drug resistance surveillance at the scale of a hospital, city, region or country. Drug susceptibility testing to first-line drugs is required for a definite case of tuberculosis (a patient with a positive culture for the MTBC or one sputum smear positive for acid-fast bacilli if a culture is not available) because of the development of worldwide resistance in tuberculosis. Drug resistance rates are higher in re-treatment cases (acquired resistance or secondary resistance) than in new cases (primary resistance), the difference varying according to the country or epidemiological situation[11]. Drug resistance during treatment, designated as 'acquired' or 'secondary resistance', results from the selection and multiplication of resistant mutant isolates pre-existing in the tubercle bacillus population before therapy. Drug resistance observed before treatment, designated as 'primary resistance', is the consequence of exposure to a drug-resistant source of infection[14,16,22].

Drug susceptibility testing to second-line drugs is mandatory for multidrug-resistant cases of TB (MDR-TB) and for patients who have been previously treated for tuberculosis with second-line drugs. Drug susceptibility testing should be repeated for a definite case when the culture is still positive after five months of treatment. It may also be repeated after three months of treatment, sufficient funds provided[30].

Identification of the mycobacterial culture or MTBC acid-fast bacilli should be done before the start of drug susceptibility testing. Non-tuberculous mycobacteria (NTM) may have similar growth patterns but will harbour intrinsic resistant characteristics that will give false-resistant information. The knowledge of the exact species within the MTBC is important when interpreting the results of pyrazinamide testing, especially of *M. bovis,* which shows inherent resistance[29].

Laboratories should use only one reliable method for drug susceptibility testing for MTBC[13,17,28]. The laboratory should have considerable experience in the chosen method, which should be carried out by skilled technicians. It is also extremely important to periodically perform an internal quality control (QC) of drug susceptibility testing. The minimum requirement for QC is to test each new batch of reagents. If the batch QC fails, all results obtained within that batch, as well as the new batch of a reagent should be thoroughly reviewed and the testing should be repeated. All laboratories performing drug susceptibility testing have to participate additionally in an external QC programme[13].

Drug susceptibility tests are usually performed on MTBC cultures (indirect testing) but may also be performed on specimens containing acid-fast bacilli known to belong to MTBC (direct testing)[7] as long as special conditions are fulfilled (see below). Results are obtained faster for direct testing; however, there is a lower rate of success due to possible contamination.

All methods described are risk level C and must be performed in a BSL3 laboratory.

7.2 Procedure 1: General method – DST by culture

7.2.1 Introduction

The methods introduced in this chapter aim not only to detect resistance but are also intended to assess susceptibility[13]. Applying these methods should lead to high sensitivity for detecting resistance (i.e. a low rate of false susceptibility results) and high specificity (i.e. a low rate of false-resistance results). Additionally, these methods should also offer high sensitivity for assessing susceptibility (i.e. a low rate of false-resistance results) and a high specificity (i.e. a low rate of false susceptible results). Studies on the performance of the various methods are referenced in each method section.

Resistance is defined as a decrease in sensitivity of sufficient degree to be reasonably certain that the strain concerned is different from wild-type strains which have never come into contact with the drug. In this case, the strain is unlikely to show clinical responsiveness to the drug. Susceptibility is defined by a level of sensitivity not

significantly different from wild-type strains which have never come into contact with the drug. In this case, the strain is likely to show clinical responsiveness to the drug[8,16,22].

Susceptibility testing methods are applied to one drug tested as a representative of a family of drugs (e.g. rifampicin results answer for rifampicin and rifapentine, prothionamide for ethionamide and vice-versa), or to one individual drug (rifampicin does not answer for rifabutin, and streptomycin does not answer for amikacin and kanamycin). First-line drugs include isoniazid (H), rifampicin (R), ethambutol (E) and streptomycin (S), although streptomycin is now not part of most of standard treatments, and pyrazinamide (Z), which is usually part of the standard treatment but is not considered a first-line *testing* drug due to the difficulty in obtaining reliable results.

Second-line drugs include the drugs used to treat MDR-TB cases, e.g. the injectables (kanamycin, capreomycin and amikacin), the fluoroquinolones (ofloxacin or moxifloxacin, ethionamide or prothionamide, and cycloserine). Due to the discrepancies in the results obtained for some of these second-line drugs (cycloserine, ethionamide and prothionamide) or due to difficulties in supply, e.g. moxifloxacin, descriptions herein will be limited to amikacin, capreomycin and ofloxacin[3,19,20]. Methods are based on growth on solid media (usually egg-based, but can be synthetic), using a manual system, or in liquid media (usually synthetic Middlebrook media), using both automated and manual systems.

7.2.2 Materials

For direct testing[7]: smear-positive specimens after classical decontamination (see Section 5.6). For indirect testing[13,17]: cultures, as a positive liquid broth or colonies on a solid media, of MTBC after checking purity.

7.2.3 Methods

Methods mostly used in Europe include the modified proportion method on solid Löwenstein-Jensen (LJ)[9,10,14] and the modified proportion method in liquid Middlebrook media[2,4,5,6,26]. A less commonly used method which is also approved for first-line and second-line drug susceptibility testing is the resistance-ratio method[13,22]. Other methods which are approved for first-line drug susceptibility testing only are: the absolute concentration method and the radiometric method in liquid medium. Debated methods amongst others are the nitrate reductase assay and the Alamar blue method[1,15,18,27].

7.2.4 Safety

All methods described are risk level C and have to be performed in a BSL3 laboratory. Cultures or smear-positive specimens should be handled under a safety cabinet using sterile and disposable tips, tubes and pipettes.

7.2.5 Report interpretation

Reporting of susceptibility results is easily done for each tested drug, if the method was applied correctly. Resistance results are preferably checked by repeated testing (clinicians should already be informed), which may in some cases determine the level of resistance. This may be done as soon as a resistance pattern is observed.

Emergency and priority reporting should be carried out for cases of smear-positive pulmonary tuberculosis and for all cases which appear to be MDR-TB. The results should be given directly to the clinician in charge of the patient. If this is not possible, it should be verified that the clinician is aware of the results. Routine reporting should contain the start date of testing and the date of reporting.

The level of resistance may be of interest for some drugs (isoniazid, streptomycin, ethambutol) if it can be determined. Usually, a report of low resistance does not imply that the drug will not be given; conversely, a report of a high-level of resistance implies that the drug is of no use to the patient.

7.3 Procedure 2: The proportion method (Canetti et al., modified) on Löwenstein-Jensen medium

7.3.1 Introduction

The proportion method was one of the first methods described for susceptibility testing of MTBC. It was first described using the egg-based Löwenstein-Jensen (LJ) media and is considered to be a reference standard against which other routine methods should be assessed.

The proportion method calculates the proportion of resistant bacilli present in a strain[12]. Below a certain proportion, the strain is classified as susceptible; above that proportion, it is classified as resistant. This method is well established for first-line and some second-line drugs. The critical proportion was assessed by a study of a cohort of patients in the 1960s who failed tuberculosis treatment[8,9]. Consequently, it can be used to predict clinical failure if the drug were to be used in monotherapy (i.e. treatment with a single drug, which would be incorrect therapy)[10].

7.3.2 Definitions

Resistance: The strain is presumed to be resistant when growth of more than a certain proportion of the inoculum occurs at a defined concentration – the critical concentration – of the drug.

Critical concentration: The lowest concentration of an anti-TB drug in the culture medium at which growth of the tubercle bacilli occurs.

Critical proportion: The percentage of tubercle bacilli in the suspension which grow on culture media containing the critical concentration of an anti-TB drug signifies the clinical ineffectiveness of that drug.

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

7.3.3 Materials

A pure, well-characterised culture of *M. tuberculosis* bacteria in the active growth phase is necessary. Pure substances (no patient tablets) of the drugs must be used at all times. No special equipment is required, only tubes (preferable with screw caps) with LJ medium, pipettes, and McFarland turbidity standard no. 1.

For direct testing, smear-positive specimens are decontaminated using the usual procedure (see Section 5.5.1.) and the pellet is used pure or diluted (see below).

7.3.4 Methods

Media preparation

LJ media with drugs incorporated before inspissation are used. Screw-capped tubes containing 5 ml of the medium are inspissated at 85 °C for 40-45 minutes. LJ medium with and without incorporated drugs can be stored at 6+/-2 °C for one month.

Drugs

The drugs to be tested should be stored according to the manufacturer's instructions. Substances, solvents and dilutions are listed in Table 7.1 along with critical concentrations.

Table 7.1. Solvents and diluents

Solvents and Diluents				
Drugs	**Substance**	**Critical concentration (mg/L)**	**Solvents**	**Diluents**
Isoniazid	Isoniazid	0.2	DW	DW
Rifampicin	Rifampicin	40	DMSO	DM
Ethambutol	Ethambutol dihydrochloride	2	DW	DW
Streptomycin	Dihydrostreptomycin sulfate	4	DW	DW
Capreomycin	Capreomycin sulfate	40	DW	DW
Amikacin	Amikacin sulfate	40	DW	DW
Ofloxacin	Ofloxacin	2	0.1N NaOH	DW
DW = Sterile distilled water; DMSO = dimethyl-sulfoxide; NaOH = Sodium-hydroxide.				

Bacterial suspension

Indirect drug susceptibility testing:

This is carried out on a primary isolate or a subculture on LJ medium. A representative portion of the bacteria is obtained by sampling as many colonies as possible within one or two weeks after the appearance of growth.

The colonies are transferred to a glass tube with physiological NaCl. Homogenisation of the suspension can be done using glass beads (3.0 mm in diameter) or with a glass rod with a molten rounded tip by rubbing the bacteria onto the glass wall. The suspension should be made without residual culture medium.

After thorough mixing and homogenisation of the suspension, the tubes should rest for ten minutes, after which the supernatant is pipetted into another tube.

The bacterial suspension used for inoculation of the culture medium should be the equivalent to approximately 1 mg wet bacterial mass/ml (about one full loop with an inner diameter of 3 mm).

Usually, two dilutions of the inoculum are inoculated onto the control LJ tubes (without drug): inoculum 1 and inoculum 2, which is a 1:100 fold dilution of the inoculum 1.

If the turbidity of the suspension is visually adjusted by comparing it to the reference suspension McFarland standard 1.0 (9.9 ml sulphuric acid [1% volume concentration] with 0.1 ml barium chloride solution [1% mass

concentration]), it will be equivalent to 10^{-2} and 10^{-4}. Serial dilutions of 10^{-1} to 10^{-4} of the standard suspension are prepared by diluting sequentially 1.0 ml of the standard suspension in tubes containing 9 ml of sterile distilled 0.9% sodium chloride.

Inoculation

Dilutions of 10^{-2} (control 1) and 10^{-4} (control 2) are inoculated onto LJ as controls. The drug containing LJ media are then inoculated with control 1 and control 2 inoculums. The volume of the inoculum is 0.1 ml.

For direct testing:

Decontaminated smear-positive specimens are inoculated directly onto LJ slopes using two inocula: inoculum 2 is a 1:100 dilution of inoculum 1. Dilutions are made according to the number of acid-fast bacilli per microscopic field (x1000 magnification):

- Undiluted (inoculum 1) and 10^{-2} (inoculum 2) if there is less than 1 AFB per field
- 10^{-1} and 10^{-3} if there are 1 to 10 AFB per field
- 10^{-2} and 10^{-4} if there are more than 10 AFB per field

Drug containing LJ media tubes are inoculated with both inocula if possible, or at least with inoculum 1 for first-line drugs (1% critical proportion) and inoculum 2 for second-line drugs CM, AMI, OFL (usually considered for a 10% critical proportion). The volume of the inoculum is 0.1 ml to 0.2 ml per tube.

There are two recommended methods of preparing the dilutions: by pipettes or by a calibrated loop. Using pipettes, serial dilutions of 10^{-1} mg/ml to 10^{-4} mg/ml of the standard suspension are prepared by diluting sequentially 1.0 ml of the standard suspension (1 mg/ml) in tubes containing 9 ml of sterile distilled 0.9% sodium chloride.

Incubation

After inoculation, the tubes are incubated at 37 °C in a slanted position, with the screw caps slightly loosened to allow for the evaporation of the inoculum. After 24 to 48 hours, screw caps are tightened and the tubes are further incubated. This does however depend on the type of screw caps; those that can be closed immediately after inoculation may be preferred.

7.3.5 Safety

See general procedure in Section 7.2.4 and Chapter 1.

7.3.6 Report interpretation

The reading of results consists of three simple steps:

- the counting of colonies grown on the different slants: dilution 1 (ex. 10^{-2}) = confluent growth; dilution 2 (ex. 10^{-4}) = 20–100 colonies;
- the calculation of the proportion of resistant bacilli by comparing counts on dilution-2 control tubes (1% of the dilution 1) and on drug containing LJ tubes inoculated with the dilution 1; and
- the ratio of the calculated proportion with the critical proportion of the drug in question, in order to determine whether the ratio is ≥ 1 (resistant strain) or < 1 (susceptible strain).

The reading of results is carried out on day 28 and 42 after inoculation. If after four weeks of incubation the proportion of resistant colonies is higher than the critical proportion, the strain can be reported as resistant. Also, if the reading on day 28 shows that there are no colonies on the drug containing media and the colonies on the control tubes are mature, the strain can be reported as susceptible. Except for these two instances, all other results should be reported after the reading on day 42.

If the number of colonies in the control tubes inoculated with inoculum 1 is below or close to 100, the test should be repeated with a less diluted inoculum.

7.4 Procedure 3: Resistance ratio method

7.4.1 Introduction

The resistance ratio (RR) method was one of the first methods described for drug susceptibility testing of *M. tuberculosis* complex[13,22]. It is calculated by the ratio of the minimal inhibitory concentration (MIC) of a drug for a patient's strain to the MIC of the same drug for the susceptible reference strain, H37Rv, both tested in the same experiment. Instead of the control strain, four known sensitive strains of *M. tuberculosis* can be used as modal control MIC. Inclusion of the reference strain in each experiment is not only serves as a quality-control measure, but also standardises results by taking into account the test variations within certain permissible limits. This feature makes the RR method the most accurate, but because of the use of large numbers of media units, it is also the most labour intensive.

$$\frac{\text{Test MIC}}{\text{Modal control MIC}} = \text{Resistance ratio}$$

7.4.2 Materials

A pure, well-characterised culture of *M. tuberculosis* bacteria in the active growth phase is necessary. Pure substances (no patient tablets) of the drugs must be used at all times. No special equipment is required, only plates or tubes with LJ medium and pipettes.

7.4.3 Methods

Calculation of modal control MIC

Four known sensitive *M. tuberculosis* strains are inoculated onto a complete set (five concentrations) of drug containing LJ media. The plates are incubated at 37 °C for two to three weeks. The modal control MIC value is calculated as the concentration where most of the four strains show no growth.

Growth on the media is recorded as follows:

- CG = confluent growth
- IC = innumerable discreet colonies
- + = 20 to 100 colonies
- – = no growth or less than 20 colonies

The modal MIC value is calculated as follows:

Table 7.2. Calculation of modal MIC value

Strain	Identifier (/)	Identifier (//)	Identifier (•)	Identifier (••)	Identifier (•••)	MIC
A	CG	CG	-	-	-	•
B	CG	IC	IC	-	-	••
C	IC	-	-	-	-	//
D	CG	IC	-	-	-	•
Mode	CG	IC	-	-	-	•

Modal MIC is the 1-dot bottle.

Inoculation and interpretation

Test strains are only inoculated onto the three highest antibiotic concentration egg media (1 dot, 2 dot, 3 dot) as the concentrations are such that the 1-dot bottle should be the modal MIC of the control strains. If the control strains show a higher or lower MIC than usual, this must be taken into consideration when reporting results. For example:

Table 7.3. Examples for MIC interpretation

Control	0.25mg/l	0.5mg/l	1.0mg/l	2.0mg/l	4mg/l	Ratio	Result
CG	CG	CG	-	-	-	MIC=1	Modal average
CG			-	-	-	1/1=1	Sensitive
CG			+	-	-		Sensitive
CG			CG	-	-	2/1=2	Sensitive
CG			CG	+	-		Borderline
CG			CG	CG	-	4/1=4	Resistant

Control	0.25mg/l	0.5mg/l	1.0mg/l	2.0mg/l	4mg/l	Ratio	Result
CG			CG	CG	+		Highly resistant
CG			CG	CG	CG	8/1=8	Highly resistant
CG			CG/+	+	+	Mixed sensitive and resistant	

Control	0.25mg/l	0.5mg/l	1.0mg/l	2.0mg/l	4mg/l	Ratio	Result
CG	CG	CG	CG	-	-	MIC=2	Modal average
CG			-	-	-	1/2=0.5	Sensitive
CG			+	-	-		Sensitive
CG			CG	-	-	2/2=1	Sensitive
CG			CG	+	-		Sensitive
CG			CG	CG	-	4/2=2	Sensitive
CG			CG	CG	+		Borderline
CG			CG	CG	CG	8/2=4	Resistant

Control	0.25mg/l	0.5mg/l	1.0mg/l	2.0mg/l	4mg/l	Ratio	Result
CG	CG	-	-	-	-	MIC=0.5	Modal average
CG			-	-	-	1/0.5=2	Sensitive
CG			+	-	-		Borderline
CG			CG	-	-	2/0.5=4	Resistant
CG			CG	+	-		Highly resistant
CG			CG	CG	-	4/0.5=8	Highly resistant
CG			CG	CG	+		Highly resistant
CG			CG	CG	CG	8/0.5=16	Highly resistant

The tests are incubated at 35–37 °C for two weeks. If growth is poor at this time the tests should be re-incubated for another week.

7.4.5 Report interpretation

Table 7.4 provides further information on resistance ratio reporting.

Table 7.4. Resistance ratio reporting

Result	Ratio	Report
- - -	1	S
G - -	2	S2
G + -		Borderline*
G G -	4	R4*
G G G	> 4	High-level resistance

7.4.4 Safety

See general procedure in Section 7.2.4 and Chapter 1

7.5 Procedure 4: Drug susceptibility testing in liquid media (MGIT 960)

7.5.1 Introduction

Egg-based medium, such as LJ, have been used for several decades, but an average of three to four weeks are required to obtain results. Thirty years ago, the first liquid-based culture media was introduced commercially, and several evaluations have demonstrated excellent correlation with the proportion method and significant time savings. One of the earlier disadvantages of this system was the use of a radioactive labelled substrate. Because of the strict regulations of handling and waste disposal of radioactive material, it became necessary to develop a non-radiometric technique based on the liquid medium.

The Becton Dickinson MGIT 960 uses 7H9 liquid media which contains an oxygen-quenched fluorochrome embedded in silicone at the bottom of the tube. During bacterial growth, the free oxygen is utilised and replaced with carbon dioxide. With the depletion of free oxygen, the fluorochrome is no longer inhibited, resulting in fluorescence and identification of bacterial growth, which can be detected manually or automatically[21,23-25].

7.5.2 Materials

A pure, well-characterised culture of *M. tuberculosis* bacteria in the active growth phase is necessary. Pure substances (no tablets) of the drugs must be used at all times. BACTEC MGIT tubes, along with supplements and drugs, and a BACTEC automated machine are required.

7.5.3. Methods

Media preparation

0.1 ml of a reconstituted drug solution and 0.8 ml supplement (available commercially) is added to each of the 7 ml of 7H9 media containing tubes.

Drugs

The drugs to be tested are stored according to the manufacturer's instructions. Commercial available drugs are: isoniazid, rifampicin, ethambutol, streptomycin, and pyrazinamide. All second-line drugs have to be prepared by the laboratories. Critical concentrations are given in Table 7.5.

Table 7.5. Critical concentrations of the most important first-line and second-/third-line drugs

Critical concentrations of the most important first line and second/third line drugs							
Drug	Isoniazid	Rifampicin	Ethambutol	Streptomycin	Capreomycin	Amikacin	Ofloxacin
Critical concentration (mg/L)	0.1	1	5	1	2.5–3	1–1.5	2

Inoculation

The indirect drug susceptibility test can be performed from liquid or solid media, according to the manufacturer's guidelines. Testing using inoculum from liquid media includes the following steps:

- A positive MGIT tube is used as the inoculum. For each set of DST, a control tube (1:100 dilution of the original inoculum) is required.
- The positive MGIT tube is inverted one to two times and then left undisturbed for about five to ten minutes to let big clumps settle to the bottom.
- The inoculum is the pure supernatant of the positive MGIT tube if positivity was observed in the last 24 hours. If positivity was observed for 48 hours, it has to be diluted two-fold; if positivity was observed for three to five days, it has to be diluted five-fold. A volume of 0.5 ml of the suspension has to be added aseptically into every drug-containing tube.
- For growth control, the suspension has to be diluted 1:100 by adding 0.1 ml of the suspension to 10 ml of sterile saline. The tube has to be well mixed before adding 0.5 ml into the growth-control tube.

Incubation

After inoculation, the tubes are incubated at 37 °C. Fluorescence will be detected automatically.

7.5.4 Safety

See general procedure in Section 7.2.4 and Chapter 1.

7.5.5 Report interpretation

Once the test is complete (four to 12 days), the instrument will indicate that the results are ready and can be reported. Results are qualitative: susceptible, resistant, or indeterminate (growth in less than four days or not enough growth within 12 days). If resistant, the drug-containing tube has to be checked for contamination, by preparing a smear and/or adding one drop onto a blood plate.

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8 Molecular typing of *Mycobacterium tuberculosis* complex isolates

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8.1 Background and principles

In the last two decades, multiple molecular typing methods for *Mycobacterium tuberculosis* complex isolates have emerged, with different levels of reproducibility, discriminative power and demands on technical expertise. Previously, phage typing could only distinguish a very limited number of strain types; while today hundreds of thousands of different strains are recognised. DNA fingerprinting currently supports routine contact tracing in many countries as well as studies on person-to-person transmission, early disease outbreak identification, high transmission risk groups, laboratory cross-contamination[19,20], and the distinction between reinfection and reactivation[24,3]. In particular, DNA fingerprinting of *Mycobacterium tuberculosis* has greatly improved the understanding of TB transmission. Moreover, the recognition of genotype families has facilitated studies on the population structure of the *Mycobacterium tuberculosis* complex and its dynamics.

The various DNA fingerprinting methods serve different purposes and have variable characteristics that enable their use in specific applications. This chapter describes the characteristics of the three most important and widely applied DNA fingerprinting methods: spoligotyping, VNTR typing, and IS*6110* RFLP typing, plus the most important applications of these typing methods. Although the application of DNA fingerprinting has improved our knowledge of the natural history of TB infections and the disease dynamics, there are still open questions. All DNA fingerprinting methods have different molecular clocks[d] and the stability of DNA profiles has been studied extensively, but is not fully understood[5]. Ideally, every transmission should effect a slight change in the DNA fingerprint, while the strains remain recognisable, which makes it possible to distinguish primary sources in a chain of transmission from secondary and tertiary ones, making for a much more detailed analysis of transmission in a given area. Unfortunately, mutations in the genome of *M. tuberculosis* complex occur according to a stochastic process and therefore DNA fingerprinting will never be a perfect tool in studies on transmission. However, the recent application of whole genome sequencing of *M. tuberculosis* isolates for the visualisation of transmission chains among isolates in RFLP clusters has shown that this approach may significantly add to the utility of our currently used fingerprinting methods.

Below is an outline of the three DNA fingerprinting methods: spoligotyping, VNTR typing and RFLP typing. Detailed protocols can be found at www.tuberculosis.rivm.nl.

8.2 Procedure 1: Spoligotyping

Spoligotyping is based on polymorphisms in the direct repeat (DR) locus in the mycobacterial chromosome[1,13]. This method utilises the amplification of variable DNA spacers in the genomic DR region of *M. tuberculosis* complex isolates as this target shows considerable strain-to-strain polymorphisms[37]. The well-conserved 36-bp direct repeats are interspersed with unique spacer sequences varying from 35 to 41 bp in size. The order of the spacers has been found to be well conserved[38]. Currently, 94 different spacer sequences have been identified, 43 of which are used in the first-generation spoligotyping for *M. tuberculosis* complex strains[10]. After amplification, the denatured PCR products are applied in the reversed line on a membrane with covalently bound multiple synthetic spacer oligonucleotides deduced from sequences of the DR regions of two control strains (*M. tuberculosis* H37Rv and *M. bovis* BCG P3). Each spoligotype can then be conveniently represented as a 43-dimensional binary vector. Clinical isolates of *M. tuberculosis* complex can be differentiated by the presence or absence of one or more spacers. Almost all strains reveal a few (up to 43) of these spacers. Clinical specimens[21] and even paraffin-embedded samples have also been evaluated[33], which makes spoligotyping useful for the detection of *M. tuberculosis* complex in a clinical microbiology setting. The obtained patterns – 43 spacers present or not – are usually characteristic of a particular genotype family, like the Beijing genotype strains[26]. Spoligotyping is therefore a simple, cheap, rapid and reproducible[15] tool to study the phylogeny of *M. tuberculosis* complex strains or to associate phenotypic features of isolates with the genotype family the bacteria represent[12]. However, the level of discrimination of spoligotyping is unfortunately generally low and one has to be cautious when using this method to examine the transmission of TB on a strain level[34,16]. It is, however, possible to use spoligotyping as a screening method in typing (strains that reveal different spoligo patterns, in the vast majority of cases, also have different

[d] A measure of evolutionary change over time at the molecular level that is based on the theory that specific DNA sequences spontaneously mutate at constant rates and that is used chiefly for estimating how long ago two related organisms diverged from a common ancestor

patterns in the other, more discriminative typing methods). On the other hand, when spoligotype patterns are identical, then no conclusion can be drawn about the epidemiological relationship between the respective patients. The SpolDB4 database is one of the largest publicly available databases on the *M. tuberculosis* complex and contains spoligopatterns from approximately 40 000 clinical isolates from 122 countries.

8.3 Procedure 2: VNTR typing

The variable-number of tandem-repeat (VNTR) method to typing bacterial strains has proven to be a suitable method for the detection of genetic polymorphisms within bacterial species[18]. Differences in the number of tandem repeats in the 24 stretches of the genome of *M. tuberculosis* complex strains are the basis for an internationally recognised typing method which offers advantages over IS*6110* typing. In multiplex PCRs, up to 24 loci are amplified using primers specific for the flanking regions of each repeat locus, after which the sizes of the amplified stretches of tandem repeats are determined to deduce the number of tandem repeats present. The number of tandem repeats detected at the different loci results in a numerical code that serves as a DNA fingerprint of the respective tuberculosis bacteria. This result format is another advantage over IS*6110* RFLP*,* which allows users of this technique to exchange results and perform inter-laboratory comparisons.

The determination of the PCR products in VNTR typing can be done manually by performing all single-locus PCRs, followed by interpretation of the product length by electrophoreses. It is recommended to use a molecular marker with multiple bands around the molecular size of interest; using a marker containing all the different numbers of repeats possible for the specific locus is even better, otherwise the determination of the number of repeats is highly inaccurate. More automated and higher throughput methods that determine the sizes of the PCR product take advantage of a DNA sequencer, which labels the primers with a particular colour dye. Although this approach is more expensive, it saves a lot of work and is considered to be more accurate.

Originally, VNTR typing used only 12 loci, which resulted in a limited level of discrimination[16,22,23,25]. Later on, more spacers were added and an international standard set of 15 and 24 loci was proposed, which made it possible to distinguish unrelated strains and provided the clonal stability to reliably identify isolates from the same transmission chain[28]. In order to have a level of discrimination comparable with RFLP typing (see below), 24 loci are needed, but this makes VNTR typing also very labour intensive.

Due to the fact that VNTR typing is based on DNA amplification and hence can be performed on early positive cultures or even on clinical material containing sufficient bacteria, the turn-around time of this typing method is much shorter when compared to RFLP typing. In addition, results are presented in an easy-to-compare format and the method is much easier to perform than IS*6110* RFLP typing, which makes it possible to implement VNTR typing in laboratories without extensive molecular experience. Consequently, VNTR typing is currently considered to be the most suitable high-discrimination typing method.

An open database to which users can add VNTR patterns would be a powerful tool for the online analysis of the clonal identification of *M. tuberculosis* complex isolates; a first step in this direction has been taken with the launch of the MIRU-VNTR*plus* database[2].

8.4. Procedure 3: RFLP typing

The restriction fragment length polymorphism (RFLP) method to typing bacterial strains[10] has been the most extensively used method in the last 15 years because of its high level of discrimination[15], reproducibility, and the fact that this was the first method that proved to be a suitable DNA-strain typing method to study transmission[27]. It is based on the fact that the number of IS*6110* insertion sequences present in the genome of strains differs from 0 to approximately 30. These genomic insertion sites themselves are also highly variable in *M. tuberculosis* complex strains, which results in highly variable banding patterns.

The generation of RFLP patterns is technically demanding and time consuming. About two micrograms (mg) of highly purified genomic DNA is needed as starting material, followed by restriction enzyme cleavage of the DNA, fragment separation by electrophoresis, the transfer of the fragments to a DNA membrane and hybridisation by a labelled probe, and final visualisation of the results on a light-sensitive film. All individual steps of the process are crucial for the final result, which also points towards the difficulties experienced with regard to inter-laboratory comparability.

In addition, the analysis of IS*6110* RFLP patterns with the Bionumerics software is complicated, requiring experienced users. However, the difficulties associated with RFLP typing have not altered the fact that RFLP typing has revolutionised our understanding of the transmission of TB. Moreover, for strains of particular genotype families like the Beijing clade, the level of discrimination of RFLP typing is still superior to that of the recently introduced 24-loci VNTR typing[11,16,17].

The three described DNA fingerprinting methods can be used for different applications. Spoligotyping is generally used in studies in which the genotype family of the respective bacteria is to be revealed; whereas this technique is

less suitable for strain typing. Both RFLP and 24-loci-VNTR typing have a high level of discrimination and reproducibility and can be used for strain typing. As the turn-around time of VNTR typing is significantly shorter than that of RFLP typing and VNTR typing is also technically far less demanding, it is now considered the international standard in Europe and the USA. VNTR typing can be used in contact tracing and source-case finding. Even in settings with good contact tracing programmes, DNA fingerprinting surveillance is far superior to contact tracing as it can be used to visualise person-to-person transmission. In the Netherlands, only about 25% of the epidemiological links detected by DNA fingerprinting are confirmed by interview-based contact tracing. 20% of the cases can be linked by DNA fingerprinting, despite the fact that there had been no reported contact between the patients. In 55% of the clustered cases, interviewees fail to report any links. This is not unexpected, as many of the transmissions will take place outside of regular contacts and will not be remembered by the persons involved.

The performance of DNA fingerprinting has also been used for the prediction of the size of future clusters following the detection of the first two cases of a new cluster. Time between the cases, age, nationality, and residence are variables that become known shortly after the diagnosis of a new TB cluster. By combining the molecular data and the patient's registration data, new cluster episodes can be predicted by using the risk factors. This information can contribute to early warning systems for the national health services[14].

DNA fingerprinting can detect possible cross-contamination in the laboratory. If a laboratory detects two isolates with identical DNA profiles in only one week, this usually indicates a sampling or laboratory mishap[19]. In that case, the clinician should be asked to review the clinical picture of the patient, and the microbiologist should check the rate of positivity of the culture and whether the cultures with the identical DNA fingerprints have been contaminated at a particular stage. Regular checks of positive cultures are recommended to detect this common problem; in the Netherlands, about 3% of all positive cultures are cross contaminated[35].

The widespread application of DNA fingerprinting has provided substantial insights into TB transmission in a given area, especially when conventional epidemiological investigation and molecular typing are combined[32]. The strong association of tuberculosis transmission with gender and lower age of the source case in a low-prevalence setting has been shown by molecular fingerprinting techniques[6]. Isoniazid (INH) resistance is a negative risk factor for transmission, while strains with a mutation at amino acid 315 of the *katG* gene are as transmissible as susceptible ones[9]. Also, studies on transmission within and outside households in South Africa[29,31] yielded important insights into the origin of TB infections in a high-prevalence setting. There is also an increased risk for previously treated and cured TB patients of developing TB again when reinfected[30].

In the European context, a study on the molecular surveillance of multidrug-resistant strains in Europe between 2003 and 2006 showed that half of the genotyped strains done by IS*6110* RFLP were derived from transmission. Moreover, 84% of these clustered MDR-TB strains were represented by the Beijing genotype, while only 6 to 7% of the susceptible strains in Europe belong to that genotype[8].

8.5 Materials

Purified DNA from *M. tuberculosis* complex bacteria is generally the best material for molecular typing. For methods based on DNA amplification, such as spoligo- and VNTR typing, only a small amount is needed as starting material. Even purified DNA from a sufficient amount of bacteria in clinical material will result in a typing pattern. However, briefly incubating these bacteria in a liquid culture medium will generally yield more reliable and reproducible results, which is advisable because of the costs and the time to perform the typing techniques. For RFLP typing, based on the specific restrictions of the isolated, unamplified DNA, a fully grown culture is needed as two micrograms of highly purified genomic DNA are required; in a diagnostic setting, it will take several weeks to achieve this amount of growth after TB detection.

Membranes, reagents and positive control DNA for spoligotyping can be purchased. RFLP typing consists of a lengthy, multi-step laboratory procedure that requires is prone to error. Poor quality in RFLP typing can be connected to laboratory technique, but very often is caused by the incorrect interpretation of results with pattern analysis software. The performance of spoligotyping is better, especially in laboratories with molecular experience, and the results are more comparable among different laboratories[4].

For 24-loci VNTR typing, a kit containing all reagents for eight multiplex amplification reactions can be obtained commercially. In addition, the PCR product fragments are analysed on an automated DNA sequencer. In the absence of an expensive analyser, the 24-loci VNTR can be used with single or multiplex amplification reactions and detection of the product, either automated or manually; see standard operating procedure on the RIVM website (www.tuberculosis.rivm.nl) (based on 28). The quality of international VNTR typing performance and the inter-laboratory reproducibility is not yet clear, but efforts are underway to provide data in this area.

8.6 Methods

Detailed protocols of all DNA fingerprinting methods and DNA isolation methods can be found on www.tuberculosis.rivm.nl/. The basis of the three fingerprinting methods have been published earlier: for spoligotyping by Kamerbeek et al. (1997), for RFLP typing by van Embden et al. (1993), and for VNTR typing by Supply et al. (2006).

8.7 Results/interpretation

Spoligo patterns are codes of 43 possible digits and can be sorted with standard software like Excel. Most institutions however use the Bionumerics software (Bionumerics, Kortrijk, Belgium), which is able to compare the results of any typing method. There is an international database of spoligo patterns[7] which holds tens of thousands of spoligo patterns that can be used to compare the locally obtained typing results with patterns that have been found elsewhere and are already labelled with a genotype family designation.

The result of VNTR typing is a numerical code of usually 12, 15 or 24 numbers which can be analysed relatively easily, as described above in the section on spoligotyping. Local comparisons for epidemiological investigations are best done with the Bionumerics software. There are several international databases with VNTR typing results in which locally obtained results can be compared with international collections. However, as large-scale VNTR typing is a relative new phenomenon, only a limited number of patterns are available.

The analysis of IS*6110* RFLP patterns by Bionumerics software is technically demanding (protocol on RFLP typing: www.tuberculosis.rivm.nl/). Most important is the inclusion of internal (a mixture of two molecular markers) and external standards (DNA of a control strain with a suitable range of bands) for normalisation and accurate reading of the band sizes.

8.8 Quality control

Quality control of DNA fingerprinting is of the utmost importance. On all occasions, first-line controls should include strains with a known DNA fingerprint. In the case of spoligotyping, DNA of H37Rv and P3 should be included in each test to assess the performance of each of the 43 spacer oligos present on the blot. In VNTR typing, a strain with a know VNTR profile should be included. In RFLP typing, a particular strain with a wide range of bands should be used in each test to check the normalisation.

It is also advised to organise a second-line control procedure, which includes the blinded exchange of a set of DNA samples with another laboratory twice a year to test the reproducibility. As a third line of control, a blinded set of DNA samples supplied by an international organisation to multiple institutes should be analysed to test proficiency in the given procedure.

8.9 Biosafety

Biosafety is a concern when carrying out DNA typing, especially when heat inactivation of *M. tuberculosis* at 80 °C is used. Some publications have expressed concern whether heat inactivation is sufficient to inactivate all bacteria. Studies have shown that even after a very long period of incubation a very limited bacterial growth is sometimes still observed[36]. This implies that it is advisable to perform the first steps of DNA isolation in a biosafety cabinet after heat inactivation at 80 °C. Boiling of a culture is generally considered a safe procedure to kill all bacteria.

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9 Information for physicians: the laboratory diagnosis of tuberculosis – first steps

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9.1 Introduction

The diagnosis of tuberculosis (TB) is often the result of a combination of clinical, epidemiological, microbiological and histological data. Therefore, optimal communication and understanding between different disciplines is of the utmost importance. The whole process of collecting clinical material for the microbiological diagnosis is delicate and each step will influence diagnosis. Therefore, diagnostic material should be collected and transported in an optimal way. The clinical material for laboratory analysis should always be accompanied by sufficient and correct patient information. In the case of suspected lung TB, the patient should be provided with the appropriate sputum pots and detailed instructions on how to produce the most suitable sputum for TB diagnostics. Only appropriate approaches for obtaining, transporting and inoculating material from extra-pulmonary sites will result in the highest probability of successful confirmation of disease. The material should be kept under well-defined conditions and transported to the laboratory within defined time limits. Although molecular methods directly applied to clinical material are increasingly reliable in diagnosing TB and can indicate possible resistance to first- and second-line anti-tuberculosis drugs, the limitations associated with this approach (e.g. in the diagnosis of drug resistance) should be indicated clearly. The *in vitro* inhibition of growth of the causative *Mycobacterium tuberculosis* bacteria remains the gold standard for resistance determination, but requires microbiological culture for drug susceptibility testing.

M. tuberculosis is a BSL3 pathogen and each country has specific regulations on the safe transportation of these bacteria to referral laboratories. Positive *M. tuberculosis* cultures usually, but not always, reliably confirm a TB diagnosis, as sample and laboratory mislabelling as well as cross-contamination can occur. Cross-contamination rates (i.e. false-positives) of up to 3 to 5% of positive cultures have been reported. If misdiagnosis is suspected, communication between the laboratory and the involved physician is imperative to avoid unnecessary treatment.

This chapter aims to increase the awareness of physicians of required quality control during the first diagnostic steps, particularly how these steps influence the quality of products/specimens sent to tuberculosis laboratories. It describes optimal sampling of clinical materials and how to yield the best microbiological results. In addition, several aspects of communication between the different disciplines are described, such as the clarification and interpretation of laboratory results given to the clinician. All laboratories should provide clearly written guidance for physicians on their requirements for optimal specimens.

9.2 General instructions regarding the diagnosis of tuberculosis

- Clinical material (such as sputum) for the diagnosis of TB and the initial drug susceptibility testing should be collected before the start of treatment.
- Clinical material should be collected aseptically in sterile containers to avoid contamination with nontuberculous mycobacteria (NTM) and other microorganisms. It is noteworthy that tap water contains multiple mycobacteria of different species and therefore should not be used in this procedure.
- Cotton wool swabs are not suitable for use in the diagnosis of mycobacteria. Due to evaporation, the yield of positive cultures will drop significantly unless the material is processed directly. Moreover, the captured material will generally be insufficient for TB diagnosis.
- The yield of positive results will increase for some specimens if a higher amount of the material is provided (in case of CSF) or multiple samples are examined. In general, and especially for dilutional fluids such as CSF, ascetic, and pleural fluid, the largest possible volume should be collected and sent to the laboratory. The WHO recommends examination of at least two sputa for the diagnosis of pulmonary TB.
- Short transportation times from the taking of clinical material to the laboratory can benefit diagnosis. Some extra-pulmonary materials may even benefit from inoculation at the bedside to increase the probability of a positive *M. tuberculosis* diagnosis. However, if inoculated at the bedside, an additional sample should be collected for microscopy and further diagnostic measures in case of contamination by *Staphylococci* or other environmental organisms.
- If clinical material cannot be sent directly to the laboratory, it should be kept in a refrigerator (4 °C).
- All clinical material for TB diagnosis sent to a laboratory should be accompanied with a completed form. For this purpose, dedicated forms should be used which provide information such as the name of the patient, date of birth, gender, patient file number, probability of resistance to anti-tuberculosis drugs (i.e. previous

history of TB, previous or current antituberculous treatment, and country of birth), required diagnostic tests, date of collection, sample taken before or during treatment, and detailed information on the submitting physician, including telephone number.

- If a rapid test is requested, e.g. for the detection of rifampicin resistance by molecular test, the physician should apply for this by a phone call to the laboratory or use a special form and provide contact details for the rapid return of results.
- It is the responsibility of the sender to pack clinical materials appropriately. Packaging in most countries is provided by the laboratories and is leakproof. *M. tuberculosis* cultures are a BSL3 microorganism, so special regulations apply. In principle, the biosafety regulations of directive 2000/54/EC[1] can be applied, but national authorities have also released individual regulations for the transportation of BSL3 microorganisms (see Chapter 1). It is recommended that the sender notifies the laboratory when the culture has been sent. Receiving laboratories should acknowledge receipt so that missing parcels can be traced.

9.3 Specific instructions on collection and treatment of clinical material in TB diagnosis

9.3.1 Pulmonary material

If there is a suspicion of pulmonary tuberculosis, 5 to 10 ml of early morning sputum should be collected in an appropriate sputum pot with a wide opening and a secure lid. This should be done on at least two consecutive days. The sputum should be freshly expectorated from the lung (rather than saliva) and the patient should be instructed on how to produce this material[2]. The collection of sputum over 24 hours is not recommended as the extended time of collection increases the chance of contamination by NTM and other bacterial microorganisms.

If the patient is unable to produce sputum, sputum can be induced by supplying an aerosol of hypertonic saline solution (5% NaCl) or by collecting an early morning gastric juice sample. In addition, an aspirate can be obtained by bronchoscopy with a bronchial lavage. However it should be emphasised that carrying out a bronchoscopy in a patient suspected of multibacillary TB can a) be a risk to the person performing the procedure, and b) requires thorough disinfecting of the bronchoscope, i.e. the procedure must be performed using appropriate safety standards.

All patient material collected should be provided at the highest quality and in sufficient quantity[3]. There is not much known about the *ex vivo* survival of mycobacteria in clinical material. In one study on ZN-positive sputa kept at 4 °C, 60% of the mycobacteria in the sputum appeared to be viable after four weeks, while at room temperature only 38% survived[4]. It is therefore advised to send pulmonary clinical material directly to the laboratory. If this is not possible, sputum samples should be kept in the refrigerator for only the minimum number of days.

The use of a transport medium can be considered, especially in difficult field conditions, and this includes the use of 1% cetyl-pyridinium chloride in 2% sodium hydroxide. This solution can be mixed in equal quantities with patient material and results in mycobacteria which remain viable for at least eight days. However, the disadvantage of this approach is that the bacteria only grow on Löwenstein-Jensen medium thereafter and not on 7H10 or other agar-based media[5].

9.3.2 Pleural material

If pleural fluid is obtained, the chance of a positive culture can be increased by taking increased volumes, which can then be concentrated. The largest volume practicable should be taken and sent. However, recent studies have shown that the transport medium, the place of inoculation, and the type of inoculation medium influence the yield of mycobacteria. Bedside inoculation in combination with a liquid *Mycobacterium spp.* medium appeared to be the best choice. Alternatively, containers with heparin should be used to avoid clotting and trapping of mycobacteria[6].

9.3.3 Lymphadenitis material

For the diagnosis of tuberculous lymphadenitis, lymph node biopsy (ideal) and fine-needle aspiration (FNA) are the first-choice diagnostic methods in both low-incidence and endemic countries[6,7,8]. In the case of a negative FNA, an excision biopsy should be considered, which often results in a higher chance of positive microscopy for mycobacteria. A fine-needle aspirate should be taken using a 19- or 21-gauge needle; the sample needs to be transported directly to the microbiological/pathological laboratory to prevent evaporation. It is important to inform both the pathologist and the microbiologist beforehand that the delicate clinical material is to be dealt with immediately.

9.3.4 Gastric juice

Investigation of gastric fluid is recommended in the diagnosis of pulmonary TB when examination of sputum or bronchial lavage fluid is not possible, e.g. in young children. The fasting gastric fluid should be collected after the administration of 20–30 ml of physiological saline in 5–10 ml of sodium carbonate (Na_2CO_3). The material should be transported to the laboratory within four hours. Also, for immunocompromised patients not yielding sputum, the investigation of gastric fluid can be rewarding[9].

9.3.5 Peritoneal fluid

If tuberculous peritonitis is suspected, at least 5–10 ml ascitic fluid can be collected and sent, or inoculated at the bedside in a liquid mycobacteria culture medium. However, in a review involving more than a thousand patients, a peritoneal biopsy appeared more sensitive[10,11]. In general, sending the largest possible volume for centrifugation at the laboratory is ideal.

9.4 Information flow from the microbiologist to the physician

When laboratory results are reported to the physician, a basic level of information should always be provided to avoid mistakes and confusion, for example the date of specimen reception at the laboratory, the test date, the patient's identifiers, the results, and a clear interpretation. Physicians should, if required, demand an unambiguous clarification of the results. In the case of microscopic examination, (semi-)quantitative results of the number of bacteria/field (WHO) scale are helpful.

For cultures, the quantitative results in terms of time to positivity (in days) for liquid medium are helpful, and the number of colonies on solid medium should be stated unless the laboratory is already using these criteria in its interpretation and advice to the clinician. A low number of colonies on a primary culture, unusual timings for positive cultures (e.g. a higher than usual positivity rate associated with inoculation on or around the same day, or a very short time to positivity in a smear-negative extra-pulmonary sample) may raise serious suspicions of cross contamination, and the laboratory needs to be aware of this. If suspected, positive cultures found on consecutive days can be subjected to VNTR typing (DNA fingerprinting, see Chapter 8); if they reveal the same profile, cross-contamination is highly likely.

In addition, the results of nucleic amplification tests (NATs) should be provided with the right nuance; overall validity depends on the use of inhibition/amplification controls, negative and positive controls, as well as on the positive and negative predictive value. For example, when a NAT is positive while the microscopic smear is negative, it should be explained that the positive predictive value of this result is highly dependent on the prevalence of tuberculosis in the respective patient category.

Molecular tests for the detection of mutations associated with resistance are indirect tests, i.e. not all mutations are associated with clinically-relevant resistance. This should always be clearly stated. Large-scale studies that confirmed molecular test results by phenotypic drug resistance testing showed close concordance for rifampicin (as a surrogate for MDR-TB[12]) and clearly demonstrated the value of such testing systems, provided appropriate controls are used. Therefore, these tests can be used to direct the therapy and the choice of anti-tuberculosis drugs; however, the true resistance profile of the causative bacteria should be measured with an *in vitro* growth inhibition method, although this requires much more time than molecular tests. For instance, the detection of rifampicin susceptibility with a molecular test (if no *rpoB* mutations are found) indicates that one can be more than 95 % sure that the strain is indeed susceptible to rifampicin. The molecular tests for the detection of isoniazid (INH) resistance are less precise. If no mutations are found in the *katG* gene or in the *inhA* promotor, there is still a 10 to 25 % chance that the respective bacteria are resistant to INH.

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