



TECHNICAL DOCUMENT

Handbook on TB laboratory diagnostic methods in the European Union

ECDC TECHNICAL DOCUMENT

Handbook on TB laboratory diagnostic methods for the European Union



This report of the European Centre for Disease Prevention and Control (ECDC) was coordinated by Csaba Ködmön with support from Marieke J. van der Werf, Francis Drobniowski and Vladyslav Nikolayevskyy.

This report was sent for consultation to the members of the ERLTB-Net network (see Appendix 1 for list of contributors).

The first version of this ECDC technical report, previously published as 'Mastering the basics of TB control: Development of a handbook on TB diagnostic methods' (Stockholm 2011) concerned the development of the handbook which was included as an annex. This report has now been revised, updated and renamed as the 'Handbook on TB laboratory diagnostic methods for the European Union'.

Suggested citation: European Centre for Disease Prevention and Control. Handbook on TB laboratory diagnostic methods for the European Union, Stockholm: ECDC; 2016.

Stockholm, March 2016

ISBN 978-92-9193-739-4

doi 10.2900/216384

Catalogue number TQ-01-16-109-EN-N

© European Centre for Disease Prevention and Control, 2016

Reproduction is authorised, provided the source is acknowledged

Contents

Abbreviations	v
Background and introduction	1
1 Biosafety in the laboratory diagnosis of tuberculosis	4
1.1 Background and principles	4
1.2 The containment laboratory (biosafety level 3)	5
1.3 The laboratory facility	6
1.4 Procedures	7
1.5 Personal protective equipment (PPE).....	7
1.6 The human resource component	8
1.7 Specific laboratory tasks related to hazards	8
2 Quality assurance.....	12
2.1 Background and principles	12
2.2 Selection and implementation of tests.....	13
2.3 Validation	13
2.4 Control and trend monitoring by key indicators	14
2.5 Physical and environmental conditions	14
2.6 Equipment.....	14
2.7 Management and staff.....	14
2.8 Documentation and registration.....	15
2.9 Sampling guidance, review of requests and service to customers.....	15
2.10 Subcontracting, services and supplies.....	15
2.11 Complaints, errors and corrective actions.....	15
2.12 Improvements.....	15
2.13 Reporting and releasing results.....	16
3. Diagnosis of latent TB infection	17
3.1 Background and principles	17
3.2 The tuberculin skin test (TST)	18
3.3 Interferon- γ release assays (IGRAs).....	18
4. Smear microscopy.....	32
4.1 Background and principles	32
4.2 Procedure 1: Ziehl-Neelsen (ZN).....	32
4.3 Procedure 2: Fluorochrome staining	34
4.4 Sample collection	35
4.5 Smear preparation	36
4.6 Staining procedures.....	36
4.7 Microscopy	37
4.8 Recording and reporting	38
4.9 Quality control	40
5. Culture tests for <i>Mycobacterium tuberculosis</i> complex	42
5.1 Background and principles	42
5.2 Biohazards and biosafety in the TB laboratory.....	42
5.3 Specimen collection, storage and transport.....	44
5.4 Homogenisation and decontamination of specimens	45
5.5 Culture media: principles	49
5.6 Solid media.....	49
5.7 Culture tube inoculation	50
5.8 Culture incubation	51
5.9 Culture examination	51
6. Identification of <i>M. tuberculosis</i> and drug resistance in cultures/sputum using molecular assays and immunoassays..	54
6.1 Background and principles	54
6.2 Procedure 1: Chromatographic immunoassay for the qualitative detection of <i>Mycobacterium tuberculosis</i> complex from cultures	54
6.3 Procedure 2: Line probe assay for identification of the genus <i>Mycobacterium</i> and 16 different mycobacterial species including <i>M. tuberculosis</i> complex from culture	55
6.4 Procedure 3: Line probe assay for identification of <i>M. tuberculosis</i> complex and detection of rifampicin resistance in <i>M. tuberculosis</i>	57
6.5 Procedure 4: Line probe assay for identifying species from genus <i>Mycobacterium</i> and detecting potential MDR- and XDR-TB from cultures and smear-positive specimens	62
6.6 Procedure 5: Nucleic acid hybridisation test for the identification of <i>M. tuberculosis</i> complex.....	69
6.7 Procedure 6: Single-use sample-processing cartridge system with integrated multicolour real-time PCR capacity for detection & identification of <i>M. tuberculosis</i> complex bacteria and detection of rifampicin resistance.....	70
7. First- and second-line drug susceptibility testing for <i>Mycobacterium tuberculosis</i> complex.....	72

7.1 Background and principles	72
7.2 Procedure 1: General method – DST by culture.....	72
7.3 Procedure 2: The proportion method on Löwenstein-Jensen medium	74
7.4 Procedure 3: Resistance ratio method	76
7.5 Procedure 4: Drug susceptibility testing in liquid media (MGIT 960)	78
7.6 Procedure 5: Other methods for first-line drug susceptibility testing	79
8. Molecular typing of <i>Mycobacterium tuberculosis</i> complex isolates	83
8.1 Background and principles	83
8.2 Procedure 1: Spoligotyping	83
8.3 Procedure 2: MIRU-VNTR typing.....	85
8.4 Procedure 3: RFLP typing.....	86
8.5 General considerations on routine genotyping methods	86
8.6 Procedure 4: whole genome sequencing	86
8.7 Materials	87
8.8 Results/interpretation	88
8.9 Quality control	88
8.10 Biosafety	88
9. Use and validation of disinfectants for <i>Mycobacterium tuberculosis</i>	92
9.1 Liquid disinfection	92
9.2 Gaseous disinfection.....	94
10. Information for physicians: laboratory diagnosis of tuberculosis – first steps.....	98
10.1 Introduction.....	98
10.2 General considerations regarding the diagnosis of tuberculosis	98
10.3 Specific considerations regarding diagnosis of tuberculosis	99
10.4 Information flow from microbiologist to physician and instructions regarding tuberculosis diagnosis.....	100

Figures

Figure 1.1 Biohazard warning sign for laboratory doors	5
Figure 3.1 Potential outcomes of human host infection by <i>M. tuberculosis</i>	18
Figure 4.1 Microscope components.....	37
Figure 4.2 AFB smear microscopy of biological specimens.....	39
Figure 4.3 AFB smear microscopy of <i>M. tuberculosis</i> cultures	39
Figure 5.1 Minimal examination schedule for solid cultures	52
Figure 6.1 Examples of chromatographic immunoassays for qualitative detection of <i>M. tuberculosis</i> complex.....	55
Figure 6.2 Interpretation scheme for INNO-LiPA	57
Figure 6.3 Interpretation of LiPA	61
Figure 6.4 Interpretation sheet for GenoType Mycobacterium CM.....	65
Figure 6.5 Interpretation sheet for GenoType Mycobacterium AS	66
Figure 6.6 Interpretation sheet for GenoType Mycobacterium MTBC.....	66
Figure 6.7 Interpretation sheet for GenoType Mycobacterium MTBDRPlus.....	67
Figure 6.8 Interpretation sheet for GenoType Mycobacterium MTBDRs	67

Tables

Table 1.1 Examples of personal protective equipment	8
Table 2.1 Overall scope of ISO 17025 and ISO 15189	12
Table 2.2 Central terms and abbreviations in quality assurance	13
Table 3.1 List of national LTBI diagnosis guidelines	19
Table 3.2 QuantiFERON-TB Gold results interpretation	24
Table 3.3 QuantiFERON-TB Gold PLUS results interpretation	24
Table 3.4 Materials provided by the manufacturer	26
Table 4.1 Equipment required for smear preparation and staining	36
Table 4.2 Reporting of microscopy smears	38
Table 4.3 Common causes of error in smear microscopy	40
Table 7.1 Solvents and diluents.....	74
Table 7.2 Calculation of modal MIC value.....	76
Table 7.3 Examples of MIC interpretation.....	77
Table 7.4 Critical concentrations of the most important first-line and second-/third-line drugs	78
Table 9.1 Differences between the two major gaseous hydrogen peroxide technologies, Steris VHP and Bioquell HPV.....	95

Abbreviations

AFB	Acid-fast bacilli
BCG	Bacillus Calmette-Guérin
BD	Becton and Dickinson
BSC	Biological safety cabinet
BSL	Biosafety level
CFU	Colony forming units
CSF	Cerebral spinal fluid
CRI	Colorimetric redox indicator
CXR	Chest X-ray
DNA	Deoxyribonucleic acid
DST	Drug susceptibility testing
EEA	European Economic Area
ENP	European Neighbourhood Policy
EPTB	Extrapulmonary tuberculosis
ERLN-TB	European Reference Laboratory Network for Tuberculosis (2010–2014)
ERLTB-Net	European Reference Laboratory Network for Tuberculosis (from 2014)
EQA	External quality assessment
EU	European Union
HEPA filter	High-efficiency particulate air filter
HPF	High-power field
IATA	International Air Transport Association
IFN- γ	Interferon-gamma
INSTAND e.V.	Society for promoting quality assurance in medical laboratories
IGRA	Interferon-gamma release assay
IQA	Internal quality assessment
ISO	International Organization for Standardization
LED	Light-emitting diode
LJ medium	Löwenstein-Jensen medium
LTBI	Latent TB infection
MGIT	Mycobacteria growth indicator tube
MIC	Minimum inhibitory concentration
MIRU	Mycobacterial interspersed repetitive units
MODS	Microscopically observed drug susceptibility
MTBC	<i>Mycobacterium tuberculosis</i> complex
NAAT	Nucleic-acid amplification test
NRA	Nitrate reductase assay
NRL	National reference laboratory
NTM	Non-tuberculous mycobacteria
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PPD	Purified protein derivative
PPE	Personal protective equipment
PPV	Positive predictive value
QA	Quality assurance
QC	Quality control
RFLP	Restriction fragment length polymorphism
RNI	Reactive nitrogen intermediates
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphism
SOP	Standard operating procedures
TB	Tuberculosis
TST	Tuberculin skin test
UKAS	United Kingdom Accreditation Service
UK NEQAS	UKAS accredited proficiency testing provider No. 4715
VNTR	Variable number tandem repeat
ZN	Ziehl-Neelsen (staining method)

Background and introduction

Francis Drobniewski

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, a survey of existing mycobacterial laboratory services and quality control practices throughout the EU confirmed the key role of national reference laboratories (NRL) for TB and their services. The main conclusion of the survey was that a network of reference laboratories for tuberculosis could contribute to improving 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 was funded and coordinated by ECDC. One or two officially nominated reference laboratories from each Member State joined the network, along with those in EEA countries and EU candidate countries.

Following the success of the ERLN-TB network, in 2014 ECDC commissioned a renewal of the network (now called European Reference Laboratory TB-Network [ERLTB-Net]) involving the same centres but anticipating the participation of institutions from the EU enlargement countries in the longer-term.

The three main goals of the new network are: to support the harmonisation of laboratory 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 by the network was the development of a handbook of key diagnostic methods for tuberculosis which was first published in 2011. This document represents the latest version of the handbook.

The aim of this handbook is to provide network members and 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 latent TB infection (LTBI). This handbook offers a single source of reference by compiling all methods, with a strong focus on standard (reference) and evidence-based methods.

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

The handbook addresses the changing technological landscape that has emerged over the last decade, particularly with regard to non-microbiological assays and platforms. Much of this technology has led to a major shift in TB diagnostic activities with the development of multiple, large and well-equipped diagnostic centres with similar capacity and skills to national reference laboratories (e.g. rapid molecular diagnostic tests). Conversely, other molecular diagnostic developments have moved us away from 'big laboratory' approaches and closer to 'point-of-care' devices. The two approaches are not mutually exclusive and both bring advanced diagnostics closer to the patient. The handbook is designed to meet the needs of both centralised and decentralised service delivery models and recognises that the role of national reference laboratories will change significantly over the next few years.

How this handbook relates to other work available in this field

This handbook presents a compilation of methods currently applied in EU/EEA Member States. It describes common work carried out and endorsed by European laboratory experts. It also features methods and procedures developed or refined by ERLTB-Net network partners.

What this document is/is not

This document is a handbook of agreed methods in the field of TB diagnostics for laboratories serving reference functions in Europe. It provides a comprehensive compilation of key methods for the diagnosis of TB. Relevant stakeholders are encouraged to use this compilation as a basis for the validation, development, updating and dissemination of information.

The current document does not contain any formal recommendations for implementation of specific methods in EU/EEA Member States. Recommendations and protocols contained within the handbook are not mandatory for EU/EEA laboratories.

Intended use and users

The report, which provides both basic- and reference-level methods for the diagnosis of TB, is intended for use by laboratory experts. It will also be of interest to public health professionals in the field of global TB control, particularly those involved in European initiatives to foster progress towards elimination of TB.

History of the handbook

The first annual meeting of the ERLN-TB was held in Stockholm in 2010. 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.

The first edition was published in 2011. The handbook was extensively revised during 2014–2015 following the formation of the new ERLTB-Net network and in light of decisions made during its first annual meeting in September 2014. A draft of the handbook was sent out to all ERLTB-Net network partners for review and endorsement in February 2015. An approved version was submitted to ECDC in March 2015.

This edition

This handbook represents the second edition of a publication on the most reliable TB diagnostic methods, endorsed by the members of the ERLTB-Net. This publication is a compilation of protocols for the laboratory diagnosis of TB, designed for laboratory experts and public health professionals. It was compiled to contribute to the harmonisation of methods in the field of TB diagnosis in the EU/EEA, EU enlargement countries and European Neighbourhood Policy (ENP) partner countries, with the goal of ensuring comparability of TB diagnoses in Europe, and provision of the best care possible for TB patients, based on a quality-assured diagnosis. This publication can also support laboratories in establishing a safe working environment for staff by minimising the risk of exposure to *M. tuberculosis*. The second edition of the ERLTB-Net handbook consists of ten 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. In order to ensure effective infection control, it is crucial that a comprehensive and strict biosafety policy is developed and followed. Such a policy includes standardised rules and regulations for containment, personal protective equipment (PPE), standard operating procedures (SOP) for different laboratory tasks and a transparent structure for regulating safe working conditions in diagnostic TB laboratories.

2 Quality assurance

National tuberculosis programmes are supported by laboratories that provide reliable and quality-assured results. The chapter provides a comprehensive overview of 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 are used for the diagnosis of latent TB infections (LTBI): tuberculin skin tests (TSTs) and Interferon- γ release assays (IGRAs). The chapter describes in detail the two commercially available IGRAs for the detection of latent TB infection and provides support for interpreting and reporting 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 for 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 extrapulmonary tuberculosis (EPTB) and in the event of treatment failure. The chapter provides an overview of key principles for sampling and transporting clinical specimens and processing sputum and other specimens before inoculation to solid and liquid culture media, culture incubation and examination. The issue of contamination is addressed, along with the measures necessary 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 susceptibility 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 to improve individual treatment management of tuberculosis cases and drug-resistance surveillance at the level of a hospital, city, region, or country. The chapter describes three different methods for drug susceptibility testing of first- and second-line drugs on solid and liquid media.

8 Molecular typing of *Mycobacterium tuberculosis* complex isolates

Various DNA fingerprinting methods are currently available that serve different purposes and have variable characteristics for specific applications. This chapter describes the three most important and widely applied methods (spoligotyping, variable number tandem repeat [VNTR] typing, IS6110 restriction fragment length polymorphism [RFLP] typing) and their application.

9 Use and validation of disinfectants for *M. tuberculosis*

In Containment Level 3 laboratories handling *M. tuberculosis*, liquid and gaseous methods are currently in use for disinfection and decontamination. The efficacy of any new disinfectants is analysed using a set of standard validation methods.

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

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

Disclaimer

Some protocols included in the handbook list specific commercial products and assays. Such instances do not constitute endorsement of relevant products by the ECDC.

1 Biosafety in the laboratory diagnosis of tuberculosis

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

Revised by Dick van Soolingen, Dimitrios Papaventsis, Melles Haile and Sven Hoffner (2014)

Note: This chapter largely consists of a summary of principles and procedures previously published by the World Health Organisation [1]. 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 laboratory examinations. Access to a safety laboratory should be restricted to staff members and accredited visitors [1]. This chapter describes the most important features of the facility, the procedures, and the personal protective equipment required to ensure biosafety.

It is well documented that *M. tuberculosis* can cause laboratory-acquired infections and the risk of TB among healthcare workers is consistently higher than the risk in the general population [2]. *M. tuberculosis* even features in the top-ten list of hazardous agents for laboratory staff, however the source of the TB infection can only be traced to a specific laboratory accident in a minority of cases [3-5]. The most important route for laboratory-acquired infections is aerosols. Thus, infection control efforts need to focus on limiting the generation of aerosols during laboratory work, for example through safe centrifugation and pipetting. The potential risk of infection depends on the type of techniques used and the way they are executed. For example, needle stick injuries are 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. Moreover, improvements in quality as a result of regular validation and proper use of biosafety cabinets have significantly enhanced infection control.

For effective infection control it is crucial that a comprehensive and strict biosafety policy is developed, accepted and followed by the laboratory staff. The policy should include standardised rules and regulations for containment; personal protective equipment (PPE); a set of standard operating procedures (SOPs) for all the different laboratory tasks and transparent and clearly defined levels of responsibility for establishing and maintaining safe working conditions in the diagnostic TB laboratory [1,3,6,7]. In addition to the necessary training, there should be regular interaction between the responsible manager and the laboratory staff on interpretation of the rules and potential biosafety hazard to verify commitment and optimise follow-up of the implemented procedures.

In the 2012 version of the WHO manual on biosafety in TB laboratories [8] more emphasis was placed on risk assessment. The risk of laboratory infections due to *M. tuberculosis* is related to the concentration of bacteria and the possible induction of aerosols. The handling of clinical specimens such as sputum (e.g. for smear examination and culture inoculation) poses a lower biohazard than working with positive cultures of the bacteria (e.g. for drug susceptibility testing). 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 possible genotyping) may be carried out in a BSL2 laboratory setting. Nevertheless, the use of a well-functioning and regularly-serviced biosafety cabinet remains important when working with suspected *M. tuberculosis* material. Techniques performed on *Mycobacterium* cultures, such as the extraction of DNA from positive cultures and phenotypic drug susceptibility testing, require a higher level of biosafety and the use of a BSL3 containment laboratory. Molecular techniques involving previously extracted DNA do not require a biosafety level standard [1,3,6].

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. In 2008, the European Committee for Standardization (CEN) published the CWA 15793 Laboratory Biorisk Management Standard, which is based on a management systems approach [9]. Both CWA 15793:2008 and its latest version CWA 15793:2011 are laboratory biorisk management system agreements, establishing the requirements necessary to control risks associated with the handling or storage and disposal of biological agents and toxins in laboratories and facilities [10,11]. These standards use risk classifications described in the WHO Laboratory biosafety manual and formed the basis for development of the minimum requirements for TB diagnostic laboratories, described in the 2012 edition of the WHO Tuberculosis Laboratory biosafety manual [8].

Appropriate infection control measures are necessary to enable laboratory staff to work safely with potentially infectious microorganisms. These measures are based on the following four main components:

- administration (management)
- environment (engineering)
- personal protective equipment (PPE)
- technical expertise and training (good microbiological practices).

If correctly implemented, each of these components will help lower the risk of exposing laboratory personnel to the pathogen.

A scheme for biosafety training and registration of laboratory staff experience levels is a useful tool for reducing the risk of laboratory infections. New staff members and regular laboratory staff should periodically undergo medical examination to document any laboratory-acquired infections and to offer preventive therapy or full treatment according to national guidelines. This will also make it possible to improve procedures that may have contributed to the LAI and will ensure that management and staff continue to focus on biosafety.

The biosafety policy and guidelines should be based on national and international regulations, as well the specific local risk assessment for each stage of clinical diagnostic work. Since international recommendations might change over time and are often not very detailed, it is of great importance to always base new biosafety instructions on the most recent sources and to implement a regular update of the biosafety policy [1,6].

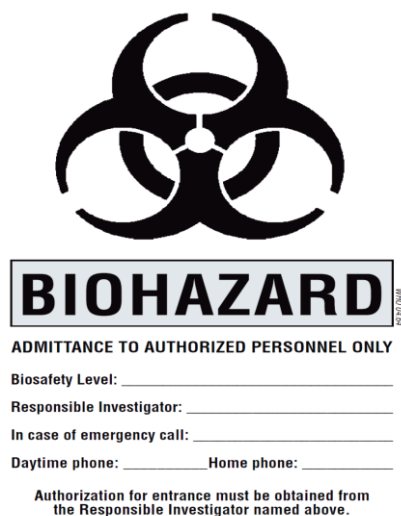
This chapter is based on available biosafety recommendations at the time of writing (September 2013), and should not be seen as a replacement of other international recommendations. The recently released WHO TB Laboratory biosafety manual [8] differs in its approach by putting less emphasis on each and every detail of a fully established BSL3 laboratory, and emphasising the importance of risk assessment in relation to the work conducted by TB laboratories at different levels. ECDC has also worked with an expert group of biosafety professionals [12], on a risk-based approach analysing work practices in TB laboratories in Europe and in 2013 it produced important recommendations for the ERLN-TB [12,13]. 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 laboratory access doors must identify the biosafety level and the name of the laboratory supervisor controlling access (Figure 1.1). Signs should also indicate the conditions for access, for example use of respirators and immunisation requirements. The best way to control access and also enable a retrospective check-up of all staff that entered the BSL3 laboratory at a particular point in time, is to use an electronic pass system. Any maintenance or technical personnel should always be accompanied by an authorised staff member and only be allowed access when the laboratory is considered relatively safe and if they are wearing personal protective equipment and the appropriate gown and shoes [1].

Figure 1.1 Biohazard warning sign for laboratory doors



Source: World Health Organization. *Laboratory biosafety manual, 3rd edition*. Geneva; 2004 [1]

Inside the laboratory, protective clothing and shoes are used in accordance with 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. When working in the biosafety cabinet, the cuffs of the gown should be covered by disposable gloves to prevent contamination of the sleeves. Laboratory protective clothing should never be worn outside the laboratory and should be decontaminated before being laundered. Potentially infectious material should always be handled in a biological safety cabinet (corresponding to specific standards – e.g. BS EN 12469:2000 or NSF/ANSI 49-2008). Respiratory protective equipment is necessary for some laboratory procedures [1].

1.3 The laboratory facility

There are many technical aspects to be considered before a BSL2 or BSL3 facility is constructed. Planning includes choice of equipment, ventilation system, technical maintenance and waste management, including the placing of the autoclave. All details of the construction should be documented and strictly follow the relevant rules and regulations at national level. If no national rules have been established, application of international rules is advised. 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 and is dependent on a risk assessment of the type of work to be carried out in the facility.

The laboratory should be separated from areas that offer unrestricted entrance to the building. If the laboratory cannot be located in a separate, dedicated building, separation may be achieved by placing the laboratory at the blind end of a corridor, or by constructing a partition and a door or only allowing access through an anteroom. The anteroom should have clearly demarcated zones, with facilities for separating clean and dirty clothing and a shower. The anteroom doors should be self-closing and should open/close in the right direction to facilitate differences in pressure. The doors should also be interlocked, so that only one door can be open at a time. Ideally, there should be an electronic delay system that only allows the opening of one of the doors when the pressure in the anteroom has been restored. Depending on its size and shape the laboratory should have a break-through panel or emergency exit for use in case of emergencies [1,3].

As a rule, the following issues should be taken into consideration [1,3]:

- Ample space should be provided around the equipment for cleaning and maintenance.
- Walls, ceilings and floors should be non-absorbent, 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 prevent leakage and facilitate decontamination of the room/s using gas.
- Bench tops should be impervious to water and resistant to disinfectants, acids, bases, organic solvents and moderate heat.
- Lighting 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 should be sealed and break-resistant.
- Storage space must be adequate to hold supplies for immediate use and thus prevent clutter on bench tops 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 the exit door.
- To contain unintended release of aerosols, a controlled ventilation system should continuously maintain the negative pressure. A visual monitoring device should be placed at the entrance and in the inside of the laboratory to enable the pressure to be checked.
- There should be an alarm to warn personnel when pressure is out of range without causing panic or undesirable reactions.
- The building ventilation system should be installed in such a way that air from the containment laboratory can never be recirculated to other sections of the building.
- Air from the BSL3 laboratory should be filtered by HEPA filters. When air extracted from the laboratory is discharged to the outside of the building, it must be dispersed away from occupied buildings and air intakes. A heating, ventilation and air-conditioning control system may be installed to prevent positive pressurisation of the laboratory.
- The HEPA filters must be installed in a manner that permits gaseous decontamination and testing. The exhausted air from biological safety cabinets, which will have been passed through HEPA filters, should be discharged so that it does not interfere with the air balance in the laboratory.
- Biological safety cabinets should be situated away from walking areas and cross-currents from doors and ventilation systems.
- There should be a programme in place for the regular testing and validation of biological safety cabinets.

- An autoclave for the decontamination of contaminated waste material should be available in the containment laboratory, ideally in the wall. In this way containers with contaminated waste can be uploaded in the BSL3 laboratory and, after the autoclave procedure, safely removed in a clean zone outside of the laboratory. In accordance with local regulations in some countries, the autoclave may be connected to the laboratory, located in the same building. In this case infectious waste must be transported in sealed, unbreakable and leak-proof containers in accordance with national or international regulations, as appropriate. These containers should preferably have vents that open when the containers are heated, so the steam can exit the containers.
- Waste water coming from the sinks installed in BSL3 should be decontaminated in kill tanks. Otherwise, if decontamination facilities are not available, sinks should not be installed.
- Safety systems should be implemented to handle the risks of fire and electrical emergencies. An emergency shower and an eyewash facility should also be installed.
- Suitably equipped and readily accessible first-aid areas or rooms should be available near the BSL3 laboratory. The personnel of a BSL3 laboratory should ideally have received first-aid training.
- A mechanical ventilation system should provide an inward flow of air without recirculation.
- There should be a reliable and adequate electricity supply and emergency lighting to permit safe exit. A back-up system is highly important (e.g. a stand-by generator and a UPS to support alarm systems and other essential equipment such as biological safety cabinets, freezers, etc.)

General requirements to the BSL3 facility are summarised in the appendix to Chapter 1.

1.4 Procedures

Based on local risk assessments, a set of detailed standard operating procedures should be developed and implemented for the safe performance of all tasks carried out in the facility. As a rule, the following procedures should be adhered to [1,6,8]:

- Pipetting by mouth must be strictly forbidden.
- All technical procedures should be performed so as to minimise the formation of aerosols and droplets. The use of any sharp objects, such as hypodermic needles and syringes, should be avoided.
- 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 action to be undertaken after any unintended release of bacteria and possible exposure of staff, including a medical surveillance programme.
- A written procedure must be developed for the cleaning-up of all spills. All staff members must be trained regularly and should have a good knowledge of, and a high level of compliance with all biosafety instructions.
- A spill emergency kit should be available with all required material to undertake immediate action in the event of a spill.
- All staff should always have an appropriate mask in their gown to put on if there is an unintended release of bacteria.
- Potentially contaminated liquids must be decontaminated (autoclaved) before being discharged into the public sewer system.

1.5 Personal protective equipment (PPE)

The use of Personal Protective Equipment (PPE) should be regulated in local standard operating procedures and be based on a local risk assessment as well as national and international regulations. It is crucial that the agreed choice of PPE is accepted, respected and followed by all staff members and visitors [1,6,8].

The following table (Table 1.1), adapted from the WHO Laboratory Biosafety Manual [1], lists examples of personal protection items for laboratory workers.

Table 1.1. Examples of personal protective equipment [1,8]

Equipment	Hazard corrected	Safety features
Laboratory coats, gowns, coveralls	Contamination of clothing	Back opening. Cover normal clothes
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 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 mycobacterial cultures.

1.6 The human resource component

Even the technically most advanced biosafety facility will fail if the laboratory staff neglects the biosafety rules. It is recommended that experienced technical staff members remain involved throughout the planning and implementation of the biosafety programme and establishment of the laboratory [1,6].

A well-designed training programme for biosafety, offered on an annual basis to all staff members, will raise the level of knowledge on the laboratory infection control plan. New staff members, as well as students and/or visiting researchers should be informed of all relevant rules and recommendations before being allowed to work in the laboratory. When they begin working in the laboratory they should never work independently, but be guided by experienced staff. It is recommended that the person responsible (often the laboratory supervisor) should inform them of all rules and regulations governing the laboratory and test their knowledge before they begin 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 and discussed extensively. This should be done in an atmosphere of cooperation to avoid staff members covering up details or becoming less inclined to report incidences/accidents in the future. A written suggestion for improvement should be prepared, describing in detail 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 a staff member claiming ignorance of the safety guidelines after an accident. There should be a clear consensus on the interpretation and application of the rules; otherwise they should be adapted so that all staff adhere to the local and international regulations.

1.7 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 [1,6,8]. Here are some examples:

- **Handling of containers with clinical specimens.** Even if it 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 therefore be handled carefully and only be opened in a biological safety cabinet.
- **Centrifugation.** Fluid may spill from centrifuge tubes or tubes may break, releasing aerosols. Only closed biosafety cups with appropriate and safe centrifugation tubes should be used. Centrifuge safety cups should therefore only be loaded/unloaded in the biological safety cabinet.
- **Pipetting.** Pipettes and Pasteur pipettes in particular are likely to generate bubbles that burst and form aerosols. Pipetting should therefore always be performed in biological safety cabinets and by using disposable pipettes. Pipetting by mouth is strictly forbidden.

- **Mechanical homogenising (vortexing, grinding, blending).** Appropriate procedures to avoid the induction of aerosols should be in place. Mechanical homogenisation should always be carried out in a biological safety cabinet and even then it still creates a risk.
- **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 directly heated in the flame of a Bunsen burner due to the risk of splashing and the creation of aerosols. Bunsen burners must not be used in a biosafety cabinet because this disturbs the flow of air. The use of disposable plastic loops is highly recommended.
- **Animal studies.** Major risks to staff are self-inoculation with material meant to infect the animals and exposure to aerosols from diseased animals. The litter of infected animals can also 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 suspected clinical samples, in particular cultured *M. tuberculosis*. These transport rules depend on arrangements within a country, between countries or with regard to air transportation. It is the responsibility of the shipping laboratory to identify the relevant regulations and follow them strictly. The use of dedicated couriers specialised in transport of BSL3 material and familiar with all regulations is highly recommended. All requested procedures must be fulfilled by the sender laboratory to ensure correct packing.
- **Storing 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. Access to the freezers should be limited to specific laboratory staff. The stock of BSL3 cultures should be registered in order to identify any illegal removal or unexplained disappearance.

References

1. World Health Organization. Laboratory biosafety manual. 3rd edition. Geneva: WHO; 2004. Available at: <http://www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf>
2. Baussano I, Williams B, Pivetta E, Bugiani M, Scano F. Tuberculosis among healthcare workers. *Emerg Infect Dis* 2011, 17:488–494.
3. Fleming DO, Hunt DL, Editors. *Biological safety: principles and practices*, 4th ed. Washington: ASM Press; 2006.
4. Collins CH, Kennedy DA. *Laboratory-acquired infections: history, incidence, causes and prevention*. 4th ed. Oxford: Butterworth-Heinemann; 1999.
5. Sewell DL. Laboratory-acquired infections: Are microbiologists at risk? *Clin Microbiol Newsl.* 2006;1:1-6.
6. US Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, National Institutes of Health. *Biosafety in microbiological and biomedical laboratories*. 5th ed. Washington: CDC/NIH; 2009. Available at: <http://www.cdc.gov/biosafety/publications/bmb15/BMBL.pdf>
7. Miller JM, Astles R, Baszler T, Chapin K, Carey R, Garcia L, et. al. Guidelines for safe work practices in human and animal medical diagnostic laboratories. Recommendations of a CDC-convened, Biosafety Blue Ribbon Panel. *MMWR Surveill Summ.* 2012 Jan 6;61 Suppl:1-102.
8. World Health Organization. *Tuberculosis laboratory biosafety manual*. Geneva: WHO; 2012. WHO/HTM/TB/2012.11. Available at: http://apps.who.int/iris/bitstream/10665/77949/1/9789241504638_eng.pdf
9. CEN. *Laboratory Biorisk Management Standard CWA 15793:2008*. Brussels; 2011. Available at: <ftp://ftp.cenorm.be/PUBLIC/CWAs/wokrshop31/CWA15793.pdf>
10. CEN. *Laboratory Biorisk Management Standard CWA 15793: 2011*. Brussels; 2011. Available at: ftp://ftp.cenorm.be/CEN/Sectors/TCandWorkshops/Workshops/CWA15793_September2011.pdf
11. CEN. *Laboratory biorisk management Standard CWA 16393- Guidelines for the implementation of CWA 15793:2008*. Brussels; 2012. Available at: <ftp://ftp.cen.eu/CEN/Sectors/List/ICT/Workshops/CWA%2016393.pdf>
12. ECDC: Biorisk Initiative for Capacity Building and Knowledge Base Development (BRICK): Available at: http://ecdc.europa.eu/en/activities/microbiology/biosafety/Documents/101117_Bioriskproject_Description.pdf
13. ECDC. *Laboratory networks and biosafety communities. How to make biosafety pan-European?* Available at: http://ecdc.europa.eu/en/publications/Publications/1108_MER_Biosafety.pdf

Appendix

The following checklist provides criteria for the planning or overhaul of BSL3 laboratories [1,6,8].

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 goes out of range, an audio-visual alert should be triggered in the anteroom and the laboratory.
12.	Air from the laboratory and the anteroom should be extracted 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 extraction system fails.
14.	The ventilating system for a BSL3 laboratory should be completely independent and separate 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 separate from the exhaust duct to prevent airflow contamination between the two ducts.
Entrance and access	
17.	The entrance to the BSL3 laboratory should be marked with a biohazard sign, information on the containment level, details of 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 should provide emergency power.
20.	If staff technicians are allowed access, they should 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 being processed and the laboratory is relatively safe after multiple air exchanges during the night and UV treatment. Equipment touched by staff technicians has to be disinfected with 80% ethanol. Maintenance personnel 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 of 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 the anteroom and the laboratory have the same ventilating system, but in fact the anteroom is a transition zone between uncontaminated and potentially contaminated areas. It is therefore recommended that the anteroom is split into 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 this 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 be 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.

Checklist for BSL3 laboratories

Biological safety cabinets

- | | |
|-----|---|
| 28. | Class I and II biological safety cabinets are acceptable. For maximum containment at the source, the installation of a Class III biological safety cabinet may be considered, although this would result in ergonomic disadvantages. |
| 29. | The biological safety cabinet should be positioned in the laboratory so that airflow would not be disturbed by personnel or open doors. A Class III biological safety cabinet is not affected by this. |
| 30. | Air extracted from a biological safety cabinet can be discharged in three ways:
1. Air can be recirculated to the room, which is not advisable because of possible biological safety cabinet filter leaks which then would introduce contaminated air to the laboratory. This is not just a hypothetical risk, especially when biological safety cabinets are not well-maintained.
2. The biological safety cabinet features a continuous airflow connection with a bypass for air treatment.
3. With a 'thimble' or 'canopy hood', extracted air can be recirculated to the room or discharged to the outside of the building via a dedicated duct or through the main extraction system.
When a biological safety cabinet is switched on, it also contributes to the negative pressure in the laboratory. However, only options 2 and 3 ensure that the negative pressure is still maintained when the safety cabinet is off. |
| 31. | Biological safety cabinets have to be tested and certified at least once a year. Between maintenance intervals safety cabinets are decontaminated by being fumigated 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 leak-proof 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 to be so low that wastewater does not have to be decontaminated. |

2 Quality assurance

Daniela Homorodean, Dimitrios Papaventsis, Tiina Kummik, Vibeke Østergaard Thomsen

Revised by Daniela Homorodean (2015)

2.1 Background and principles

National tuberculosis programmes are to 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) [1].

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. National policies regarding the quality of laboratory results constitute an essential component of national tuberculosis programmes, and provision of sustainable high-quality services is one of the key roles of any NRL. Guidance for national tuberculosis programmes and NRLs on establishing a standardised assessment system for the laboratory network focused on microscopy, was published by GLI (Global Laboratory Initiative), and proposed eleven standards for TB Microscopy Network Accreditation [2]. It is recommended that every laboratory develops, establishes and strictly follows quality assurance procedures 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). In 2012, the revised version of ISO 15189 (Medical laboratories - Requirements for quality and competence) was issued, replacing the second edition (ISO 15189:2007) [1]. 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 be based 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

ISO 17025. General requirements for the competence of testing and calibration laboratories	ISO 15189:2012 Medical laboratories - Requirements for quality and competence
<p>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.</p> <p>It is applicable to all organisations 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.</p> <p>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.</p> <p>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.</p> <p>ISO/IEC 17025:2005 is not intended to be used as the basis for certification of laboratories.' [1]</p>	<p>In this new version some chapters have been renamed and some requirements are more detailed both in management and technical requirements. There are new normative sections (Reporting and Release of results), and annexes B and C from the former became new sections (Laboratory information management and Ethical conduct, respectively).</p>

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 publication 'International vocabulary of metrology – basic and general concepts and associated terms' (VIM) [3] and the 'IUPAC Compendium of Chemical Technology – the gold book' [4]. This chapter outlines the most important requirements from ISO/IEC 17025 and ISO 15189:2012. It 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 not only test patient samples, but also offer advisory, interpretative and educational services. The full text of these standards is available for purchase online [1].

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 assessment	A process of regular performance checks to ensure that a method is performing as expected. Internal quality assessment (IQA) 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 assessment (EQA) 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 should apply international, regional, or national standards by reputable technical organisations, from scientific journals, or as specified by a kit/equipment manufacturer. 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 [6,7].

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 specification of written requirements, determination of the assay characteristics, checking that the requirements are fulfilled by the assay and a statement on the validity. For standardised methods it is only necessary to validate that the method works 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 introduction of the 'in vitro diagnostic directive' (IVD) [5] in 2003, manufacturers have been 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 a scientific understanding of the theoretical principles of the method and practical experience.

An overview of validation principles can be found in Westgard (2008) [6] and Westgard (2010) [7]. In addition, 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, the range of values has been simplified due to lack of information.

2.4 Control and trend monitoring by key indicators

Appropriate internal quality assessments (IQAs) 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 [1,2,3,5,6].

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). The NRL from each country provides guidelines for IQAs in accordance with the ISO standard requirements, the methods used and local conditions (infrastructure, workload, equipment and staff). Examples of IQAs can be found in Chapters 3 to 7.

External quality assessment (EQA) programmes are essential parts of quality assurance. Programmes of relevance for mycobacteriology laboratories are available through INSTAND e.V., UK NEQAS, US CDC, LabQuality and the WHO supranational TB reference laboratories, to which all national TB reference laboratories have links. If EQA programmes for a test are not available, exchanges with other laboratories can be arranged. A minimum of three laboratories should participate; however, EQA programmes do not replace IQA [2].

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-amplification areas) and precautions should be taken to prevent cross-contamination. Access to and use of laboratories must be controlled if this will affect the quality of tests. Measures should also be taken to ensure good housekeeping in the laboratory, thus improving biosafety (Chapter 1) [1,2,3,5,6].

2.6 Equipment

Equipment and related software must achieve the required level of accuracy and comply with specifications relevant to the tests. Procedures for handling, storage, use and maintenance should ensure the proper functioning of the equipment 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 and date of last and next recalibration [1,2,3,5,6].

2.7 Management and staff

Managerial and technical staff must be authorised to carry out tests to the required standards. All necessary resources must be available to them. Management and staff should be free of any conflicts of interest and key personnel should have appointed deputies. One person should be appointed quality officer and laboratory staff should understand that quality is everyone's responsibility. Management must document the competencies of the staff to ensure that equipment is properly handled, and that they know how to 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 [7].

2.8 Documentation and registration

Laboratories document their policies and procedures for testing and equipment use (in their local language) to ensure the quality of results. All documents must be reviewed and approved by authorised staff before being issued. They must also be read and understood by the relevant staff and revised on a regular basis. The use of expired/amended documents must be prevented. All documents should be easily accessible. Handwritten corrections and annotations should be incorporated quickly into the official version of the document. Handwritten corrections should be dated and signed with initials [1,2,3,5,6].

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 signed or initialed and dated by the staff member who corrected the information.

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

2.9 Sampling guidance, review of requests and service to customers

The laboratory provides the customer with standardised operating procedures (based on existing guidelines) for sampling that are easily accessible at the sampling location (e.g. online). The sampling guidelines 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 sub-optimal or unacceptable for testing, the results report should indicate this and/or this should be communicated to the appropriate staff members [1,2,3,5,6].

Procedures must be implemented for the review of requests, tenders and contracts for testing according to the national guidelines. For routine diagnostics, recommendations from the laboratory and the completed request form is sufficient. The laboratory will identify customer needs, including guidance and analysis of results. In the event 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 work undertaken by subcontractors is completed to meet the required standards. Examples are the calibration of equipment or second-line drug susceptibility testing of *M. tuberculosis* at another laboratory. There is a need for policies and procedures for services and supplies that directly influence diagnostic quality: procedures cover the purchase, receipt and storage of reagents, as well as compliance of supplies with standards and regulations. The laboratory is advised to 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 corrective actions have to be authorised first [1,2,3,5,6].

2.12 Improvements

Senior management should be committed to developing, implementing and improving the quality assurance system. The laboratory should revise 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 should be 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 [1,2,3,5,6].

2.13 Reporting and releasing 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 method used, a description of specimens/isolates (including sampling date), patient identification, specimen type, date of receipt, test result, release date and name of the person(s) authorising the report [1,2,6,7].

References

1. International Organization for Standardization. ISO Standards. Geneva: ISO; 2011. Available at: http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=39883 and http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=42641
2. TB Microscopy Network Accreditation – An assessment tool. First English Edition, October 2013. Available at: <http://www.stoptb.org/wg/gli/>
3. International Organization of Legal Metrology (OIML), Joint Committee for Guides in Metrology. International vocabulary of metrology – basic and general concepts and associated terms (VIM). Paris: International Organization of Legal Metrology; 2007. [Accessed 28 July 2015.] Available at: http://www.oiml.org/en/files/pdf_v/v002-200-e07.pdf/view
4. International Union of Pure and Applied Chemistry. IUPAC Compendium of chemical terminology – the gold book. Research Triangle Park, NC: IUPAC; 2005-2009 [Accessed 28 July 2015.] Available at: <http://goldbook.iupac.org>
5. Directive 98/79/EC of the European Parliament and of the Council of 27 October 1998 on *in vitro* diagnostic medical devices. Available at: <http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:31998L0079:EN:HTML>
6. Westgard, JO. Basic method validation. 3rd edition. Madison, WI: Westgard QC; 2008.
7. Westgard, JO. Tools, technologies and training for healthcare laboratories. [Accessed 28 July 2015.] Available at: <http://www.westgard.com>

3. The diagnosis of latent TB infection

Dimitrios Papaventsis and Vladyslav Nikolayevskyy

Revised by Dimitrios Papaventsis and Vladyslav Nikolayevskyy (2015)

3.1 Background and principles

Most people who are initially infected with *Mycobacterium tuberculosis* do not develop active tuberculosis. This state (when a person infected with the TB bacillus has not developed active TB) is called latent TB infection (LTBI) [1]. It is characterised by persistence and a low-rate multiplication of viable *M. 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/other immunosuppressive medications, or advanced age), reactivation of latent infection may occur [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 [5]:

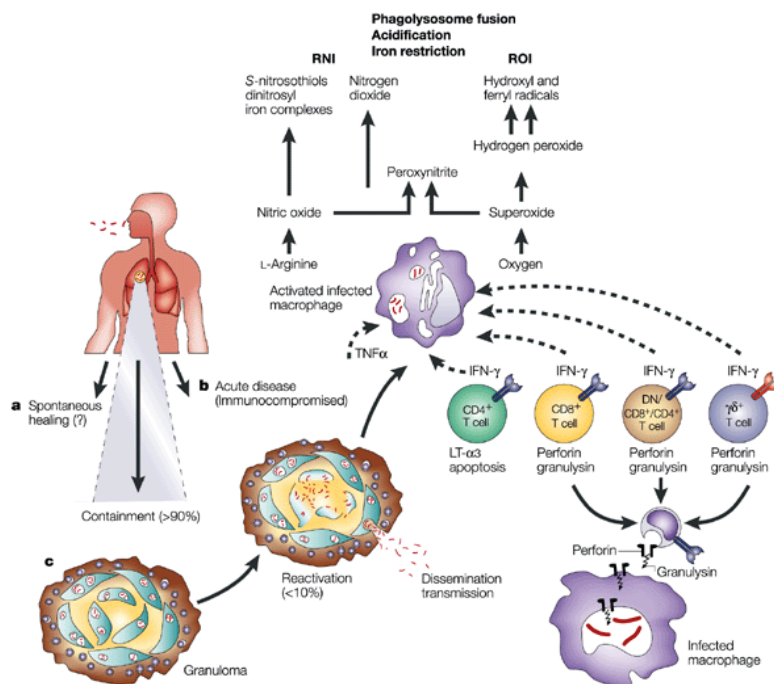
- High sensitivity in all populations at risk;
- High specificity regardless of BCG vaccination and infection with environmental mycobacteria;
- Reliability and stability over time;
- Objective criteria for positive result, affordability and easy administration;
- Ability to identify recently infected 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]. When the human host is infected by *M. tuberculosis* there are three potential outcomes (see also Figure 3.1):

- Spontaneous healing
- 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.
- 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 active TB develops. 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 human host infection by *M. tuberculosis*

Reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Immunology*, S. Kaufmann, *How can immunology contribute to the control of tuberculosis?* 2001 Oct;1 [17]

Although the host response is essential to controlling 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 intradermal 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.

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 non-tuberculous mycobacteria infection;
- technical problems in administration;
- immune response boosting after repeated TST;
- complicated and subjective interpretation; and
- a need for a second visit.

3.3 Interferon- γ release assays (IGRAs)

3.3.1 Introduction

The QuantiFERON-TB Gold (QFT-G, by Qiagen GmbH, Hilden, Germany) 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) (used only in QFT-G) are absent from all BCG strains and from most non-tuberculous mycobacteria 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. canettii) have mononuclear cells in their blood that recognise these mycobacterial antigens. This recognition process leads in vitro to the stimulation and secretion of Interferon- γ (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]. 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: greater sensitivity; higher specificity (less influenced by BCG vaccination and non-tuberculous mycobacteria infection; less influence by technical problems in administration and interpretation and the need for only one visit.

3.3.2 Current national guidelines and the clinical use of IGRAs

With the growing evidence, many national guidelines for LTBI diagnosis now include IGRAs although most countries continue to recommend and use TST. A recent review [18], based on thirty-three guidelines and policy papers from 25 countries and two international organisations, demonstrated considerable diversity in the approaches. Guidelines are predominantly available in high-income countries with established LTBI screening programmes. Four approaches are generally adopted:

- two-step approach of TST first, followed by IGRA, either when the TST is negative (to increase sensitivity, mainly in immunocompromised individuals), or when the TST is positive (to increase specificity, mainly in BCG-vaccinated individuals);
- either TST or IGRA, but not both;
- IGRA and TST together (to increase sensitivity)
- IGRA only, replacing the TST.

There is also a trend towards using IGRAs alone prior to anti-TNF- α therapy. Some guidelines are still not proposing IGRA use in children under five years of age. Most of the current guidelines do not use objective, transparent methods to grade evidence and recommendations, and rarely disclose conflicts of interests. Existing national guidelines on LTBI diagnosis in EU/EEA countries are listed in Table 3.1.

Table 3.1 List of national LTBI diagnosis guidelines

Country	Guideline
Austria	Schmidgruber B. Guidelines for the diagnosis of latent tuberculosis in canton Vienna; 2011; Gesundheitsdienst (Department of Public Health) of Vienna; Austria.
Bulgaria	Markova R. Guidelines for the diagnosis of latent tuberculosis in Bulgaria; 2011; Dept. Immunology and Allergology, National Centre of Infections and Parasitic Diseases; Sofia, Bulgaria.
Croatia	Katalinic-Jankovic V. Guidelines for the diagnosis of latent tuberculosis in Croatia; personal communications with Vera Katalinic-Jankovic. 2011; Croatia National Institute of Public Health; Zagreb, Croatia
Czech Republic	Czech Thoracic Society. Recommendation of Czech Thoracic Society for QuantiFERON-TB Gold test; 2005; Research Institute for Tuberculosis and Respiratory Diseases; Prague, Czech Republic.
Denmark	Diagnos af tuberkulose, 2011; Dansk Lungemedicinsk Selskab, Denmark
Finland	Suositus tuberkuloosin kontaktiselvityksen toteuttamiseksi, 2011; Terveyden Ja Hyvinvoinnin Laitos, Finland
France	Test de détection de la production d'interféron- γ pour le diagnostic des infections tuberculeuses, 2006; Haute Autorité de Santé, France
Germany	Neue Empfehlungen für die Umgebungsuntersuchungen bei Tuberkulose Deutsches Zentralkomitee zur Bekämpfung der Tuberkulose. New Recommendations for Contact Tracing in Tuberculosis German Central Committee against Tuberculosis. Germany
Ireland	Guidelines on the Prevention and Control of Tuberculosis in Ireland, 2010; Health Protection Surveillance Centre, Ireland
Italy	Aggiornamento Delle Raccomandazioni Per Le Attività Di Controllo Della Tuberculosis: Gestione dei contatti e della tubercolosi in ambito assistenziale, 2009; Ministero del Lavoro della Salute e delle Politiche Sociali, Italy
Netherlands	Interferon Gamma Release Assays bij de diagnostiek van tuberculose, 2011; IGRA-werkgroep Commissie voor Praktische Tuberculosebestrijding, Netherlands
Norway	Tuberkuloseveilederen som e-bok, 2011; Folkehelseinstituttet, Norway
Poland	Zalecenia postępowania w zapobieganiu i leczeniu gruźlicy u chorych leczonych antagonistami TNF- α / Recommendations for prophylaxis and management of tuberculosis in patients treated with TNF- α antagonists, 2008; Poland

Country	Guideline
Portugal	Tuberculose Latente: Projecto de expansão dos testes IGRA, 2010; Programa Nacional de Luta Contra a Tuberculose (PNT), Portugal
Slovakia	Slovakian Guidelines on Latent Tuberculosis Testing, 2010; Ministerstva Zdravonictva Slovenskej Republiky, Slovakia
Spain	Consensus Document on the Diagnosis, Treatment and Prevention of Tuberculosis, 2010; Spanish Society of Pneumology and Thoracic Surgery (SEPAR), Spain
Switzerland	Handbuch Tuberkulose (Rohfassung), 2011; Kompetenzzentrum Tuberkulose, Switzerland
United Kingdom	Tuberculosis: NICE Guideline, 2011; NICE, UK

Information included in the different national guidelines and recommendations suggests that IGRAs are increasingly being recommended, primarily in low-incidence settings, as they offer a higher specificity combined with logistical advantages [19]. TST is still favoured in high-incidence and low-resource settings.

In general, evidence suggests that screening for LTBI (using both TST and IGRA) should ideally be confined to those who are at sufficiently high risk of progressing to disease and who will benefit from chemoprophylaxis should they test positive.

The clinical use of IGRAs in different groups has been recently reviewed and policy papers have been published by international organisations including WHO and ECDC [20-23]. WHO generally discourages use of IGRAs and recommends using TST, but only in low- and middle-income settings, regardless of HIV status. The ECDC approach is based on TB incidence. In high-incidence settings, the ECDC suggests not to use IGRAs to diagnose LTBI since the focus of prevention and control is on identifying and treating active TB cases. In low-incidence settings a two-step approach is suggested. For active TB diagnosis, ECDC suggests that IGRAs should not be a replacement for standard diagnostic methods and generally do not have an added value in most clinical situations, when combined with standard methods for diagnosing active TB. However, in certain clinical situations (e.g. patients with extrapulmonary TB, patients who test negative for acid-fast bacilli in sputum and/or negative for *M. tuberculosis* after culture, TB diagnosis in children, or in the differential diagnosis of infection with non-tuberculous mycobacteria), ECDC suggests that IGRAs could contribute supplementary information as part of the diagnostic process and laboratory management. Overall, the contact tracing practices in adults appear to suggest a clear trend towards an increased use of IGRAs, especially in low-incidence countries, mostly as a two-step strategy [18].

3.3.3 Predictive value of IGRA for progression to active TB

Existing evidence suggests that both TST and IGRA are acceptable but imperfect tests and neither test can accurately differentiate between LTBI and active TB, distinguish reactivation from reinfection, or accurately predict progression from LTBI to active disease [19]. In a recent meta-analysis [24] based on a combined sample size of 26 680 individuals and data derived from 15 longitudinal studies it was concluded that neither IGRA nor TST accurately predict the risk of developing active TB, although use of IGRAs in certain groups might reduce the number of people considered for chemoprophylaxis.

Overall, evidence currently available suggests that the predictive value of IGRAs for progression to active TB disease is low and only marginally (non-significantly) higher than that of the TST [19]. There is also limited evidence suggesting that IGRA conversion detected using multiple testing may have a greater predictive value than single IGRA results as it may indicate recent infection [25]. With regard to high-risk populations (e.g. HIV-infected individuals) there are currently no data suggesting that IGRAs are better in predicting active TB in this group than the TST [19,26].

To conclude, evidence available to date suggests that both TST and IGRA have limited predictive values and their usefulness is restricted to identification of those who would potentially benefit from preventive therapy. Current and future studies will help establish the place and role of IGRAs in TB clinical and laboratory management and potentially identify novel, highly-predictive biomarkers.

Existing evidence suggests that both TST and IGRA are acceptable but imperfect tests and neither of the tests can accurately differentiate between LTBI and active TB; distinguish reactivation from reinfection, or accurately predict progression from LTBI to active disease [19]. In a recent meta-analysis [24] based on a combined sample size of 26 680 individuals and data derived from 15 longitudinal studies, it was concluded that neither IGRA nor TST accurately predict the risk of developing active TB, although use of IGRAs in certain groups might reduce the number of people considered for chemoprophylaxis.

Overall, current evidence suggests that the value of IGRAs for predicting progression to active TB disease is low and only marginally (non-significantly) higher than that of the TST [19]. There is also limited evidence suggesting that IGRA conversion detected using multiple testing may have a greater predictive value than single IGRA results as it may indicate recent infection [25]. With regard to high-risk populations (e.g. HIV-infected individuals) there are currently no data suggesting that IGRAs are better in predicting active TB in this group than the TST [19,26].

To conclude, evidence available to date suggests that both TST and IGRA have limited predictive values and their usefulness is restricted to identification of those who would potentially benefit from preventive therapy. Current and future studies will help establish the place and role of IGRAs in TB clinical and laboratory management and potentially identify novel, highly predictive biomarkers.

3.3.4 IGRA performance and reproducibility

Performance characteristics (sensitivity and specificity) of the two IGRAs currently available on the market (QFT-G and the T-SPOT) have recently been extensively reviewed [19,27,28]. In the absence of a gold standard, surrogate markers are used to estimate performance characteristics. Specificity of both assays (>95%) is not affected by BCG vaccination and is similar to that of the TST in non BCG-vaccinated individuals; in populations where BCG vaccination is administered, specificity of TST is significantly lower (60%). Existing evidence suggests that sensitivity of T-SPOT is slightly higher (~90%) than that of QFT-G (~80%) and is usually lower in children and immunocompromised individuals.

IGRAs have certain reproducibility issues and variability can be due to natural sources (immunomodulation and functionality of T-cells), manufacturing issues, pre-analytical and analytical deviations. This may require the reconsideration of cut-off values and the introduction of borderline zones (especially for the QFT-G assay) which might help to improve the reproducibility and diagnostic value of the assays [19].

3.3.5 Procedure 1: QuantiFERON-TB Gold and QuantiFERON-TB Gold PLUS

The QuantiFERON-TB Gold (QFT-G) and QuantiFERON-TB Gold PLUS (QFT-GP) made by Qiagen GmbH, Hilden, Germany)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 for QFT-G and nil control, two antigen tubes, and a mitogen tube for QFT-GP), 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 adding normal mouse serum to the green diluent and using F(ab')₂ monoclonal antibody fragments as the IFN-γ capture antibody coated to the microplate wells.

Baseline epidemiological data

Before performing the QuantiFERON-TB Gold IT test, baseline epidemiological data should be recorded: name, full address, contact information, 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 chest X-ray (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).

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.

Materials provided by the manufacturer

Blood collection tubes

QFT-G:

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

¹ Descriptions of laboratory procedures are based on the manufacturer's recommendations (Qiagen) and international safety, quality control and laboratory management regulations. QuantiFERON-TB Gold package insert available from: <http://www.quantiferon.com/irm/content/PI/QFT/PLUS/UK.pdf>

QFT-GP:

- Nil control (grey cap with white ring)
- TB 1 antigen (green cap with white ring)
- TB 2 antigen (yellow cap 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 H₂O₂, 3,3',5,5' tetramethylbenzidine);
- Enzyme stopping solution (contains 0.5M H₂SO₄).

Required materials (not provided)

- 37°C \pm 1°C incubator (with or without CO₂);
- Calibrated variable-volume pipettes for delivery of 10 μ l to 1000 μ l with disposable tips;
- Calibrated multichannel pipette capable of delivering 50 μ l and 100 μ l 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: 2 litres;
- Microplate washer (for safety reasons, an automated washer is recommended);
- Microplate reader fitted with 450 nm filter and 620nm to 650 nm reference filter;
- Variable speed vortex;
- Timer;
- Measuring cylinder: 1 litre or 2 litre;
- Reagent reservoirs.

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 on which 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.

Methods**Sample collection and handling**

The contents of the tubes should be thoroughly mixed with the blood. Incubation at 37°C \pm 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 1 ml 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 that another blood sample be obtained. 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 1 ml transferred to each of the three tubes, ensuring appropriate safety procedures. Alternatively, blood may be collected in a single generic blood collection tube and then transferred to QFT tubes. The generic collection tube must only contain lithium heparin as an anticoagulant, other anticoagulants such as EDTA may interfere with the assay.

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 \pm 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

The tubes will have to be re-mixed if the blood is not incubated immediately after collection.

Incubate the tubes upright at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ 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

Before use, plasma samples and reagents, except for conjugate 100x concentrate, must be brought to room temperature ($22^{\circ}\text{C} \pm 5^{\circ}\text{C}$). Allow at least 60 minutes for equilibration.

Allow at least two strips on the ELISA frame 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.

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.

Reconstitute dried conjugate 100x concentrate with 0.3 ml 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 the package insert. Working strength conjugate should be used within six hours of preparation.

Prior to assay, mix plasmas thoroughly.

Add 50 μl of freshly prepared working strength conjugate to each ELISA well.

Add 50 μl of test plasma samples to appropriate wells. Add 50 μl each of the standards 1 to 4. The standards should be assayed at least in duplicate (triplicate preferred).

Mix the conjugate and plasma samples/standards thoroughly using a microplate shaker for one minute at 500 to 1 000 rpm.

Cover each plate and incubate at room temperature ($22^{\circ}\text{C} \pm 5^{\circ}\text{C}$) for 120 ± 5 minutes. Plates should not be exposed to direct sunlight during incubation. Deviation from specified temperature range can lead to erroneous results.

During incubation, dilute one part wash buffer 20x concentrate with 19 parts deionised or distilled water and mix thoroughly. Wash wells with 400 μl 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.

Tap the plates face down on absorbent towel to remove residual wash buffer. Add 100 μl of enzyme substrate solution to each well and mix for one minute at 500 to 1 000 rpm using a microplate shaker.

Cover each plate with a lid and incubate at room temperature ($22^{\circ}\text{C} \pm 5^{\circ}\text{C}$) for 30 minutes. Plates should not be exposed to direct sunlight during incubation.

Following the 30-minute incubation, add 50 μl 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.

Measure the optical density (OD) of each well within five minutes of stopping the reaction using a microplate reader fitted with a 450 nm filter and with a 620 nm to 650 nm reference filter. OD values are used to calculate results.

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 epidemiological, historical, 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. QuantiFERON-TB Gold IT results are interpreted using the following criteria (Tables 3.2 and 3.3).

Table 3.2 QuantiFERON-TB Gold 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

Table 3.3 QuantiFERON-TB Gold PLUS results interpretation

Nil (IU/ml)	TB1 minus Nil or TB2 minus Nil (IU/ml)	Mitogen minus Nil (IU/ml)*	QFT-Plus Result	Report/interpretation
≤8.0	≥0.35 and ≥ 25% of Nil	Any	Positive	<i>M. tuberculosis</i> infection likely
≤8.0	<0.35	≥0.5	Negative	<i>M. tuberculosis</i> infection NOT likely
≤8.0	≥0.35 and <25% of Nil	≥0.5	Negative	<i>M. tuberculosis</i> infection NOT likely
≤8.0	<0.35	<0.5	Indeterminate	Results are indeterminate for TB-antigen responsiveness
≤8.0	≥0.35 and <25% of Nil	<0.5	Indeterminate	Results are indeterminate for TB-antigen responsiveness
>8.0	Any	Any	Indeterminate	Results are indeterminate for TB-antigen responsiveness

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. Please note that responses to the mitogen positive control (and occasionally TB antigen) can be outside the range of the microplate reader. This has no impact on test results.

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 $\leq 15\%$;
- 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)

Extensive quality management, including both internal and external quality assurance, is a keystone of TB laboratory diagnosis and is essential for the laboratory accreditation. The UK National External Quality Assessment Service (UK NEQAS, www.ukneqas.org.uk) has recently established an EQA scheme for QuantiFERON-TB Gold tests, which is also available for customers outside the UK. Qiagen offers the QFT-G test panel (Cat No 0594-0805), comprising three sets of interferon-gamma (IFN- γ) controls made up of recombinant human IFN- γ . Each complete set consists of three individual controls representing different IFN- γ concentration levels (levels 1, 2, and 3) within the linear range of QuantiFERON assays. New schemes are being developed based on previous experience [15,16] and should be established as soon as possible.

3.3.6 Procedure 2: T-SPOT procedure²

General principles

T-SPOT (Oxford Immunotec, Abingdon, UK), unlike QuantiFERON-TB Gold, uses an enzyme-linked immunospot (ELISPOT) technique based on enumeration of activated specific T-cells responding to stimulation by specific antigens (ESAT-6 and CFP10) and 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 at 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.

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 TST, 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).

Safety

This diagnostic procedure involves the handling of human blood samples and plasma, potentially infected with blood-borne 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 standard operating procedures should be developed and regularly updated.

² 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, it is extremely important to use an aseptic technique in order to avoid contamination of reagents, wells, cell cultures, and nourishing media;
- Blood should be progressed into the assay within eight hours of 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.

Materials

Provided by the manufacturer with the kits (Table 3.4):

Table 3.4 Materials provided by the manufacturer

	T-SPOT TB 96 kit	T-SPOT TB 8 kit
1	One microtitre plate: 96 wells coated with a mouse monoclonal antibody to IFN- γ .	One microtitre plate: 96 wells, supplied as 12x8-well strips in a frame, coated with a mouse monoclonal antibody to IFN- γ
2	Two vials (0.7 ml each) Panel A: contains ESAT-6 antigens	Two vials (0.8 ml each) Panel A: contains ESAT-6 antigens
3	Two vials (0.7 ml each) Panel B: contains CFP10 antigens	Two vials (0.8 ml each) Panel B: contains CFP10 antigens
4	Two vials (0.7 ml each) Positive control: contains phytohaemagglutinin (PHA)	Two vials (0.8 ml each) Positive control: contains phytohaemagglutinin (PHA)
5	One vial (50 μ l) 200x concentrated conjugate reagent: mouse monoclonal antibody to IFN- γ conjugated to alkaline phosphatase.	One vial (50 μ l) 200x concentrated conjugate reagent: mouse monoclonal antibody to IFN- γ conjugated to alkaline phosphatase
6	One bottle (25 ml) substrate solution: ready to use BCIP/NBT (plus) solution.	One bottle (25 ml) 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 μ l 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 \pm 1°C with a 5% CO₂ 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 8 ml or two 4 ml CPT tubes or one 6 ml lithium heparin tube;
- Children 2–9 years old: one 4 ml CPT or lithium heparin tube;
- Children up to 2 years old: one 2 ml paediatric tube.

After collection, blood should be stored at room temperature (no refrigeration or freezing) and assayed within eight hours. This time period can be prolonged to 32 hours if T-Cell *Xtend* is used. The T-Cell *Xtend* reagent should be added prior to PBMC separation using standard separation techniques. Whole blood samples should be stored at room temperature (18–25 °C) between 23 and 30 hours post venipuncture with the use of T-Cell *Xtend* reagent.

If the T-Cell *Xtend* reagent is to be used, immediately before cell separation remove the cap from the blood collection tube and add 25 µl of the T-Cell *Xtend* reagent solution per ml of blood sample.

Replace the cap and invert the blood collection tube gently eight to ten times to mix. Incubate for 20 ± 5 minutes at room temperature (18–25°C) and then proceed to isolate the PBMC layer using Ficoll density gradient centrifugation.

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. Leucosep tubes are now validated for use with the T-SPOT.TB assay, and can be used with T-Cell *Xtend* simplifying Ficoll preparation of peripheral blood mononuclear cells (PBMCs). The Leucosep tube eliminates the time-consuming and laborious layering of the sample material over FICOLL-PAQUE PLUS. For details of the specimen preparation procedures involving T-Cell *Xtend* please refer to the T-SPOT TB Technical handbook available at <http://www.oxfordimmunotec.com/UK%20Technical%20Handbook>.

CPT tubes (with gel plug)

- Centrifuge 8 ml CPT tubes at 1600xg for 28 minutes or 4 ml CPT tubes at 1800xg for 30 minutes at 18°C if 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 15 ml conical centrifuge tube. Make up the volume to 10 ml 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 15 ml conical centrifuge tube. Make up the volume to 10 ml with cell culture medium AIM V or RPMI 1640;
- Centrifuge at 600xg for seven minutes. Pour off the supernatant and re-suspend the pellet in 1 ml AIM V or RPMI medium;
- Make up the volume to 10 ml with fresh AIM V or RPMI medium and centrifuge at 350g for seven minutes;
- Pour off the supernatant and resuspend the pellet in 0.7 ml AIM V culture medium.

Cell counting and dilution

The T-SPOT.TB assay requires 2.5 x 10⁵ 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 10 µl of the final cell suspension to 40 µl 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 500 µl of the final cell suspension at a concentration of 2.5x10⁵ cells/100 µl. 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 50 µl AIM V culture medium to each nil control well;
 - Add 50 µl Panel A solution to each well required;
 - Add 50 µl Panel B solution to each well required;
 - Add 50 µl positive control solution to each positive control well.
- To each of the four wells to be used for a patient sample, add 100 µl of the patient's final cell suspension (containing 250 000 viable cells).
- Incubate the plate in a humidified incubator at 37°C with 5% CO₂ 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 200 µl 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 50 µl working strength conjugate reagent solution to each well and incubate at 2–8°C for one hour.
6. Discard the conjugate and perform four PBS washes as described in steps 2 and 3 above.
7. Add 50 µl 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 highest 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 epidemiological information should be used to help determine TB infection status of the patient.

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*

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 with local and governmental regulations. This can 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 T-SPOT TB tests. These should be established and implemented as soon as possible based on previous experience.

References

1. Tufariello JM, Chan J, Flynn JL. Latent tuberculosis: mechanisms of host and bacillus that contribute to persistent infection. *Lancet Infect Dis*. 2003 Sep;3(9):578-90.
2. Dye C, Scheele S, Dolin P, Pathania V, Raviglione MC. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. *JAMA*. 1999 Aug 18;282(7):677-86.
3. Styblo K. The relationship between the risk of tuberculosis infection and the risk of developing infectious tuberculosis. *Bull Int Union Tuberc*. 1985;60:117-9.
4. Flynn JL, Chan J. Immunology of tuberculosis. *Annu Rev Immunol*. 2001;19(1):93-129.
5. World Health Organization; Stop TB Partnership. The Stop TB Strategy. Geneva: World Health Organization; 2006. Available at: http://whqlibdoc.who.int/hq/2006/WHO_HTM_STB_2006.368_eng.pdf
6. Lee E, Holzman RS. Evolution and current use of the tuberculin test. *Clin Infect Dis*. 2002 Feb 1;34(3):365-70.
7. Harboe M, Oettinger T, Wiker HG, Rosenkrands I, Andersen P. Evidence for occurrence of the ESAT-6 protein in *Mycobacterium tuberculosis* and virulent *Mycobacterium bovis* and for its absence in *Mycobacterium bovis* BCG. *Infect Immun*. 1996 Jan;64(1):16-22.
8. Behr MA. Comparative genomics of BCG vaccines. *Tuberculosis (Edinb)*. 2001;81(1-2):165-8.
9. Andersen P, Munk ME, Pollock JM, Doherty TM. Specific immune-based diagnosis of tuberculosis. *Lancet*. 2000 Sep 23;356(9235):1099-104.
10. Andersen P, Doherty TM, Pai M, Weldingh K. The prognosis of latent tuberculosis: can disease be predicted? *Trends Mol Med*. 2007 May;13(5):175-82.
11. Pai M. Guidelines on IGRAs: Concordant or discordant? 2nd Global Symposium on IGRAs, May 30–June 1, 2009. Dubrovnik, Croatia.
12. Nyendak MR, Lewinsohn DA, Lewinsohn DM. New diagnostic methods for tuberculosis. *Curr Opin Infect Dis*. 2009 Apr;22(2):174-82.
13. Mazurek GH, Jereb J, Lobue P, Iademarco MF, Metchock B, Vernon A. Guidelines for using the QuantiFERON-TB Gold test for detecting *Mycobacterium tuberculosis* infection, United States. *MMWR Recomm Rep*. 2005 Dec 16;54(RR-15):49-55. Erratum in: *MMWR Morb Mortal Wkly Rep*. 2005 Dec 23;54(50):1288.

14. National Collaborating Centre for Clinical Conditions. Royal College of Physicians. Tuberculosis: clinical diagnosis and management of tuberculosis, and measures for its prevention and control. London: Royal College of Physicians; 2006. Available at: <http://www.nice.org.uk/nicemedia/live/10980/30020/30020.pdf>
15. Westgard JO, Barry PL, Hunt MR, Groth T. A multi-rule Shewhart chart for quality control in clinical chemistry. *Clin Chem*. 1981 Mar;27(3):493-501.
16. Hang NT, Ishizuka N, Keicho N, Hong LT, Tam DB, Thu VT, et al. Quality assessment of an interferon-gamma release assay for tuberculosis infection in a resource-limited setting. *BMC Infect Dis*. 2009 May 18;9:66.
17. Kaufmann SH. How can immunology contribute to the control of tuberculosis? *Nat Rev Immunol*. 2001 Oct;1(1):20-30.
18. Denkinger CM, Dheda K, Pai M. Guidelines on interferon- γ release assays for tuberculosis infection: concordance, discordance or confusion? *Clin Microbiol Infect*. 2011 Jun;17(6):806-14
19. Pai M, Denkinger CM, Kik SV, Rangaka MX, Zwerling A, Oxlade O, et al. Gamma interferon release assays for detection of *Mycobacterium tuberculosis* infection. *Clin Microbiol Rev*. 2014 Jan;27(1):3-20
20. European Center for Disease Prevention and Control. Use of interferon-gamma release assays in support of TB diagnosis. Stockholm. 2011. Available at: http://ecdc.europa.eu/en/publications/Publications/1103_GUI_IGRA.pdf
21. World Health Organization. Guidelines for intensified tuberculosis case-finding and isoniazid preventive therapy for people living with HIV in resource-constrained settings. Geneva, 2011. Available at: http://whqlibdoc.who.int/publications/2011/9789241500708_eng.pdf
22. World Health Organization. Use of tuberculosis interferon-gamma release assays (IGRAs) in low-and middle-income countries: policy statement. Available at: http://www.who.int/tb/features_archive/policy_statement_igra_oct2011.pdf
23. World Health Organization. Guidelines on the management of latent tuberculosis infection. Available at: http://apps.who.int/iris/bitstream/10665/136471/1/9789241548908_eng.pdf?ua=1&ua=1
24. Rangaka MX, Wilkinson KA, Glynn JR, Ling D, Menzies D, Mwansa-Kambafwile J.. Predictive value of interferon-gamma release assays for incident active tuberculosis: a systematic review and meta-analysis. *Lancet Infect. Dis*. 2012;12:45–55
25. Machingaidze S, Verver S, Mulenga H, Abrahams DA, Hatherill M, Hanekom W, et al. Predictive value of recent QuantiFERON conversion for tuberculosis disease in adolescents. *Am. J. Respir. Crit. Care Med*. 2012;186:1051–1056.
26. Kim YJ, Kim SI, Kim YR, Wie SH, Park YJ, Kang MW.. Predictive value of interferon-gamma ELISPOT assay in HIV 1-infected patients in an intermediate tuberculosis-endemic area. *AIDS Res. Hum. Retroviruses* 2012; 28:1038–1043
27. Cattamanchi A, Smith R, Steingart KR, Metcalfe JZ, Date A, Coleman C, Marston BJ, Huang L, Hopewell PC, Pai M. Interferongamma release assays for the diagnosis of latent tuberculosis infection in HIV-infected individuals—a systematic review and meta-analysis. *J. Acquir. Immune Defic. Syndr*. 2011;56:230–238
28. Diel R, Loddenkemper R, Nienhaus A. Predictive value of interferon-gamma release assays and tuberculin skin testing for progression from latent TB infection to disease state: a meta-analysis. *Chest* 2012;142:63–75.

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 a BCG vaccination? Yes No Don't know
- If yes, please specify age: _____
- BCG scar: Yes No
- TB skin 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 Katalinić-Janković, Daniela Cirillo

Revised by Emanuele Borroni and Enrico Tortoli, 2015

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 conditions, these are not easily decolourised, 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 to detect 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. Kinyoun staining is not as effective as 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 less time is required to screen the slides when compared to Kinyoun or ZN staining because slides are screened at lower magnification [6].

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) and the high number of bacilli required for positivity (in the range of 5×10^3 – 10^4 bacilli per ml). Sensitivity and the positive predictive value (PPV) of smear microscopy are 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 smear preparation, staining and reading process. 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 lower numbers of AFB.

4.2 Procedure 1: Ziehl-Neelsen (ZN)

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 for detecting 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 ($\geq 95\%$);
 - 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 basic fuchsin content should represent 85–88% of the 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 $3\text{ g}/0.75 = 4$ grams will be weighed for the 0.3% stain. If 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 the 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 50 g of phenol and swirl the flask until it is dissolved.
- Add 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 continuing to swirl.

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-litre 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-litre conical flask.
- Measure 30 ml of concentrated hydrochloric acid into 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 3g 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 of 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, with every batch clearly identified by the name of the reagent and the preparation date on the bottle labels. Perform quality control by using one or more freshly-prepared staining reagents, carrying out the usual staining procedure as described for positive controls. Test the performance of cabol-fuchsin by staining and examining two scanty or 1+ smears stained once, and two negative smears stained three times [2].

4.3 Procedure 2: Fluorochrome staining

4.3.1 Quality control of freshly-prepared staining reagents

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

Dissolve phenol crystals in water.

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. When left standing turbidity may develop 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 are needed.

C. Counterstains

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

Potassium permanganate	
Potassium permanganate (KMnO ₄)	0.5 g
Distilled water	100 ml

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 ₂ HPO 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 three months.

4.3.2 Safety measures

Never add water to acid! To reduce exposure to toxic phenolic fumes, reagents and staining solution containing phenol should be prepared 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 the event 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, at least two specimens should be collected and processed for mycobacterial microscopy and culture [7,15].

Country guidelines will provide information on 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 leak-proof 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 in accordance with 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, preferably in open spaces where possible, or in rooms with negative pressure and adequate air exchange [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 [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 and water-like, 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. Blood-streaked sputum is suitable, but pure blood should not be examined [1,2].

4.5 Smear preparation

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

- Smears should be prepared using new, clean, grease-free and unscratched slides. Using a pencil, 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.
- If smear is prepared directly from a fresh sample (without prior centrifugation) use an applicator stick or wire/disposable loop, select and pick up the yellowish purulent particles of sputum. For re-suspended pellets (after the centrifugation) a disposable loop is advisable.
- 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, press the stick firmly perpendicular to the slide and move in small concentric circles or coil-like patterns.
- Place the used stick in 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) one or two drops of sediment should be smeared on the slide.
- Allow the smear to air-dry completely at room temperature within the biological safety cabinet.
- 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 biological safety cabinet.

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 loop with an inner diameter of 3 mm to spread sputum on the slide
Microscope slide (grease-free and unscratched)
Marking pen to put the identification number on the microscopy slide
Forceps to hold smear slide
Bunsen burner to fix the smear slide and flame the smear during staining
Staining rack to hold the smear slide
Slide rack in which to place stained smear slide for air-drying

4.6 Staining procedures

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 vapour 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 wall.
- 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 methylene blue counterstain 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 it to quench 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.

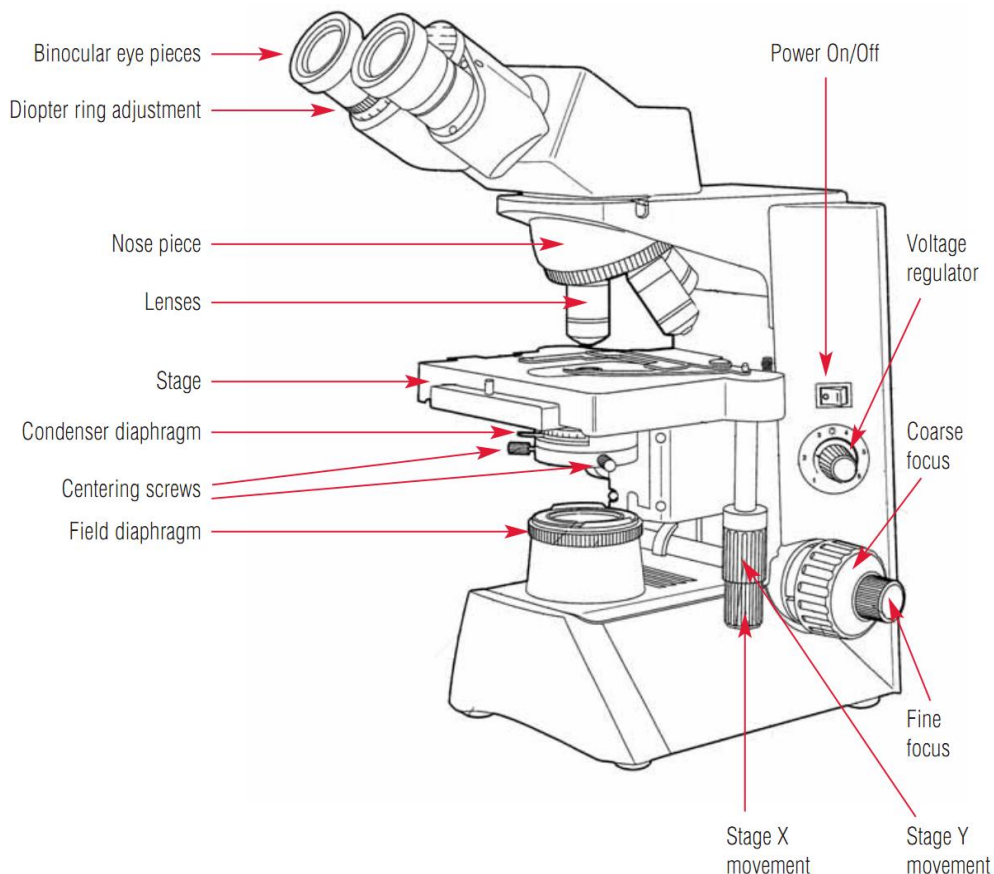
4.6.3 Automatic staining

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 and fluorochrome staining. Accurate and appropriate maintenance after each staining session is required to maintain consistent, high-quality staining.

4.7 Microscopy

Figure 4.1 shows the requirements of a microscope for smear examination.

Figure 4.1 Microscope components



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

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 the 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 has 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. Moreover, 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 over 10 000 hours), has low power requirements, and can be easily introduced in microscopy centres, including peripheral facilities. In addition, light intensity can easily be regulated.

Since LED-based microscopy has been acknowledged as an important development in direct fluorescence microscopy, 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 are of importance: training requirements, validation during the introductory phase, monitoring of trends in case detection and treatment outcomes. Adapted systems may need to 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)	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)
Result			
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 in at least 50 fields	10–100 AFB/1 field on average	5–50 AFB/1 field on average
3+	>10 AFB/1 HPF in at least 20 fields	>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.

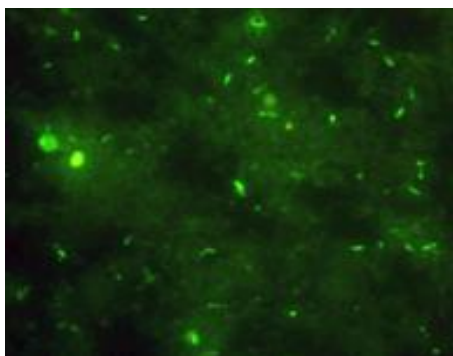
Negative report: Negative for acid-fast bacilli where no organisms observed in 100 fields.

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

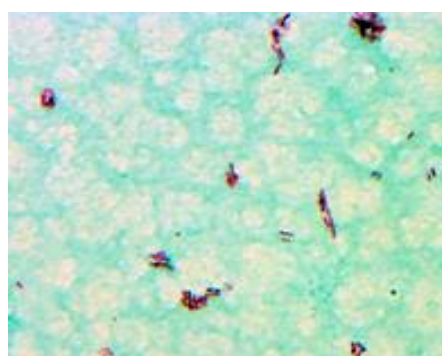
The results should be recorded in the TB laboratory register, and recorded on the sample examination request form as well as forwarded to the person requesting the sample examination [10].

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

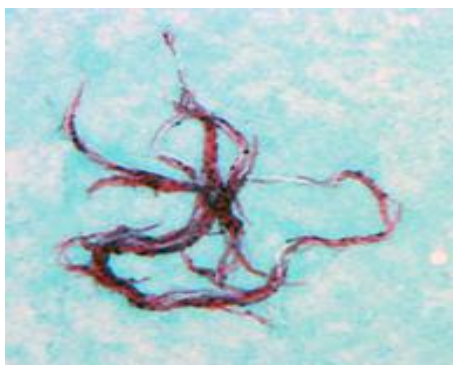


(a) auramine stain

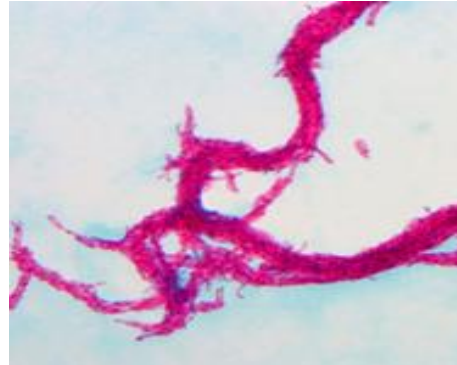


(b) Ziehl-Neelsen stain

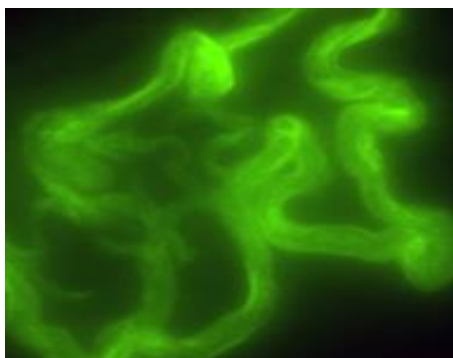
Figure 4.3 AFB smear microscopy of *M. tuberculosis* cultures (images show cords)



(c) Ziehl-Neelsen stain



(d) Ziehl Neelsen stain



(e) auramine stain

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 Institute, Warsaw Poland.

4.9 Quality control

4.9.1 Quality control parameters

Quality control in microscopy is a process for internally monitoring the performance of bench work in the laboratory. It consists of 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 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 error in smear microscopy. Control slides should be assessed prior to reading the patient smears to confirm the correctness of staining. If quality control slides are acceptable, patient smears can be read and reported. If the control slide(s) are unacceptable, the procedures and reagent preparations should be reviewed. 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 error in smear microscopy

Errors	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 or make smear in 1x2 cm area only
	Poor staining	Check quality control of reagents, prepare new reagents, check dilution.
False positive	Cross-contamination	Avoid contact between slides during staining procedure, do not use staining jars. Clean objective lens after reading each slide. Check water/solutions for environmental contamination.
	Red precipitates	Prepare new solution. Filter before use.

References

1. Kent PT, Kubica GP. Public health mycobacteriology: a guide for the level III laboratory. Atlanta, GA: United States Department of Health and Human Services, Centers for Disease Control;1985.
2. World Health Organization. Laboratory services in tuberculosis control. Part II: Microscopy. Geneva: World Health Organization; 1998. Available at: [http://whqlibdoc.who.int/hq/1998/WHO_TB_98.258_\(part2\).pdf](http://whqlibdoc.who.int/hq/1998/WHO_TB_98.258_(part2).pdf) (WHO/TB/98.258).
3. World Health Organization. Maintenance and repair of laboratory, diagnostic imaging and hospital equipment. Geneva: World Health Organization; 1994. Available at: <http://whqlibdoc.who.int/publications/1994/9241544635.pdf>
4. World Health Organization. Maintenance manual for laboratory equipment. 2nd ed. Geneva: World Health Organization; 2008. Available at: www.who.int/entity/diagnostics_laboratory/documents/guidance/guidance2/en/.
5. World Health Organization. Manual of basic techniques for a health laboratory, 2nd ed. Geneva: World Health Organization; 2003.
6. Rieder HL, Van Deun A, Kam KM, Kim SJ, Chonde TM, Trébuq A, et al. Priorities for tuberculosis bacteriology services in low-income countries. 2nd ed. Paris: International Union against Tuberculosis and Lung Disease; 2007.
7. Centers for Disease Control [homepage on the internet]. Atlanta, Georgia, USA: CDC. Acid-fast direct smear microscopy training package [cited 14 February 2011]. Available at: <http://stacks.cdc.gov/view/cdc/31282>
8. Health Protection Agency, Standards Unit, Evaluations and Standards Laboratory. Investigation of specimens for *Mycobacterium* species. London: Health Protection Agency; 2006. Available at: http://www.hpa-standardmethods.org.uk/pdf_sops.asp.

9. Collins C, Grange J, Yates M. Organization and practice in tuberculosis bacteriology. London: Butterworths; 1985.
10. World Health Organization. Revised TB recording and reporting forms and registers – version 2006. Geneva: World Health Organization; 2006. Available at: http://www.who.int/tb/dots/r_and_r_forms/en. (WHO/HTM/TB.2006.373)
11. World Health Organization: Fluorescent Light Emitting Diode (LED) microscopy for diagnosis of tuberculosis: WHO policy statement. Geneva: World Health Organization; 2010. Available at: <http://www.stoptb.org/wg/gli/assets/documents/WHO%20Policy%20Statement%20on%20LED%20Microscopy%20FINAL%20March%202010.pdf>
12. World Health Organization. A roadmap for ensuring quality tuberculosis diagnostics services within national laboratory strategic plans. The Global Laboratory Initiative, Advancing TB Diagnosis. 2010. Available at: http://www.tbvidence.org/documents/rescentre/books/GLI_Roadmap_2010.pdf
13. Stop TB Partnership and World Health Organization. New Laboratory Diagnostic Tools for TB Control. Geneva, World Health Organization, 2008. Available at: <http://apps.who.int/tdr/publications/non-tdr-publications/diagnostic-tool-tb/pdf/diagnostic-tool-tb.pdf>
14. Isenberg HD. Clinical microbiology procedures handbook. Washington: ASM Press; 1992
15. Cirillo D, Piana F, Vincent V, Angra P, Barrera L, Boulahbal F, et al. Training package on culture of acid-fast bacilli and drug-susceptibility testing of *Mycobacterium tuberculosis*. In: Tuberculosis Control Assistance Program. TB CAP Toolbox – TB CAP Laboratory Tools [CD ROM]. n.p.; 2009. Available at: http://www.tbcta.org/Uploaded_files/Zelf/NewLabTools1297631626.zip
16. Vincent V, van Deun A, Feldmann K, Barrera L, Paramasivan CN, Weyer K, et al. Guidance for countries on the preparation and implementation of TB laboratory standard operating procedures (WHO SOPs). In: Tuberculosis Control Assistance Program. TB CAP Toolbox – TB CAP Laboratory Tools 2009 [CD ROM];
17. Lumb R, Bastian I. Laboratory diagnosis of tuberculosis by sputum microscopy. Adelaide: Institute of Medical and Veterinary Science; 2005. p. 38.

5. Culture tests for *Mycobacterium tuberculosis* complex

Susana David, Vera Katalinić-Janković, Lanfranco Fattorini, Daniela Cirillo

Revised by Emanuele Borroni, Enrico Tortoli, and Vlad Nikolayevskyy (2015).

5.1 Background and principles

Bacteriological cultures can provide a definitive diagnosis of tuberculosis. The primary advantage of culture tests over sputum microscopy is their 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 for diagnosing 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].

As the EU has adopted the culture-based case definition, the main distinction in TB cases is between culture-positive and -negative, and not sputum smear status. The first section of the European Standards for Tuberculosis Care (ESTC) 2012 [1] dedicated to the Standards for Diagnosis (ESTC 1 through ESTC 6) specifies the minimum requirements for obtaining a valid TB diagnosis in different settings or with various suspect types, both microbiological and clinical [1,2]. If this is not feasible, culture tests should at least be performed for:

- 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:

- selection of the correct type of specimen;
- the quality of the sample; and
- 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 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 or accidental inoculation – are essential for minimising biohazards [13]. Nosocomial transmission of *M. tuberculosis* from specimens is a major concern for laboratory workers. All specimens suspected of containing *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) of homogenised sputa and during culture handling, must be minimised and therefore processed and contained in a biological safety cabinet.

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 biological safety cabinet 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 biological safety cabinet in laboratories complying with BSL3 standards. All aerosol-generating procedures should be performed in a class I or II biological safety cabinet [9-11].

The health of laboratory workers should be regularly monitored by the employer. They should be educated about the symptoms of TB and, if symptoms arise, they should be provided with readily accessible free medical care in accordance to the national regulations.

5.2.1 Minimum WHO recommendations for TB culture/drug susceptibility testing facilities

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 biological safety cabinet and an autoclave or other means of decontamination available in the same building. More information on biosafety is given in Chapter 1 [9-14].

Identification, subculturing, and drug susceptibility testing 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 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. Biological safety cabinets have to be ducted to the outside or vented through a thimble. Recirculation of air from biological safety cabinets 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 [14].

The decision to use additional PPE should be based on risk assessments. Risk assessments should be reviewed routinely and revised when necessary [7].

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 to prevent the spread of microorganisms from the wearer to others by capturing the large wet particles in the exhaled air.

Although biological safety cabinets and airflow in the laboratory are the main means of protection against exposure to contaminated aerosols generated during culture and drug susceptibility testing activities, the need for additional personal protection must be 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 [14].

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 in 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 involving the release of viable organisms into the work area. Respirators should be stored in a convenient, clean and sanitary location and discarded after eight hours of (cumulative) 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 [12,14].

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 biological safety cabinet, gloves must be pulled off and discarded in a waste container in the safety cabinet [7,12,14].

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 corn-starch 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 [14].

Gowns

Always wear a gown inside the laboratory (never outside) and change at least weekly. Long-sleeved back-opening gowns or overalls with narrow cuffs 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. Extra clothing should be available suitable for visitors, maintenance and emergency response personnel [7,12,14].

Always remove personal protective equipment 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, leak-proof, 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 [1-8].

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 laboratory analysis of two sputum smear samples will improve case-finding, reduce time to diagnosis, accelerate initiation of treatment and decrease the number of patients lost during the diagnostic process. 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 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 and biosecurity guidelines or international rules. For international transfer of infectious substances, the International Air Transport Association (IATA, www.iata.org) should be contacted [7].

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

- a leak-proof primary receptacle(s);
- a leak-proof 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 reasonably expected to contain pathogens [7,8]. Category A (UN2814) 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 as well as human biological specimens belong to Category B (UN3373).

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 aspirates, urine, skin, autopsy 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 and therefore to minimise the number of dead mycobacteria, the digestion/decontamination procedure must 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. At present, new commercial kits are available, containing transportation media supplemented with decontamination solutions for

longer shipments between countries or cities. These may help to reduce the proportion of contaminated cultures due to long shipments and increase the proportion of positive cultures among those samples which are not inoculated at the point of collection. 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/l, 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°C for 20 minutes.

Phosphate buffer, 0.067 mol/l, pH 6.8:

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

Dissolve 9.47 g of anhydrous Na₂HPO₄ in 1 litre of distilled water.

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

Dissolve 9.07 g of KH₂PO₄ 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 the 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°C 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.
- Re-suspend 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 only kills about 30% of the tubercle bacilli in clinical specimens; a lower NaOH concentration means that contamination rates may be higher than for other decontamination methods. The time needed to process 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 that the sediment is re-suspended 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 ₂ O 2.94%	
Trisodium citrate 3H ₂ O	29.4g
Distilled water	1000 ml

Dissolve trisodium citrate 3H₂O 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/l, pH 6.8

See above for preparation.

Sputum processing

Sputum specimens are not to 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 are not to 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 to decontaminate.
- Fill the tube to within 2 cm of the top (e.g. the 50 ml mark on the tube) with 0.067 mol/l 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.
- Re-suspend 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 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/l, 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, re-suspend 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 cavitory 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 in 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 while liquid media promotes a faster growth of mycobacteria.

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 (or potentially contaminated) specimens (sputum, abscess contents, bronchial washings, gastric lavage fluid, urine, etc.) [1,2].

The most commonly used non-selective 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.

Other commonly used selective media 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, polymixin 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).

5.6 Solid media

5.6.1 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 geographical regions where patients may be infected with either organisms [3]. Ogawa medium is LJ without asparagine. Non-selective egg-based media can be stored in the refrigerator for several months provided that the tube caps are tightly closed to minimise evaporation.

A disadvantage of egg-based media is 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.

5.6.2 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 the addition of Middlebrook oleic acid-albumin-dextrose-catalase (OADC) enrichment. Because of the transparency of 7H10 and 7H11 plates, *M. tuberculosis* micro colonies with typical cord formation can be detected and counted using a microscope as early as one week after incubation. Moreover, visibility of colonial morphology on agar plates is better than on egg-containing slants, aiding the identification of mycobacteria. Middlebrook 7H11 is preferable 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.

5.6.3 Liquid media

Liquid media offer a considerable time advantage over solid media: 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 colorimetric sensor detects the production of CO₂ dissolved in the culture medium.

5.6.4 Quality control of media

Quality control 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 mainly applies to quality control of solid media, as it is on such media that colonies are visible to the eye and species identification is therefore possible.

Commercially prepared media do not need to be quality controlled for sterility, growth and selectivity, provided that documentation of the manufacturer's quality control procedures is 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 1–3% of each batch at 35–37°C in 5 to 10% CO₂ 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 and *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 10 µl of the control suspension using a pipette or a calibrated loop. To test selective properties of the media, inoculate them with 10 µl of 1:10 suspension in sterile 0.85% NaCl. Incubate all media at 35–37°C in 5–10% CO₂ for up to 21 days.
- Expected results are as follows:

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

5.7 Culture tube inoculation

5.7.1 Solid media

In media that is purchased ready-to-use, condensed moisture is frequently observed on the culture slants and it is advisable to remove it before use. Each slant should be inoculated with 0.2–0.4 ml (2–4 drops or four 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. Using a ready-made commercially available egg-based media supplemented with antibiotic mixture may help to reduce contamination rates. Too little 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.

5.7.2 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.

5.8 Culture incubation

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. Caps should then 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 the media drying out.

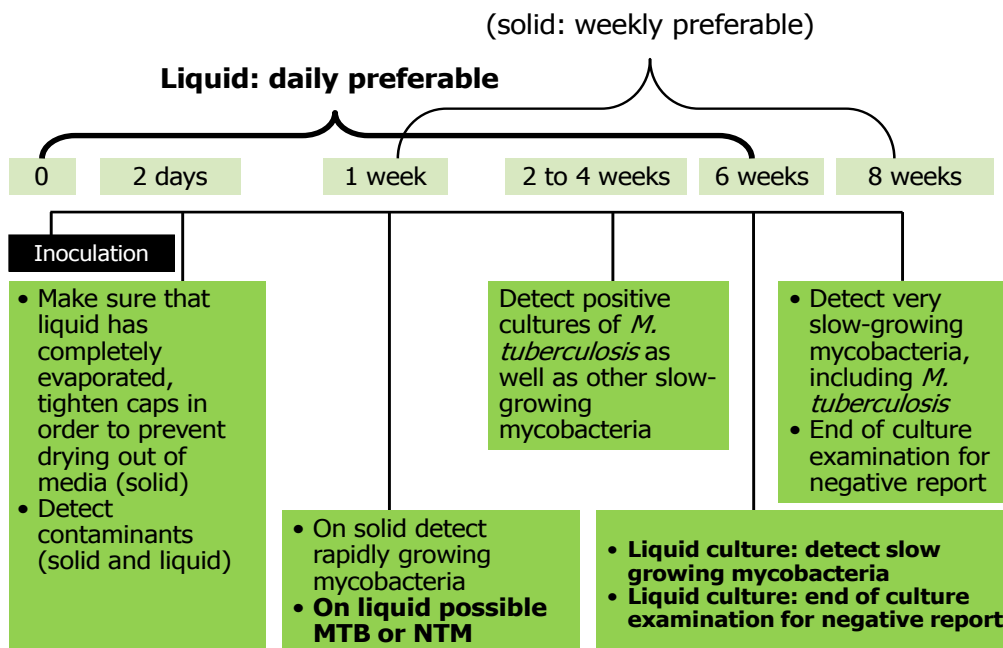
5.9 Culture examination

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.

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

- Seven-day check: To detect rapidly growing mycobacteria.
- Three-to-four-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]

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 AFBs only in the deposit, with no non-AFBs, indicates pure growth of mycobacteria – the deposit should be processed for identification and drug susceptibility testing (inoculation of a non-selective agar plate, such as blood agar, can be used to check for purity).
- Presence of AFBs with non-AFBs in the deposit indicates contamination of the 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 three to 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:

- 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*.

References

1. Migliori GB, Zellweger JP, Abubakar I, Ibraim E, Caminero JA, De Vries G, et al. European Union standards for tuberculosis care. *Eur Respir J*. 2012 Apr;39(4):807-19
2. De Kantor IN, Kim SJ, Frieden T, Laszlo A, Luelmo F, Norval PY, et al. Laboratory services in tuberculosis control. WHO Global Tuberculosis Programme. WHO/TB/98.258. Geneva: WHO; 1998
3. Leão SC, Martin A, Mejia GI, Palomino JC, Robledo J, Telles MAS, et al. Practical handbook for the phenotypic and genotypic identification of mycobacteria. Antwerp: TropMed Central Antwerp; 2004. Introduction available at: <http://www.esmycobacteriology.eu/PDF%20files/foreword.pdf>
4. Iseman MD. A clinical guide to tuberculosis. Philadelphia: Lippincott Williams & Wilkins, 2000.
5. Mase SR, Ramsay A, Ng V, Henry M, Hopewell PC, Cunningham J, et al. Yield of serial sputum specimen examinations in the diagnosis of pulmonary tuberculosis: a systematic review. *Int J Tuberc Lung Dis*. 2007 May;11(5):485-95.
6. Cirillo D, Piana F, Vincent V, Angra P, Barrera L, Boulahbal F, et al. Training package on culture of acid-fast bacilli and drug-susceptibility testing of *Mycobacterium tuberculosis*. In: Tuberculosis Control Assistance Program. TB CAP Toolbox – TB CAP Laboratory Tools [CD ROM]. n.p.; 2009. Available at: http://www.tbcta.org//Uploaded_files/Zelf/NewLabTools1297631626.zip
7. World Health Organization. Guidance on regulations for the Transport of Infectious Substances 2013–2014. Geneva: WHO; 2012 Available at: http://apps.who.int/iris/bitstream/10665/78075/1/WHO_HSE_GCR_2012.12_eng.pdf?ua=1
8. Vincent V, van Deun A, Feldmann K, Barrera L, Paramasivan CN, Weyer K, et al. Guidance for countries on the preparation and implementation of TB laboratory standard operating procedures (WHO SOPs). In: Tuberculosis Control Assistance Program. TB CAP Toolbox – TB CAP Laboratory Tools 2009 [CD ROM].
9. Health Protection Agency, Standards Unit, Evaluations and Standards Laboratory. Investigation of specimens for *Mycobacterium* species. London: Health Protection Agency; 2006. Available at: <https://www.gov.uk/government/publications/smi-b-40-investigation-of-specimens-for-mycobacterium-species>
10. Richmond JY, McKinney RW, editors. Primary containment for biohazards: selection, installation and use of biological safety cabinets. 2nd ed. Washington, D.C.: US Department of Health and Human Services; 2000.
11. Drobniewski FA, Hoffner S, Rusch-Gerdes S, Skenders G, Thomsen V; WHO European Laboratory Strengthening Task Force. Recommended standards for modern tuberculosis laboratory services in Europe. *Eur Respir J*. 2006 Nov;28(5):903-9.
12. World Health Organization. Laboratory biosafety manual. Third Edition. WHO/CDS/CSR/LYO/2004.11. Geneva: WHO; 2004.
13. Tuberculosis Control Assistance Program. TB CAP Toolbox – TB CAP Laboratory Tools 2009 [CD ROM]. Available at: http://www.tbcta.org//Uploaded_files/Zelf/NewLabTools1297631626.zip
14. World Health Organization. Tuberculosis laboratory biosafety manual. WHO/HTM/TB/2012.11. Geneva: WHO; 2012. Available at: http://apps.who.int/iris/bitstream/10665/77949/1/9789241504638_eng.pdf

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

Doris Hillemann, Sarah Mitchell, Francis Drobniewski

Revised by Doris Hillemann (2014)

6.1 Background and principles

Historically, mycobacteria have been directly detected in patient specimens 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 to culture mycobacteria in special liquid and solid media, a method which 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 cannot/should not replace the currently endorsed standard methods of detecting mycobacteria and determining drug-susceptibility patterns. Instead they should be used to support 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 specimens.

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

Assays used: 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 identifying *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 sub-strains of *M. bovis* BCG) but not non-tuberculous mycobacteria specifically and predominantly secrete the MPB64 protein (mycobacterial protein fraction from BCG of Rm 0.64), this can be used to discriminate between *M. tuberculosis* complex and non-tuberculous mycobacteria. 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 use the test plates provided by the manufacturer, 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.

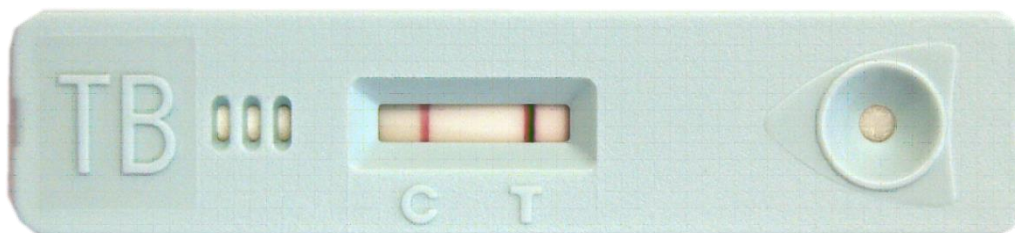
The tests can be used with positive liquid media tubes or visible colonies grown on solid media. In the case of liquid cultures, a 100 µl volume is dropped on to the test device. For solid cultures, 1 µl bacteria (=1 mm loop) or 1 AFB+ colony (at least 1 mm) are re-suspended in the respective buffer,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

6.2.4 Results/interpretation

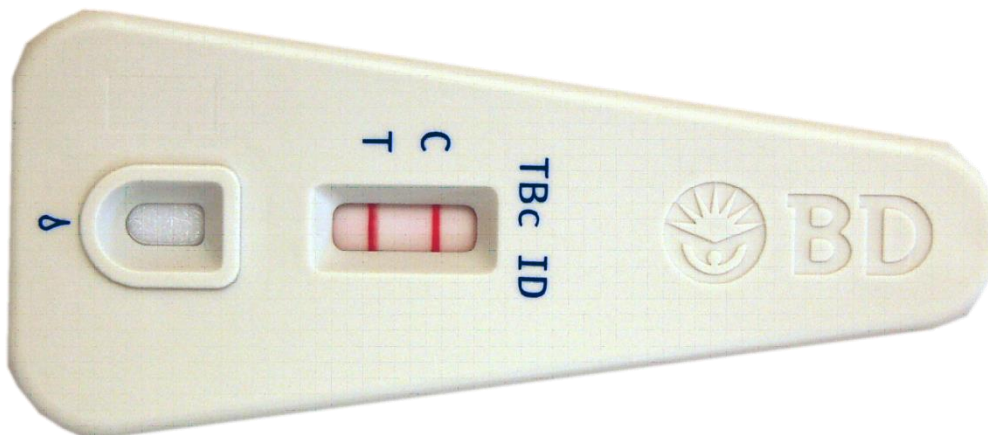
For a specific MPB64 antigen-antibody reaction, a red-purple colour band becomes visible within 15 minutes. The culture is interpreted as positive for *M. tuberculosis* complex if the colour reaction takes place in the test and control area. The intensities of the bands may vary. The specimen is interpreted 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 gives examples of chromatographic immunoassays.

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

Capilia TB-neo



BD MGIT TBc identification test



Although most *M. tuberculosis* complex 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 [2,3]. 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].

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 biological safety cabinet 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

Assays used: INNO-LiPA MYCOBACTERIA v2, Fujirebio Europe, 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

The materials required are 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, MgCl₂ solution, reverse line blots, hybridisation solution, stringent wash solution, denaturation solution, conjugate solution, conjugate buffer, substrate solution and 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 re-suspended 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 MgCl₂ 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.

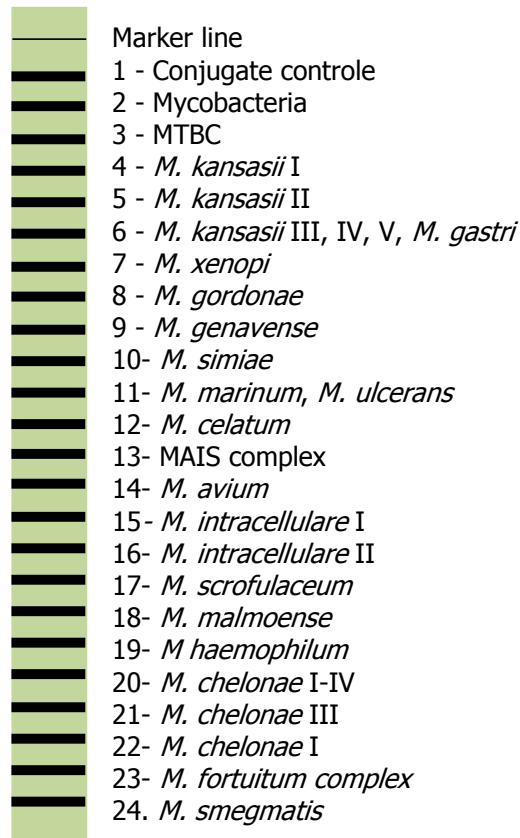
The amplification profile is as follows:

4 min	95°C
30 sec	95°C
30 sec	62°C repeat steps 35 times
30 sec	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 pre-warmed 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 one 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 the strips have been dried 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*, *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 biological safety cabinet in a BSL3 laboratory. After heating, further steps can be performed outside the safety cabinet.

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

Assay used: INNO-LiPA Rif. TB kit, Fujirebio Europe, 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 – Fujirebio Europe 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 small amount of DNA.

DNA extraction

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

Amplification

A one step amplification 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 the laboratory scientist's clothing, reagents, equipment, etc. unless thorough decontamination has been carried out to remove DNA/RNA.

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:
 - 10 μl purified water
 - 20 μl 2X buffer
 - 10 μl primer mix
 - 0.2 μl Taq DNA polymerase
- Mix by inverting the tube 10 times, then briefly centrifuge before distributing 30 μl aliquots into labelled 0.2 ml 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 10 μl of DNA extract or control to the appropriately labelled PCR tube. Once all extracts and controls have been transferred, add 1 μl of inhibition control to the inhibition control tubes.
- Close tubes tightly and transfer to the PCR product lab. Note: Remove coat and carry tubes using gloves. Do not take anything into the PCR product lab.
- Place tubes in the thermal cycler and close lid. The PCR cycles should be:

15 min	95°C	
60 s	95°C	
30 s	58°C	
90 s	72°C	repeat steps 35 times
10 min	70°C	
End	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:
 - 10 μl purified water
 - 20 μl 2X buffer
 - 10 μl primer mix
 - 0.2 μl Taq DNA polymerase

- Mix by inverting the tube 10 times and distribute 40 µl aliquots into labelled 0.2 ml 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 1 µl 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:

15 min	95°C	
30 s	95°C	
30 s	65°C	
30 s	72°C	repeat steps 35 times
10 min	70°C	
End	4°C	∞

Hybridisation

In PCR product lab:

- Remove INNO-LiPA Rif.TB box from fridge. Preheat the hybridisation solution and stringent wash (SW) solution to 62°C. Allow other contents to warm up to room temperature while bath heats to 62°C. Check using a thermometer and adjust accordingly. Temperature must be $\pm 0.5^\circ\text{C}$ for accurate results.
- Place required number of hybridisation trays in plastic rack.
- Mix the denaturation solution. Using a plugged 20 µl pipette tip and a P20 pipette, place 10 µl of denaturation solution into all trays to be used in the assay.
- Using a new 20 µl plugged pipette tip for each sample, add 10 µl of PCR product and mix (by pipetting up and down six times) with the denaturation solution 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 hybridisation solution to ensure all contents are dissolved. Using a P1000 pipette and a plugged pipette tip, place 1 ml of hybridisation solution 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 hybridisation solution using the tap vacuum to discard into the sink and add 1 ml of SW solution. Agitate gently for one 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 10 µl of conjugate concentrate and 990 µl of conjugate diluent to a plastic universal tube 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 1 ml of rinse solution and agitate for 1 minute. Repeat this wash step. Remove all the rinse solution and add 1 ml 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 10 µl of substrate concentrate and 990 µl 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 50 ml 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 *M. tuberculosis* complex 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 *M. tuberculosis* complex 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.

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

If the LiPA is negative for *M. tuberculosis* complex, the test is negative for *M. tuberculosis* complex 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 *M. tuberculosis* complex and has a wild-type genotype, the *M. tuberculosis* complex 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 *M. tuberculosis* complex and has a mutant genotype, the *M. tuberculosis* complex is likely to be rifampicin resistant and the report should read:

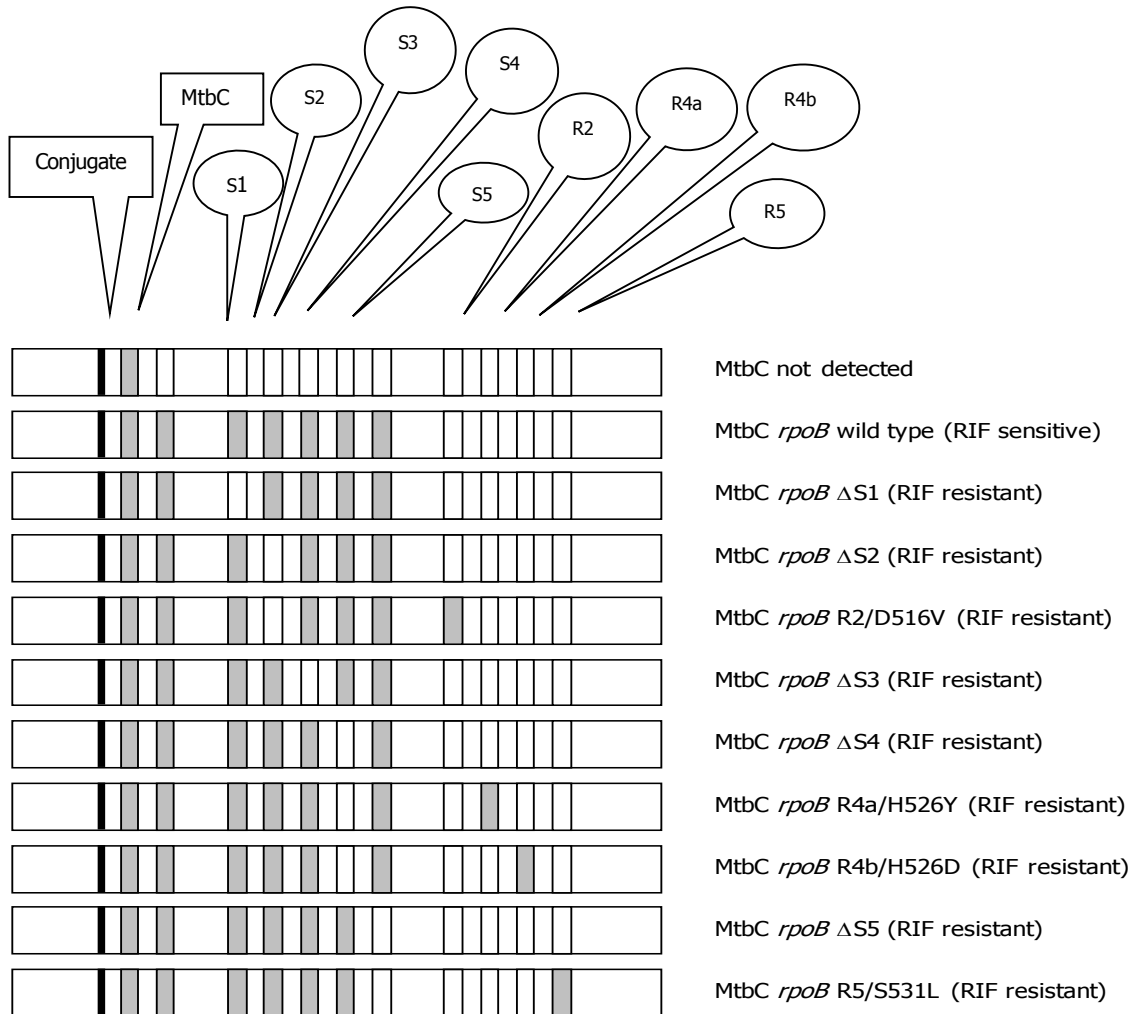
'The performed test indicated:

- 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 *M. tuberculosis* complex, record the test as negative for *M. tuberculosis* complex (see above). If positive for *M. tuberculosis* complex 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.'

Figure 6.3 Interpretation of LiPA



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 identifying species from *Mycobacterium* genus and detecting potential multidrug and extensively multidrug-resistant tuberculosis (MDR- and XDR-TB) from cultures and smear-positive specimens

Assays used: Hain Lifescience GenoType CM, AS and *M. tuberculosis* complex tests and 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 differentiate additional species. The GenoType MTBC test differentiates between the species of the *M. tuberculosis* complex. 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 *M. tuberculosis* complex and simultaneously its resistance to rifampicin and/or isoniazid by mutations in the *rpoB* and *katG/inhA* (high/low isoniazid resistance) genes, respectively [9]. The GenoType MTBDR*sl* simultaneously detects *M. tuberculosis* complex and 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. The currently available GenoType MTBDR*plus* v.2 assay has better sensitivity characteristics.

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; or
- AM-A and AM-B mixes (GenoType MTBDRplus v.2 only)
- 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 10 µl CON-C to 1 ml CON-D per test. Note: this must be made fresh each time.
- Substrate containing 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 biological safety cabinet.

- DNA extraction from solid cultures: Using a 1 µl loop, transfer a small amount of growth to a 1.5 ml screw-top Eppendorf tube containing 100 µl molecular-grade water.
- DNA extraction from liquid cultures and primary specimens: For very large volumes of culture (over 2 ml) centrifuge at 3000g for 20 minutes and discard the supernatant, leaving about 1 ml. Re-suspend the deposit in the remaining fluid and add 100 µl to a 1.5 ml screw-top Eppendorf tube. Note: Primary specimens should not be used with AS and CM strips. For smear-positive sputum specimens, centrifuge 500 µl of decontaminated sample (see NALC-NaOH decontamination – see Section 5.4.1) as above and re-suspend in 100 µl molecular-grade water in a 1.5 ml screw-top centrifuge tube. Add 100 µl to a 1.5 ml screw-top centrifuge tube; if there is a very small volume, make up to 0.25 ml with sterile Middlebrook.
- Alternatively, GenoLyse kit (Hain Lifescience) could be used for the DNA extraction from cultures and primary specimens.
- 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) and therefore, it is necessary to treat those samples prior to DNA extraction:
 - Pipette 0.5 of the culture into a plastic universal.
 - Add 0.25 ml Triton X and 0.25 ml 1N NaOH.
 - Vortex.
 - Add 9 ml 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.
 - Re-suspend the pellet by adding 0.25 ml sterile Middlebrook medium and vortexing.
 - Add 100 µl of this deposit to a 1.5 ml screw-top centrifuge tube.

For the negative control: Add 100 µl of molecular water to a 1.5 ml centrifuge tube. Negative controls must be prepared and included in every run of the test.

All tubes:

- Add 100 µl 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 and can be considered safe. Processing can therefore 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 the PCR mix.

PCR amplification

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

PCR reaction mix

Make up PCR reaction mix:

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

Pipette 45 µl into a 0.2 ml 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.

For GenoType MTBDR*plus* v.2 assay, mix the following (please note: ready-made mixes AM-A and AM-B contain Taq polymerase):

- 10 µl AM-A
- 35 µl AM-B
- 5 µl extracted DNA

(Please refer to the Operator's manual for detailed instructions).

Amplification

This procedure should be conducted in the DNA-specimen preparation room.

- Centrifuge thawed samples (DNA preparation) at 13000g for three minutes.
- Label 0.2 ml x 8-strip thin-wall tubes with the corresponding worksheet number (1 to N).
- After centrifugation carefully pipette 5 µl 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 0.2 ml x 8-strip thin-wall tube (+ individual caps) into a diagnostic thermocycler, close the lid and screw tight.
- Run the amplification cycle:

15 min	95°C	
30 s	95°C	
2 min	58°C	repeat steps 10 times
25 sec	95°C	
40 sec	53°C	
40 sec	70°C	repeat steps 20 times
8 min	70°C	
End	4°C	∞

The reaction time is approximately 1 hour and 50 minutes.

Hybridisation of PCR amplicon onto the Hain strips³

Hybridisation 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:

- Pre-warm shaking water bath/TwinCubator to 45°C. Prewarm HYB and STR solutions 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 10 µl 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.
- 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 pre-warmed 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.

³ Descriptions of laboratory procedures are based on the manufacturer's instructions (Hain Lifescience). The original text is available from: <http://www.thevidence.org/documents/rescentre/sop/MTBDRsl.pdf>.

- 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 from three to 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

Interpretation rules are available in the manufacturer's inserts (Figures 6.4–6.8)

Figure 6.4 Interpretation sheet for GenoType Mycobacterium CM™

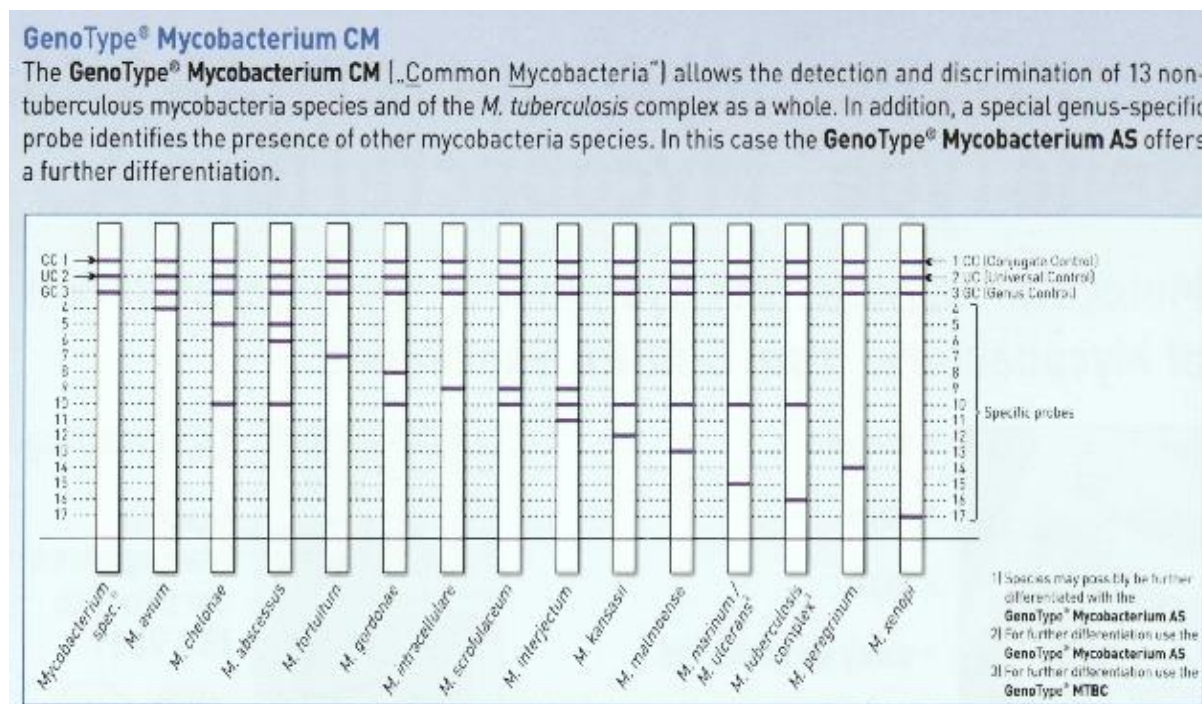


Figure 6.5 Interpretation sheet for GenoType Mycobacterium AS™

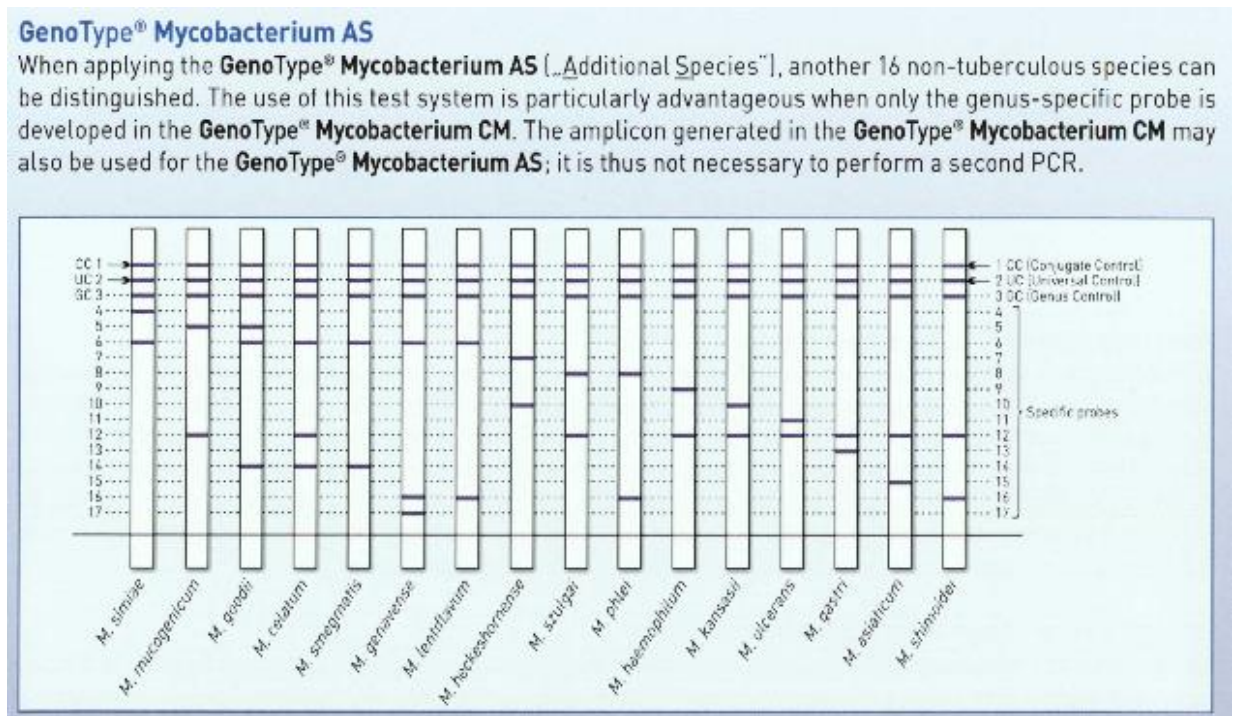


Figure 6.6 Interpretation sheet for GenoType Mycobacterium MTBC™

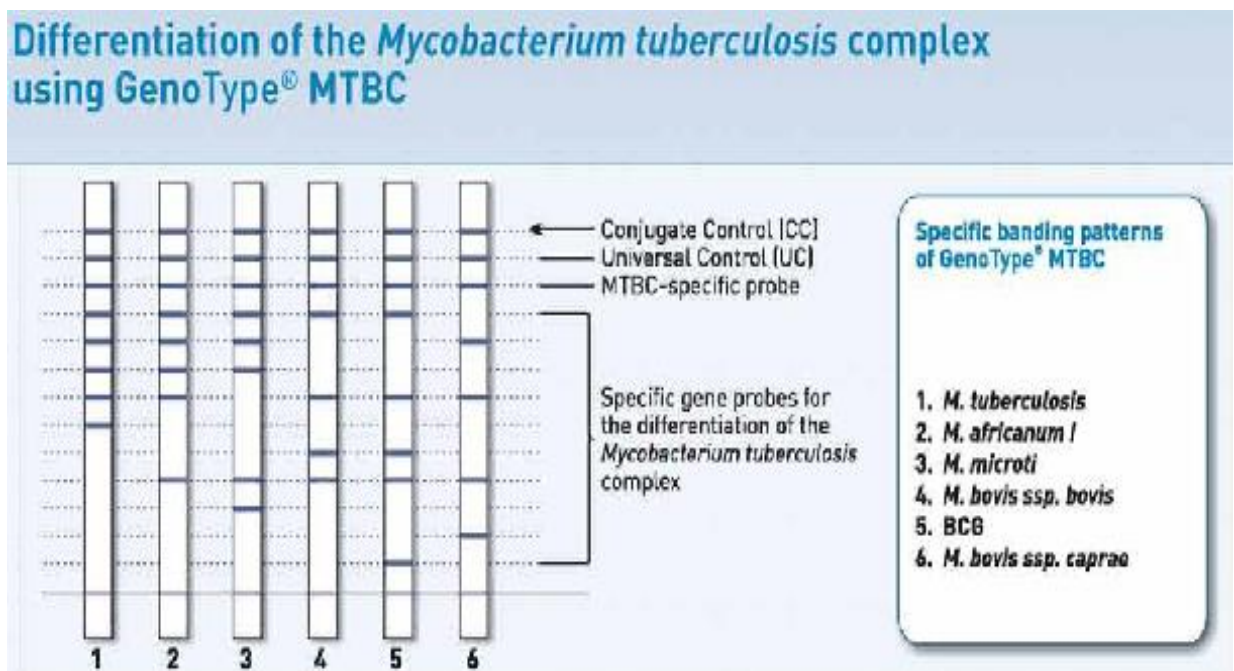


Figure 6.7 Interpretation sheet for GenoType *Mycobacterium* MTBDRPlus™

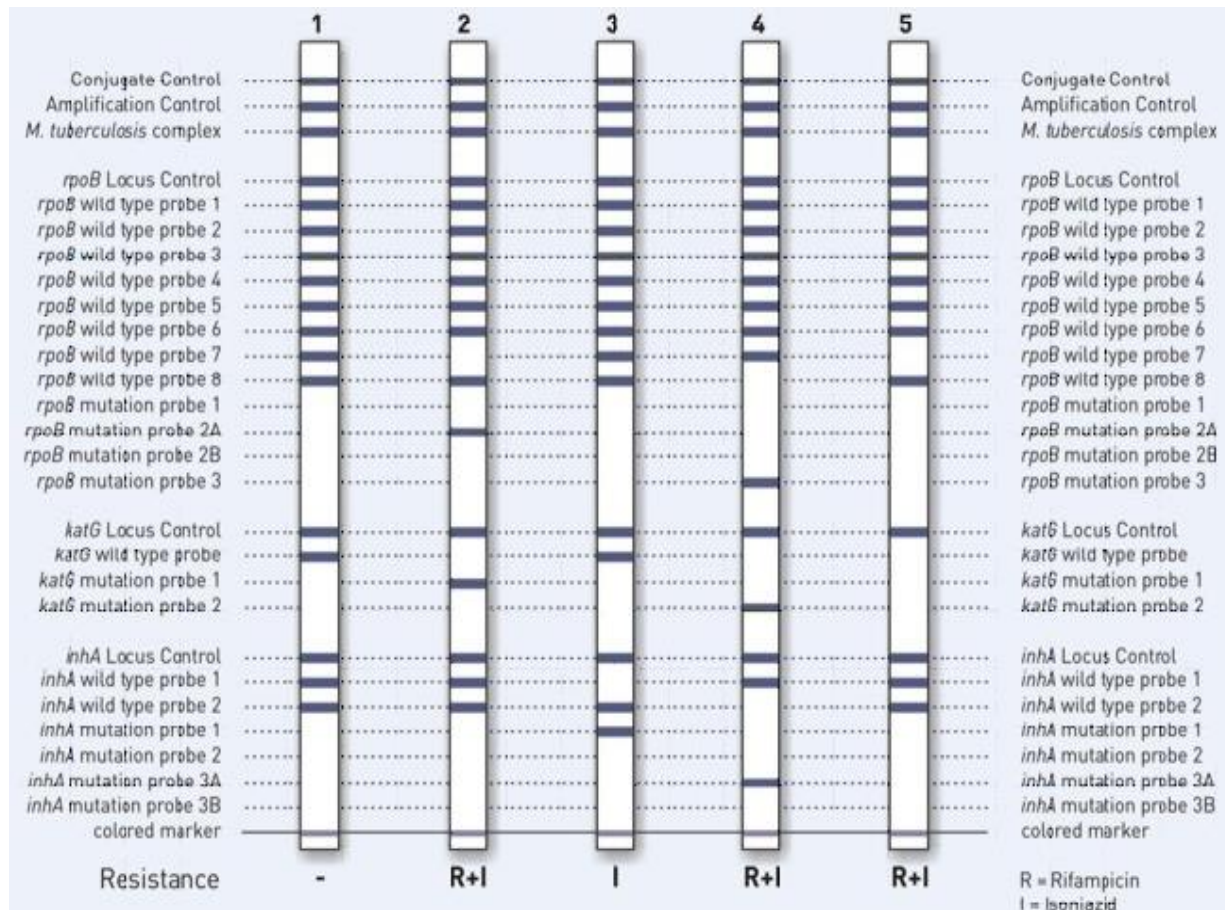
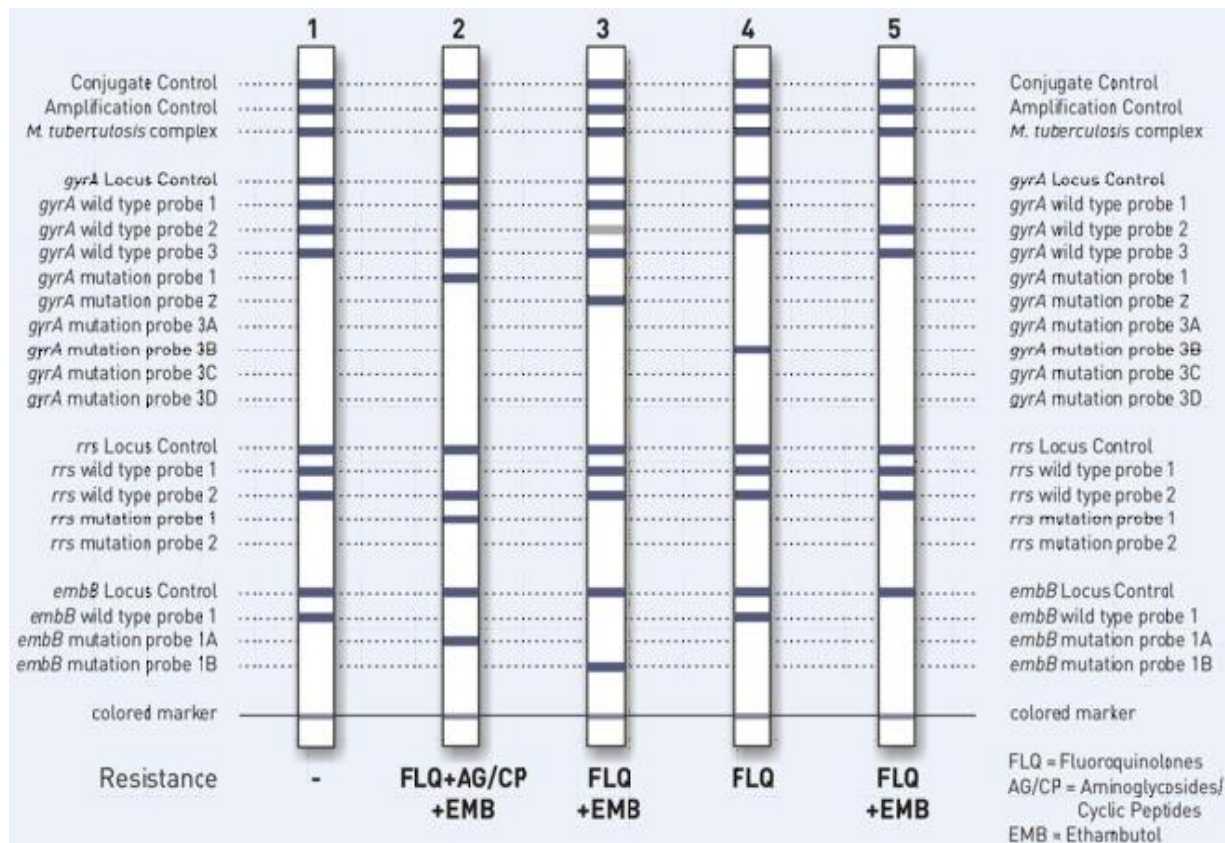


Figure 6.8 Interpretation sheet for GenoType *Mycobacterium* MTBDRsl™



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 6.4 to 6.8). 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 *M. tuberculosis* complex members.

No hybridisation, 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 *M. tuberculosis* complex if only the conjugate control 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 *M. tuberculosis* complex and has a wild type *rpoB*, *katG* and *inhA*, as in strip 1 (Figure 6.7), the *M. tuberculosis* complex 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 MTBDRplus strip is positive for *M. tuberculosis* complex and has a mutant *rpoB*, but wild type *katG* and *inhA* the *M. tuberculosis* complex is likely to be rifampicin resistant, 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 *M. tuberculosis* complex and has a wild type *rpoB*, but mutant *katG* and/or *inhA* (e.g. strip 3, Figure 6.7) the *M. tuberculosis* complex 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 *M. tuberculosis* complex probe is negative but multiple locus control probes are visible or the *M. tuberculosis* complex 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

Assay used: 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 non-tuberculous mycobacteria and does not differentiate between members of the *M. tuberculosis* complex, but the result of *M. tuberculosis* complex is sufficient for most clinical laboratories since *M. tuberculosis* is the most prevalent of the *M. tuberculosis* 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, 100 µl of reagent 1 (lysis reagent) and 100 µl of reagent 2 (hybridisation buffer) are transferred into a reagent tube. For liquid cultures, only 100 µl of reagent 2 is needed. Samples (a loopful of bacteria or 100 µl 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 100 µl volume of the lysed specimens is 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, 300 µl 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 non-radioactive DNA probes for the identification of *M. tuberculosis* complex were 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

Assay used: 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, 2 ml 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 in accordance with the institutional guidelines.

References

1. Abe C, Hirano K, Tomiyama T. Simple and rapid identification of the *Mycobacterium tuberculosis* complex by immunochromatographic assay using anti-MPB64 monoclonal antibodies. J Clin Microbiol. 1999 Nov;37(11):3693-7.
2. Hillemann D, Rüscher-Gerdes S, Richter E. Application of the Capilia TB assay for culture confirmation of *Mycobacterium tuberculosis* complex isolates. Int J Tuberc Lung Dis. 2005 Dec;9(12):1409-11.
3. Hirano K, Aono A, Takahashi M, Abe C. Mutations including IS6110 insertion in the gene encoding the MPB64 protein of Capilia TB-negative *Mycobacterium tuberculosis* isolates. J Clin Microbiol. 2004 Jan;42(1):390-2.
4. Lebrun L, Espinasse F, Poveda JD, Vincent-Levy-Frebault V. Evaluation of non-radioactive DNA probes for identification of mycobacteria. J Clin Microbiol. 1992 30(9):2476-2478.
5. Reisner BS, Gatson AM, Woods GL. Use of Gen-Probe AccuProbes to identify *Mycobacterium avium* complex, *Mycobacterium tuberculosis* complex, *Mycobacterium kansasii*, and *Mycobacterium goodii* directly from BACTEC TB broth cultures. J Clin Microbiol. 1994 32(12):2995-2998.
6. Sam IC, Drobniewski F, More P, Kemp M, Brown T. *Mycobacterium tuberculosis* and rifampin resistance, United Kingdom. Emerg Infect Dis. 2006 May;12(5):752-9.
7. Scarparo C, Piccoli P, Rigon A, Ruggiero G, Nista D, Piersimoni C. Direct identification of mycobacteria from MB/BacT alert 3D bottles: comparative evaluation of two commercial probe assays. J Clin Microbiol. 2001 39(9):3222-3227.
8. Tortoli E, Mariottini A, Mazzarelli G. Evaluation of INNO-LiPA MYCOBACTERIA v2: improved reverse hybridisation multiple DNA probe assay for mycobacterial identification. J Clin Microbiol. 2003 41(9):4418-4420.
9. Nikolayevskyy V, Balabanova Y, Simak T, Malomanova N, Fedorin I, Drobniewski F. Performance of the GenoType MTBDRplus assay in the diagnosis of tuberculosis and drug resistance in Samara, Russian Federation. BMC Clin Pathol. 2009 Mar 10;9:2.
10. Boehme CC, Nabeta P, Hillemann D, Nicol MP, Shenai S, Krapp F, et al. Rapid molecular detection of tuberculosis and rifampin resistance. N Engl J Med. 2010 Sep 9;363(11):1005-15.
11. Rossau R, Traore H, De Beenhouwer H, Mijs W, Jannes G, De Rijk P, et al. Evaluation of the INNO-LiPA Rif. TB assay, a reverse hybridisation assay for the simultaneous detection of *Mycobacterium tuberculosis* complex and its resistance to rifampin. Antimicrob Agents Chemother. 1997 Oct;41(10):2093-8.

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

Emmanuelle Cambau, Sabine Rüscher-Gerdes

Revised by Emmanuelle Cambau (2015)

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* being the most common) to anti-tuberculous drugs [11].

Major objectives for drug susceptibility testing (DST) in tuberculosis include: (i) ensuring effective individual treatment and management of a tuberculosis case, (ii) anti-tuberculosis drug resistance surveillance at the level of a hospital, city, region or country; (iii) identification of the need for institutional isolation of patients; and (iv) determination of the scope of institutional and community outbreak investigations required [13]. Because of the development of drug resistance in tuberculosis worldwide, drug susceptibility testing to first-line drugs is required for a definite case of tuberculosis (a patient with a positive culture for the *M. tuberculosis* complex or one sputum smear positive for acid-fast bacilli if a culture is not available). 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) – i.e. those resistant to at least rifampicin and isoniazid – and for patients who have previously been treated for tuberculosis with second-line drugs. Drug susceptibility testing should be repeated for a definite case if the culture is still positive after five months of treatment. It may also be repeated after three months of treatment if sufficient funds are available [30].

The mycobacterial culture or *M. tuberculosis* complex acid-fast bacilli should be identified before the start of drug susceptibility testing. Non-tuberculous mycobacteria 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 *M. tuberculosis* complex 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 *M. tuberculosis* complex [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 of drug susceptibility testing. The minimum requirement for quality control is to test each new batch of reagents. If the batch quality control 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 quality control programme [13].

Drug susceptibility tests are usually performed on *M. tuberculosis* complex cultures (indirect testing), but may also be performed on specimens containing acid-fast bacilli known to belong to *M. tuberculosis* complex (direct testing) [7] as long as special conditions are fulfilled (see below). Results are obtained more quickly for direct testing; however, there is a lower success rate 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 also to assess susceptibility [13]. Applying these methods will result in 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 will also result in 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 reference strains. 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 cover rifampicin and rifapentine, prothionamide covers ethionamide and vice-versa), or to one individual drug (rifampicin does not cover rifabutin and streptomycin does not cover amikacin and kanamycin). First-line drugs include isoniazid (H), rifampicin (R), ethambutol (E), streptomycin (S), although streptomycin is now not part of most 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 levofloxacin, and moxifloxacin), ethionamide or prothionamide, and cycloserine and linezolid. Imipenem, clofazimine and clarithromycin, used sometimes for last-chance treatment of extensively-drug resistant TB cases (XDR-TB), i.e. MDR-TB strains with additional resistance to fluoroquinolones and to injectables, are not regularly tested since acquired resistance during treatment is unknown. For the new drugs, bedaquiline and delamanid, that became available in 2014, critical breakpoints were proposed by EUCAST/EMA but the methods of testing are still under investigation. In general, second-line DST is less standardised than first-line DST. This is due to insufficient knowledge, drug instability resulting in variation of the active concentration achieved in the media after preparation, and other factors.

Due to the discrepancies in the results observed for some of the second-line drugs (cycloserine, ethionamide and prothionamide) or discussion about their critical concentrations, the descriptions appearing in this report 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. Results can rely on a direct observation of growth, such as counting of colonies or visible liquid broth, or an indirect measure of growth through oxygen consumption, CO₂ production or enzymatic modification of the media.

7.2.2 Materials

For direct testing [7]: smear-positive specimens after classic decontamination (see Section 5.6). For indirect testing [13,17]: cultures of *M. tuberculosis* complex, as a positive liquid broth or colonies on a solid media, 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 were shown to be concordant with reference methods 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 Colorimetric redox indicator (CRI) assay using resazurin; microscopically observed direct susceptibility testing (MODS) [1,15,18,27] and the Thin Layer Agar methods. Although these methods are simpler to implement than reference methods, they are less reproducible than proportion methods, probably because the inoculum is not monitored.

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 drug tested, 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. Resistance results can now also be complemented by molecular detection of mutations conferring resistance, as described in Chapter 6. If a mutation known to confer resistance (e.g. *rpoB* mutation leading to S531L substitution for rifampicin resistance) is observed, this confirms resistance. Conversely, if no mutation is observed in the genes known to confer resistance, this implies that the strain needs to be retested with a reference method. If the strain still appears resistant but no mutation is found, colonies growing in the tube containing antibiotic should be retested for molecular detection.

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 might be of interest for some first-line drugs (isoniazid, streptomycin, ethambutol) and second-line drugs (amikacin, capreomycin, ofloxacin, moxifloxacin) 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. However, more clinical studies should be done correlating patient outcome with levels of resistance.

7.3 Procedure 2: The proportion method on Löwenstein-Jensen medium

7.3.1 Introduction

The proportion method was one of the first methods described for susceptibility testing of *M. tuberculosis complex*. 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 Definition

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 drug concentration at which wild type strains do not grow.

Critical proportion: The lowest percentage of the strain population that may grow at the critical concentration and which is associated with clinical ineffectiveness of that drug.

Growth control: Culture yielded after inoculation of a tubercle bacilli suspension on a culture medium without any anti-TB 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 (preferably with screw caps) containing LJ medium, pipettes, and McFarland turbidity standard no. 1.

For direct testing, smear-positive specimens are decontaminated as per the usual procedure (see Section 5.4.1.) and the pellet is used pure or diluted (see below).

7.3.4 Methods

Media preparation

Löwenstein-Jensen tubes are used with incorporated drugs. For that, egg-based LJ is prepared according to the standard protocol (see standard operating procedure for preparation of LJ egg-based media) and mixed with drugs at the recommended quantities (Table 7.1) before inspissation. Screw-capped tubes containing 5 ml of the preparation 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 and inoculation

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. The suspension can be homogenised using glass beads (3.0 mm in diameter) or 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. The supernatant should then be pipetted into another tube.

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

Serial dilutions of 10⁻¹ to 10⁻⁵ 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.

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 sequentially diluting 1.0 ml of the standard suspension in tubes containing 9 ml of sterile distilled 0.9% sodium chloride.

Final dilutions of 10⁻² (control 1) and 10⁻⁴ (control 2) are inoculated onto LJ as controls. The drugs containing LJ media are then inoculated with control 1 and control 2 inoculums. The volume of the inoculum in control and drug containing tubes is 0.1 ml.

Direct testing using primary specimens:

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.

Drugs 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: using pipettes or a calibrated loop. Using pipettes, serial dilutions of 10⁻¹ mg/ml to 10⁻⁴ mg/ml of the standard suspension are prepared by sequentially diluting 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 are preferable.

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:

- Counting of colonies grown on the different slants: for dilution 1 (e.g. 10⁻²) a confluent growth is often observed since around 10⁴ Colony Forming Units (CFU) have been inoculated; for dilution 2 (e.g. 10⁻⁴) since around 10² (about 20–100 colonies are counted. This number may differ from strain to strain since some are dysgonic and consequently the same dilution will grow less colonies;
- Calculation of the proportion of resistant bacilli by comparing counts on dilution-2 control tubes (1% of the dilution 1) and on drug containing tubes inoculated with the dilution 1; and
- Ratio of the calculated proportion and the critical proportion of the drug in question, in order to determine whether the ratio is ≥ 1 (resistant strain) or < 1 (susceptible strain).

Results are read 28 days (early reading) and 42 days (final reading) 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. Moreover, 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. With the exception of 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 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):

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 dots, 3 dots) 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 (Table 7.3). 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
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.

7.4.4 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. Four weeks are required to obtain results and the preparation of LJ tubes, although cheap, is cumbersome. Thirty years ago, the first liquid-based culture media were introduced commercially, and several evaluations have demonstrated good 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. BACTEC MGIT tubes, along with supplements and drugs are required. Drugs are supplied as pure powder with appropriate solvent.

7.5.3 Methods

Media preparation

A total of 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. Commercially available drugs are first-line drugs (isoniazid, rifampicin, ethambutol, streptomycin and pyrazinamide) and from 2015 onwards also second-line drugs (ofloxacin, moxifloxacin, capreomycin, amikacin, ethionamide and PAS). Critical concentrations are given in Table 7.4.

Table 7.4 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. The positive MGIT tube is inverted once or twice 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 is observed within two or three days. If positivity is observed for four 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 each set of DST, the control tube will be 1:100 dilution of the original inoculum (1% control): the inoculum is 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 in the automated BACTEC/MGIT where fluorescence is detected automatically. In manual operation, tubes are incubated at 37°C and are read under UV light every day.

7.5.4 Safety

See general procedure in Section 7.2.4 and Chapter 1.

7.5.5 Report interpretation

Once the growth control (i.e. 1% inoculum) is positive, after a minimum of four days (otherwise the positive signal is due to contamination) and a maximum of 12 days for the MGIT SIRE protocol, the instrument will compare growth in the drug tubes. Results are qualitative: if the drug containing tube is negative when the growth control is positive, the strain is reported susceptible; if the drug-containing tube is positive before or on the day the growth control is positive, the strain is reported resistant. If the growth control is still negative after 12 days, the test should be redone, checking the inoculum protocol and taking into account the time to growth of the strain (dysgonic).

All positive tubes should be checked for contamination, by preparing a Gram-stained smear and/or adding one drop to a blood plate, and for mycobacterial growth by preparing a Ziehl-Neelsen stained smear.

Note: A novel procedure to extend incubation for up to 30 days – where several growth controls are tested simultaneously to measure different proportions of resistance, and several concentrations able to determine the level of resistance – is now proposed using the same tubes and automatically updated with the TB eXiST module. A protocol has been established at several European reference laboratories for tuberculosis although this still needs to be validated³⁸.

7.6 Procedure 5: Other methods for first-line drug susceptibility testing

Many other methods or protocols have been proposed to simplify the DST procedure and circumvent the long duration before reporting susceptibility results for tuberculosis [1,15,18,27]. Their main advantages are relative simplicity, robustness and lower cost. Some are also less labour-intensive and time-consuming, which is useful when testing large numbers of patients in resource-limited settings. However, these methods are less reproducible, have not been extensively evaluated in multicentre validation studies, and are not proportion methods. For example, the growth is not compared to that of a 1% growth control and the inoculum is therefore not monitored so closely, exposing it to the risk of false resistance if the inoculum is too rich (more than 10^5 CFU in contact with the drug), or false susceptibility if the inoculum is too poor (less than 100 CFU in contact with the drug). In addition, critical concentrations may be different from reference methods since they were set according to the media. Selected methods described here are the absolute concentration method; the nitrate reductase assay; the colorimetric redox indicator (CRI) assay using resazurin and the microscopically observed drug susceptibility assay (MODS).

7.6.1 Absolute concentration method

This method is in use in Russia and some eastern European countries. The absolute concentration method is performed with LJ tubes containing antibiotics at the same critical concentrations as in the proportion method on LJ (see above). One suspension is inoculated into each tube with a control drug-free tube. After 28 days incubation, resistance is indicated by more than 20 colonies growing on the drug-containing LJ tube.

7.6.2 Nitrate reductase assay

The nitrate reductase assay (NRA) is a technique based on the capacity of *M. tuberculosis* to reduce nitrate to nitrite, which is detected by adding the Griess reagent to the medium. By incorporating 1 mg/ml potassium nitrate (KNO_3) in the LJ medium, the reduction of nitrate can be detected using the Griess reagent, which produces a coloured reaction. In the presence of rifampicin or isoniazid at the critical concentration, the appearance of a red-pink colour indicates strain growth, which is interpreted as resistance to the drug. Results can be obtained faster than by macroscopic detection of colonies, as the NRA uses the detection of nitrate reduction as an indicator of growth.

The protocol is detailed in Angeby et al, 2002 [1] as follows:

- The LJ medium, into which 1 mg/ml potassium nitrate (KNO_3) is incorporated, is prepared in 10 ml portions in 75- by 25-mm screw-cap glass tubes.
- Two 1 μl loops of bacteria, from fresh cultures on Löwenstein-Jensen medium, are dispensed and vortexed in 3 ml of phosphate-buffered saline (pH 7.4) in 7.5-ml screw-cap bottles containing a few 3-mm-diameter glass beads, in order to obtain the approximate turbidity of McFarland standard no. 1.
- Part of the suspension is diluted 1:10 in phosphate-buffered saline.
- For each strain, 0.2 ml of the undiluted suspension is inoculated into the tubes containing Löwenstein-Jensen medium with KNO_3 and the antibiotics and 0.2 ml of the 1:10 dilution is inoculated into three drug-free tubes containing Löwenstein-Jensen medium with KNO_3 incorporated. The three drug-free tubes serve as growth controls.
 - The tubes are incubated at 37°C, and 0.5 ml of a mixture of three reagents (one part 50% (vol/vol) concentrated hydrochloric acid (HCl), two parts 0.2% (wt/vol) sulfanilamide, and two parts 0.1% (wt/vol) *n*-1-naphthylethylenediamine dihydrochloride, mixed shortly before use) is added to one drug-free control tube after seven days.

- If any colour change (strong or weak) can be seen, the corresponding antibiotic-containing tubes are also tested and susceptibility results are read.
- If no colour change is seen in the growth control tube, this tube is discarded and the other two control tubes and the antibiotic tubes are re-incubated.
- The procedure is then repeated on Day 10, using the second growth control, and if needed, also on Day 14, using the last growth control tube.

Interpretation

The results are classified as negative (no colour change) or \pm (pale pink) to 5+ (deep red to violet). An isolate is considered resistant to a certain drug if there is a colour change in the antibiotic tube in question greater than that in the 1:10-diluted growth control on the same day.

7.6.3 Microscopically Observed Drug Susceptibility Assay (MODS)

The microscopically observed drug susceptibility (MODS) assay is a method for direct testing of resistance to anti-tuberculous drugs. The sputum pellet is inoculated in microplate wells containing liquid media after decontamination. Cultures are examined on a daily basis using an inverted microscope [34,37].

Protocol

Inoculum: one vial containing 500 ml of sample pellet is re-suspended with 2 ml of Middlebrook 7H9 broth (BD), oleic acid, albumin, dextrose, and catalase (OADC) (BD), and polymyxin, amphotericin B, nalidixic acid, trimethoprim and azlocillin (PANTA, BD), taken from one pre-prepared tube containing 5.1 ml (as per MODS standard operating procedure: www.modsperu.org). Then 1 ml of the re-suspended pellet is removed and stored as a backup. The remaining 1.5 ml is added to the remaining 3.1 ml of 7H9-OADC-PANTA and 900 ml of this final sample suspension is inoculated into each of the four wells in a 24-well tissue-culture plate (BD) as per MODS standard operating procedure [6].

For each sample, four wells are used: in two drug-free (diagnostic) wells no drug is added and each of the remaining two wells contain critical concentrations of either 1 mg/ml rifampicin or 0.4 mg/ml isoniazid, as per the MODS standard operating procedure.

To minimise cross-contamination and occupational exposure, plates are permanently sealed inside plastic Ziploc bags after inoculation, and placed in an incubator (non-CO₂ enriched) at 37°C.

Wells are subsequently examined using an inverted light microscope at 400 magnification every day from Day 4 through to Day 21. Positive MODS cultures are identified by presence of characteristic cord formations in the drug-free control wells.

Interpretation

Growth, as observation of cord formations, in drug-free control wells but not in drug-containing wells indicates susceptibility. Growth observed in both the drug-free wells and the drug containing wells on the same day is interpreted as resistance.

The drug concentrations to be used are as follows: isoniazid 0.4 mg/l; rifampin, 1 mg/l.

7.6.4 Colorimetric redox indicator (CRI) assay [31,32]

This protocol is also used for MIC determination in microplate.

Protocol

- The inoculum is prepared from a three-week-old LJ culture re-suspended in 7H9-S medium (Middlebrook 7H9 broth/0.1% casitone/0.5% glycerol/10% oleic acid, albumin, dextrose and catalase), adjusted to a turbidity equivalent to that of a 1 McFarland standard and diluted 1:10 (100 μ l of the 1:10 dilution).
- The drug concentrations used are the following: rifampicin, 0.5 and 1.0 mg/l; isoniazid, 0.25 and 0.5 mg/l; ofloxacin, 2.0 and 4.0 mg/l; kanamycin, 2.5 and 5.0 mg/l; and capreomycin, 2.5 and 5.0 mg/l.
- A growth control containing no antibiotic and a control without inoculation are also prepared for each plate.
- The plate is covered with its lid, sealed in a plastic bag and incubated at 37°C in a normal atmosphere. After seven days of incubation, 30 μ l of 0.02% resazurin solution is added to each well and the plate is re-incubated for 24–48 hours. (Resazurin sodium salt is prepared at 0.02% (w/v) in distilled water, sterilised by filtration and stored at 48°C for no more than two weeks).

Interpretation

A change in colour from blue (oxidized state) to pink (reduced) indicates the growth of bacteria. When the growth control is positive, if wells containing drugs are positive, this indicates resistance. If wells containing drugs are negative, this indicates susceptibility.

References

1. Angeby KA, Klintz L, Hoffner SE. Rapid and inexpensive drug susceptibility testing of *Mycobacterium tuberculosis* with a nitrate reductase assay. *J Clin Microbiol.* 2002 Feb;40(2):553-5.
2. Angeby KA, Werngren J, Toro JC, Hedström G, Petrini B, Hoffner SE. Evaluation of the BacT/ALERT 3D system for recovery and drug susceptibility testing of *Mycobacterium tuberculosis*. *Clin Microbiol Infect.* 2003 Nov;9(11):1148-52.
3. Bai GH, Kim SJ, Chang CL; National and Regional Tuberculosis Reference Laboratories. Proficiency analysis of drug susceptibility testing by national-level tuberculosis reference laboratories from 1995 to 2003. *J Clin Microbiol.* 2007 Nov;45(11):3626-30.
4. Bemer P, Palicova F, Rüscher-Gerdes S, Drugeon HB, Pfyffer GE. Multicenter evaluation of fully automated BACTEC Mycobacteria Growth Indicator Tube 960 system for susceptibility testing of *Mycobacterium tuberculosis*. *J Clin Microbiol.* 2002 Jan;40(1):150-4.
5. Bergmann JS, Woods GL. Mycobacterial growth indicator tube for susceptibility testing of *Mycobacterium tuberculosis* to isoniazid and rifampin. *Diagn Microbiol Infect Dis.* 1997 Jul;28(3):153-6.
6. Bergmann JS, Woods GL. Reliability of mycobacteria growth indicator tube for testing susceptibility of *Mycobacterium tuberculosis* to ethambutol and streptomycin. *J Clin Microbiol.* 1997 Dec;35(12):3325-7.
7. Bwanga F, Hoffner S, Haile M, Joloba ML. Direct susceptibility testing for multi drug resistant tuberculosis: a meta-analysis. *BMC Infect Dis.* 2009 May 20;9:67.
8. Canetti G. Present aspects of bacterial resistance in tuberculosis. *Am Rev Respir Dis.* 1965 Nov;92(5):687-703.
9. Canetti G, Fox W, Khomenko A, Mahler HT, Menon NK, Mitchison DA, et al. Advances in techniques of testing mycobacterial drug sensitivity, and the use of sensitivity tests in tuberculosis control programmes. *Bull World Health Organ.* 1969;41(1):21-43.
10. Canetti G, Froman S, Grosset J, Hauduroy P, Langerova M, Mahler HT, et al. Mycobacteria: laboratory methods for testing drug sensitivity and resistance. *Bull World Health Organ.* 1963;29:565-78.
11. Canetti G, Rist N, Grosset J. Primary Drug Resistance in Tuberculosis. *Am Rev Respir Dis.* 1964 Nov;90:792-9.
12. David HL. Probability distribution of drug-resistant mutants in unselected populations of *Mycobacterium tuberculosis*. *Appl Microbiol.* 1970 Nov;20(5):810-4.
13. Drobniowski F, Rüscher-Gerdes S, Hoffner S; Subcommittee on Antimicrobial Susceptibility Testing of *Mycobacterium tuberculosis* of the European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID). Antimicrobial susceptibility testing of *Mycobacterium tuberculosis* (EUCAST document E.DEF 8.1) – report of the Subcommittee on Antimicrobial Susceptibility Testing of *Mycobacterium tuberculosis* of the European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID). *Clin Microbiol Infect.* 2007 Dec;13(12):1144-56.
14. Grosset J, Truffot-Pernot C, Cambau E. Bacteriology of tuberculosis. In: Reichman LB, Hershfield ES, editors. *Tuberculosis, a comprehensive international approach.* New York: Marcel Dekker; 2000. p. 157-85.
15. Hausdorfer J, Sompek E, Allerberger F, Dierich MP, Rüscher-Gerdes S. E-test for susceptibility testing of *Mycobacterium tuberculosis*. *Int J Tuberc Lung Dis.* 1998 Sep;2(9):751-5.
16. Heifets L. Drug susceptibility tests in the management of chemotherapy of tuberculosis. In: *Drug susceptibility in the chemotherapy of mycobacterial infections.* Boca Raton: CRC Press; 1991. p. 89-121
17. Heifets L. Qualitative and quantitative drug-susceptibility tests in mycobacteriology. *Am Rev Respir Dis.* 1988 May;137(5):1217-22.
18. Hoffner S, Jimenez-Misas C, Lundin A. Improved extraction and assay of mycobacterial ATP for rapid drug susceptibility testing. *Luminescence.* 1999 Sep-Oct;14(5):255-61.
19. Kim SJ. Drug-susceptibility testing in tuberculosis: methods and reliability of results. *Eur Respir J.* 2005 Mar;25(3):564-9.
20. Kim SJ, Espinal MA, Abe C, Bai GH, Boulahbal F, Fattorin L, et al. Is second-line anti-tuberculosis drug susceptibility testing reliable? *Int J Tuberc Lung Dis.* 2004 Sep;8(9):1157-8.
21. Krüüner A, Yates MD, Drobniowski FA. Evaluation of MGIT 960-based antimicrobial testing and determination of critical concentrations of first- and second-line antimicrobial drugs with drug-resistant clinical strains of *Mycobacterium tuberculosis*. *J Clin Microbiol.* 2006 Mar;44(3):811-8.

22. Mitchison, DA. Sensitivity testing. In: Heaf FRG (editor). Recent advances in respiratory tuberculosis. London: Churchill J & A; 1968. p. 160.
23. Pfyffer GE, Bonato DA, Ebrahimzadeh A, Gross W, Hotaling J, Kornblum J, et al. Multicenter laboratory validation of susceptibility testing of *Mycobacterium tuberculosis* against classical second-line and newer antimicrobial drugs by using the radiometric BACTEC 460 technique and the proportion method with solid media. J Clin Microbiol. 1999 Oct;37(10):3179-86.
24. Pfyffer GE, Palicova F, Rüschi-Gerdes S. Testing of susceptibility of *Mycobacterium tuberculosis* to pyrazinamide with the non-radiometric BACTEC MGIT 960 system. J Clin Microbiol. 2002 May;40(5):1670-4.
25. Rüschi-Gerdes S, Domehl C, Nardi G, Gismondo MR, Welscher HM, Pfyffer GE. Multicenter evaluation of the mycobacteria growth indicator tube for testing susceptibility of *Mycobacterium tuberculosis* to first-line drugs. J Clin Microbiol. 1999 Jan;37(1):45-8.
26. Werngren J, Klintz L, Hoffner SE. Evaluation of a novel kit for use with the BacT/ALERT 3D system for drug susceptibility testing of *Mycobacterium tuberculosis*. J Clin Microbiol. 2006 Jun;44(6):2130-2.
27. Wilson SM, al-Suwaidi Z, McNERNEY R, Porter J, Drobniewski F. Evaluation of a new rapid bacteriophage-based method for the drug susceptibility testing of *Mycobacterium tuberculosis*. Nat Med. 1997 Apr;3(4):465-8.
28. Woods GB, Brown-Elliott BA, Desmond EP, Hall GS, Heifets L, Pfyffer GE, et al. Susceptibility testing of mycobacteria, *Nocardiae* and other aerobic actinomycetes; approved standard, vol. 23 (18). CLSI, Wayne, PA.
29. Woods GL. Susceptibility testing for mycobacteria. Clin Infect Dis. 2000 Nov;31(5):1209-15.
30. World Health Organization. Guidelines for the programmatic management of drug-resistant tuberculosis. Geneva: WHO; 2008.
31. Martin A, Panaiotov S, Portaels F, Hoffner S, Palomino JC, Angeby K. Nitrate reductase assay for the rapid detection of isoniazid and rifampicin resistance in *Mycobacterium tuberculosis*: a systematic review and meta-analysis. Journal of Antimicrobial Chemotherapy (2008) 62, 56–64
32. Martin A, Paasch F, Docx S, Fissette K, Imperiale B, Ribon W. Multicentre laboratory validation of the colorimetric redox indicator (CRI) assay for the rapid detection of extensively drug-resistant (XDR) *Mycobacterium tuberculosis*. J Antimicrob Chemother 2011; 66: 827–833
33. Cambau EM, Viveiros D, Machado L, Raskine C, Ritter E, Tortoli V, et al. Revisiting susceptibility testing in MDR-TB by a standardized quantitative phenotypic assessment in a European multicentre study. J Antimicrob Chemother 2014
34. Martin L, Coronel J, Faulx D, Valdez M, Metzler M, Crudder C, et al. A field evaluation of the Hardy TB MODS Kit™ for the rapid phenotypic diagnosis of tuberculosis and multi-drug resistant tuberculosis. PLoS One. 2014 Sep 16;9(9):e107258.
35. Martin A, Montoro E, Lemus D et al. Multicenter evaluation of the nitrate reductase assay for drug resistance detection of *Mycobacterium tuberculosis*. J Microbiol Methods 2005; 63: 145–50.
36. Montoro E, Lemus D, Echemendia M, et al. Comparative evaluation of the nitrate reduction assay, the MTT test, and the resazurin microtiter assay for drug susceptibility testing of clinical isolates of *Mycobacterium tuberculosis*. J Antimicrob Chemother 2005; 55: 500–5.
37. Moore D, Evans AWC, Gilman RH, Caviedes L, Aldo Vivar JC, Sanchez E, et al. Microscopic-Observation Drug-Susceptibility Assay for the Diagnosis of TB. N Engl J Med. 2006 October 12; 355(15): 1539–1550.
38. Springer B, et al. Quantitative drug susceptibility testing of *Mycobacterium tuberculosis* by use of MGIT 960 and EpiCenter instrumentation. J Clin Microbiol, 2009. 47(6):1773-80.

8. Molecular typing of *Mycobacterium tuberculosis* complex isolates

Girts Skenders, Dick van Soolingen

Revised by Stefan Niemann and Vlad Nikolayevskyy (2015)

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. At present, DNA fingerprinting 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 [3,24]. In particular, DNA fingerprinting of *M. tuberculosis* has greatly improved the understanding of TB transmission. Moreover, the recognition of genotype families has facilitated studies on the population structure of the *M. tuberculosis* complex and its transmission dynamics.

The various DNA fingerprinting methods available serve different purposes and have variable characteristics that enable their use in specific applications. This chapter aims to describe the characteristics of the three most important and widely applied DNA fingerprinting methods: spoligotyping, MIRU-VNTR (Mycobacterial Interspersed Repetitive Units – Variable Number of Tandem Repeats) typing and IS6110 RFLP (Restriction Fragment Length Polymorphism) typing and the most important applications these typing methods can be used for. In addition, a perspective will be presented of using whole genome sequencing based on Next Generation Sequencing (NGS) technology for molecular epidemiology of the *M. tuberculosis* complex in the future.

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 clocks⁴ and the stability of DNA profiles has been studied extensively, but is not fully understood [5]. Ideally, every transmission results in 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, enabling 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 the current fingerprinting methods.

Below is an outline of the three DNA fingerprinting methods: spoligotyping, VNTR typing and RFLP typing.

8.2 Procedure 1: Spoligotyping

Spoligotyping is based on polymorphisms in the direct repeat (DR) locus of 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 DR region sequences of the 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, such as 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

⁴ 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. This measure is chiefly used for estimating how long ago two related organisms diverged from a common ancestor.

features of isolates with the genotype family the bacteria represent [12]. However, unfortunately with spoligotyping the level of discrimination is generally low and it is necessary to be cautious when using this method to examine the TB transmission at strain level [34,16]. It is, however, possible to use spoligotyping as a screening method in typing (in the vast majority of cases strains that reveal different spoligopatterns also have different patterns when using other, more discriminative typing methods). On the other hand, when spoligotype patterns are identical, then no conclusion can be drawn regarding 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 spoligo patterns from approximately 40 000 clinical isolates representing 122 countries.

Protocol:

PCR:

1. Reconstitute dry primers Dra and DRb (Ocimum Biosolutions, India) in 2.2 ml purified water. Keep primer Dra at +4°C, and primer DRb at -20°C.
2. Prepare MasterMix for two membranes (38 test specimens plus two control DNAs) as follows:
 - DMSO (Dimethylsulfoxide) – 50 ul
 - PCR buffer (x10) – 200 ul
 - MgCl₂ (50 mM) – 60 ul
 - dNTPs (100 mM) – 4 ul each
 - Dra (reconstituted) – 100 ul
 - DRb (reconstituted) – 100 ul
 - Purified water – 475 ul
3. Set up PCRs as follows:
 - MasterMix – 10 ul per tube
 - Purified water – 8 ul per tube
 - Taq Polymerase – 0.1 ul per tube
 - Add 2 ul of crude DNA extract
4. Run the PCR using the following parameters:

5 min	94°C	
30 s	94°C	
60 s	55°C	
60 s	72°C	repeat steps 30 times
5 min	72°C	
End	4°C	∞

Use PCR products in hybridisation step immediately or keep for up to 72 hours at +4°C

Hybridisation:

The following buffers should be prepared for one membrane:

- 2x SSPE/0.1% SDS (sodium dodecyl sulfate) – 300 ml
- 2x SSPE/0.5% SDS – 1000 ml
- 2x SSPE – 500 ml

All buffers should be pre-warmed before use.

5. Add 20 ul of PCR products to 150 ul of 2x SSPE/0.1% SDS.
6. Heat denature the diluted PCR products for 10 minutes at 100°C and cool on ice immediately.
7. Wash the membrane for five minutes at 60°C in 250 ml 2x SSPE/0.1% SDS once.
8. Place the membrane and the support cushion into the miniblotted, in such a way that the slots are perpendicular to the line pattern of the applied oligonucleotides, remove residual fluid from the slots of the miniblotted by aspiration.
9. Fill the slots with the diluted PCR products (avoid air bubbles). The first two slots should be filled with 2x SSPE/0.1% SDS. The two positive controls (*M. tuberculosis* H37Rv and *M. bovis* BCG DNA) should then be added, followed by two more slots of buffer and finally the PCR products can then be added.
10. Hybridise for 90 minutes at 60°C on a horizontal surface (no shaking). Avoid contamination of neighbouring slots. Before hybridisation, cover the holes at the top and bottom of the miniblotted with tape to avoid evaporation (e.g. autoclave indicator tape).

11. Remove the samples from the miniblotted by aspiration and take the membrane from the mini blotter using forceps.
12. Wash the membrane twice in 250 ml 2x SSPE/0.5% SDS for 10 minutes at 60°C and allow the membrane to cool down to prevent inactivation of the peroxidase in the next step.
13. Add 5 µl streptavidin-peroxidase conjugate (500U/ml) to 20 ml of 2x SSPE/0.5% SDS. Incubate the membrane in this solution for 60 minutes at 42°C in a rolling bottle. Wash the membrane twice in 250 ml of 2x SSPE/0.5%SDS for 10 minutes at 42°C and rinse the membrane twice with 250 ml 2x SSPE for five minutes at room temperature.
14. For chemiluminescent detection of hybridising DNA, incubate the membrane for one minute in 20 ml of ECL detection liquid prepared by mixing equal volumes of ECL detection reagents (GE Lifesciences).
15. Cover the membrane with transparent plastic sheet or Saran-wrap and expose a light sensitive film (e.g. Amersham ECL hyperfilm available from GE Lifesciences) to the membrane for five minutes (exposure will depend on signal intensity and may need to be adjusted for different types of films).
16. Develop and fix hyperfilm, wash extensively in tap water and air dry. If the signal is too weak or too strong the membrane can be used again directly to expose another film for a shorter or longer period.
17. Binary codes could be entered into cluster analysis software (BioNumerics or similar) for further analysis.

8.3 Procedure 2: MIRU-VNTR typing

MIRU-VNTR typing of 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 IS6110 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 IS6110 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, and then interpreting the product length using 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 also makes VNTR typing 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 for 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 IS6110 RFLP typing, making it possible to implement VNTR typing in laboratories which do not have extensive molecular experience. Consequently, VNTR typing is currently considered to be the most suitable high-discrimination typing method. The discriminatory power of MIRU-VNTR typing for highly conserved genotypes, including Beijing, could be further increased by using hypervariable loci [39].

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]. Cluster analysis could be done locally using BioNumerics software (Applied Maths, Ghent, Belgium). National molecular epidemiology databases with embedded cluster analysis tools exist in several EU countries (e.g. United Kingdom). Cluster analysis tools are also available within international databases and epidemiology reporting tools, e.g. TESSY.

Detailed protocols on multilocus MIRU-VNTR typing are available online and can be accessed at <http://www.miru-vntrplus.org/MIRU/files/MIRU-VNTRtypingmanualv6.pdf>

8.4 Procedure 3: RFLP typing

The restriction fragment length polymorphism (RFLP) method for typing bacterial strains has been the method most extensively used over the last 15 years because of its high level of discrimination [15] and reproducibility, and the fact that this was the first method that proved to be a suitable DNA-strain typing method for studying transmission [27]. It is based on the fact that the number of IS6110 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, resulting 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 are needed as starting material. The process involves 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. Each individual step of the process is crucial for the final result, which also gives an idea of the difficulties experienced with regard to inter-laboratory comparability.

In addition, the analysis of IS6110 RFLP patterns with the BioNumerics software is complex, requiring experienced users. However, the difficulties associated with RFLP typing have not altered the fact that RFLP typing has revolutionised our understanding of TB transmission. 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].

8.5 General considerations on routine genotyping methods

The three DNA fingerprinting methods described can be used for different applications. Spoligotyping is generally used in studies to reveal the genotype family of the respective bacteria; 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 to be the international standard in Europe and the USA. VNTR typing can be used in contact tracing and source-case finding and can reliably rule out transmission. Contact tracing complemented by genotyping is considered to be important for understanding person-to-person transmission. It is less clear, however, whether genotyping itself is cost-effective and has any added value beyond contact tracing from an immediate public health point of view [41].

The performance of DNA fingerprinting has also been used for predicting 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 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 this case, the clinician should be asked to review the clinical picture of the patient, and the microbiologist should check the positivity rate of the culture and whether the cultures with 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, 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]. Generally isoniazid-resistant strains have a lower potential for transmission, while strains with a mutation at amino acid 315 of the *katG* gene (one of the major mutations conferring resistance to INH) are as transmissible as susceptible ones [9]. Moreover, studies on transmission within and outside households in South Africa [29,31] has yielded important insights into the origin of TB infections in a high-prevalence setting. There is also an increased risk that previously treated and cured TB patients will develop 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 strains genotyped by IS6110 RFLP were derived from transmission. Moreover, 84% of these clustered MDR-TB strains were represented by the Beijing genotype, while only 6–7% of the susceptible strains in Europe belong to that genotype [8].

8.6 Procedure 4: whole genome sequencing

Although classic genotyping methods target highly variable genetic elements, they only interrogate a small fraction of the genome. Therefore, they cannot capture microevolution potentially occurring in other genomic regions. In contrast, NGS-based approaches give access to nearly complete genome sequences. The extended use of such approaches for research and epidemiological control has become possible through the rapid improvements and increasing affordability of NGS technologies. So-called bench top NGS systems (e.g. Illumina MiSeq) can be integrated into a routine workflow, with a throughput adapted to a routine microbiological laboratory [42,43]. In addition, commercially available easy-to-use kits for DNA library preparation and sample multiplexing now require only small amounts of genomic DNA, which limits the time needed to culture the slow-growing mycobacteria.

Several studies demonstrated that, in accordance with the wider spectrum of analysis to detect genetic variation, whole genome-based approaches provide more resolution than classical genotyping (e.g. 24-locus MIRU-VNTR typing and IS6110 fingerprinting) [42,44,46-47]. In particular, whole-genome sequence analysis allows for a more precise differentiation of isolates belonging to a specific recent transmission chain from other, closely but not directly related isolates, which is especially useful in resolving complex outbreak situations [42,45,47]. In such situations, the phylogenetic trees based on the obtained genome-wide single nucleotide polymorphism (SNP) data of the isolates correlate much better with the available epidemiological data, and the spatio-temporal distribution patterns of the corresponding TB cases, than trees based on classic typing data [42,47]. In addition to a better time-dependent signal, the higher resolution offered by whole genome-based analysis also provides other valuable information. For example, the presence of a particularly contagious case (i.e. a super-spreader), leading to multiple secondary cases, can be inferred by the observation of star-like topologies in the phylogenetic tree, where clonal variants only differing by a few SNPs and corresponding to secondary cases branch directly from a central node, representing the same single source case.

Furthermore, the identification of vacant nodes in the tree topology is suggestive of undiagnosed cases in the population. The unidirectional accumulation of SNPs allows a more clear-cut association of new cases with previous cases in a longitudinal outbreak, which may allow for more targeted contact tracing investigations [42,47]. This can be of use for the precise determination of the source cases for patient isolates of particular importance, such as MDR isolates.

To identify and delineate recent transmission chains based on whole genome sequences, a key parameter to calibrate is the level of genome-wide variation that occurs in *M. tuberculosis* strains within and between infected individuals over time. In different studies, the observed levels of divergence between such longitudinal isolates collected from chronically-infected patients or from epidemiologically-linked cases rarely exceeded three to five SNPs, thus defining a cut-off range for predicting recent transmission [42,46,47]. The calculated mean rate of change in DNA sequence was approximately 0.5 SNPs per genome per year, providing a quantitative estimate of the short-term evolution rate of *M. tuberculosis* in the human host population [42,47]. Interestingly, this rate matches the mutation rate estimated for *M. tuberculosis* in a macaque infection model [48].

Compared to classic genotyping, another major advantage of whole genome sequencing is that it simultaneously provides direct and valuable information for predicting drug resistance, as well as highly precise phylogenetic identification [43]. This combination of diagnostic and epidemiological information in a single assay is a great benefit, especially with *M. tuberculosis* for which early detection of drug resistance is important. Analysis based on whole genome sequencing goes far beyond conventional molecular tests, focusing on known selected mutations in hotspot regions of genes involved in resistance to first and second-line anti-tuberculous drugs. In principle, whole genome sequencing captures most, if not all the gene sequences determining the so-called *M. tuberculosis* resistome, which allows interrogation of known mutations associated with drug resistance [43]. Moreover, whole genome sequencing at high coverage may detect the emergence and co-existence of different drug resistance-conferring mutations before selection and fixation of a final mutant, in possible combination with compensatory mutations [49,50]. Such detection is of clinical relevance as the co-existence of wild-type and mutant subpopulations resulting in hetero-resistance might confound the current phenotypic and molecular drug resistance tests [49,50], as well as conclusions on transmission or secondary acquisition of drug resistance.

Although NGS-based molecular tracing of *M. tuberculosis* has a vast potential, its generalised use is still hampered by some remaining challenges. Despite rapid cost decreases, NGS analyses still remain too costly for many microbiological/mycobacteriological laboratories. In addition, the bioinformatics analysis and interpretation of the data require adapted computer and data storage infrastructure. In contrast to classic genotyping, technical and analytical modalities (e.g. the precise delimitation of the genome sequence that is taken into account in the analysis; the minimal sequence coverage; etc.) have not yet been standardised. Consequently, datasets generated by different laboratories are not yet directly comparable, and universal databases are not yet available (e.g. for multicentric longitudinal epidemiological studies and surveillance.) For optimal prediction of drug resistance, although some database tools are already available such as TBDRaMDB [50], more comprehensive and well-curated databases are needed to better and more quantitatively correlate genetic data with drug susceptibility phenotypes. These challenges are being addressed by large international consortia (e.g. <http://patho-ngen-trace.eu/>) in order to accelerate the implementation of NGS-based epidemiology and diagnostics.

8.7 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 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 (for example, see standard operating procedure on MIRU-VNTRplus website: <http://www.miru-vntrplus.org>). The quality of international VNTR typing performance and the inter-laboratory reproducibility has significantly improved over the last years [40].

8.8 Results/interpretation

Spoligo patterns are codes of 43 possible digits and can be sorted with standard software such as Excel. However, most institutions use the BioNumerics software (Applied Maths, 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. In epidemiological investigations local comparisons are best done using the BioNumerics software for which special plugins are available. There are several international databases with VNTR typing results in which locally obtained results can be compared with international collections (e.g. www.miru-vntrplus.org or http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/).

The analysis of IS6110 RFLP patterns by BioNumerics software is technically demanding [10]. The most important aspect 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.

8.9 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 oligonucleotide probes present on the blot. In VNTR typing, a strain with a known 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 [4].

A second-line control procedure is also advised and should include 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 [4].

8.10 Biosafety

Biosafety is a concern when carrying out DNA typing, especially when this involves heat inactivation of *M. tuberculosis* at 80°C. In some publications concern is expressed as to 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 a culture is generally considered to be a safe procedure to kill all bacteria.

References

1. de Jong BC, Antonio M, Awine T, Ogungbemi K, de Jong YP, Gagneux S, et al. Use of spoligotyping and large sequence polymorphisms to study the population structure of the *Mycobacterium tuberculosis* complex in a cohort study of consecutive smear-positive tuberculosis cases in the Gambia. *J Clin Microbiol.* 2009 Apr;47(4):994-1001.
2. Allix-Béguec C, Harmsen D, Weniger T, Supply P, Niemann S. Evaluation and strategy for use of MIRU-VNTR_{plus}, a multifunctional database for online analysis of genotyping data and phylogenetic identification of *Mycobacterium tuberculosis* complex isolates. *J Clin Microbiol.* 2008 Aug;46(8):2692-9.
3. Barnes PF, Cave MD. Molecular epidemiology of tuberculosis. *N. N Engl J Med.* 2003 Sep 18;349(12):1149-56.
4. Braden CR, Crawford JT, Schable BA. Quality assessment of *Mycobacterium tuberculosis* genotyping in a large laboratory network. *Emerg Infect Dis.* 2002 Nov;8(11):1210-5.
5. de Boer AS, Borgdorff MW, de Haas PE, Nagelkerke NJ, van Embden JD, van Soolingen D. Analysis of rate of change of IS6110 RFLP Patterns of *Mycobacterium tuberculosis* based on serial patient isolates. *J Infect Dis.* 1999 Oct;180(4):1238-44.
6. Borgdorff MW, Nagelkerke NJ, de Haas PE, van Soolingen D. Transmission of *Mycobacterium tuberculosis* depending on the age and sex of source cases. *J Epidemiol.* 2001 Nov 15;154(10):934-43.
7. Brudey K, Driscoll JR, Rigouts L, Prodinger WM, Gori A, Al-Hajj SA, et al. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. *BMC Microbiol.* 2006 Mar 6;6:23.
8. Devaux I, Kremer K, Heersma H, Van Soolingen D. Clusters of multidrug-resistant *Mycobacterium tuberculosis* cases, Europe. *Emerg Infect Dis.* 2009 Jul;15(7):1052-60.
9. van Doorn HR, de Haas PE, Kremer K, Vandenbroucke-Grauls CM, Borgdorff MW, van Soolingen D. Public health impact of isoniazid-resistant *Mycobacterium tuberculosis* strains with a mutation at amino-acid position 315 of *katG*: a decade of experience in The Netherlands. *Clin Microbiol Infect.* 2006 Aug;12(8):769-75.
10. van Embden JD, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, et al. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *J Clin Microbiol.* 1993 Feb;31(2):406-9.
11. Gori A, Esposti AD, Bandera A, Mezzetti M, Sola C, Marchetti G, et al. Comparison between spoligotyping and IS6110 restriction fragment length polymorphisms in molecular genotyping analysis of *Mycobacterium tuberculosis*. *Mol Cell Probes.* 2005 Aug;19(4):236-44.
12. de Jong BC, Antonio M, Awine T, Ogungbemi K, de Jong YP, Gagneux S, et al. Use of spoligotyping and large sequence polymorphisms to study the population structure of the *Mycobacterium tuberculosis* complex in a cohort study of consecutive smear-positive tuberculosis cases in The Gambia. *J Clin Microbiol.* 2009 Apr;47(4):994-1001.
13. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, et al. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *J Clin Microbiol.* 1997 Apr;35(4):907-14.
14. Kik SV, Verver S, van Soolingen D, de Haas PE, Cobelens FG, Kremer K, et al. Tuberculosis outbreaks predicted by characteristics of First patients in a DNA fingerprint cluster. *Am J Respir Crit Care Med.* 2008 Jul 1;178(1):96-104.
15. Kremer K, van Soolingen D, Frothingham R, Haas WH, Hermans PW, Martín C. Comparison of methods based on different molecular epidemiological markers for typing of *Mycobacterium tuberculosis* complex strains: interlaboratory study of discriminatory power and reproducibility. *J Clin Microbiol.* 1999 Aug;37(8):2607-18.
16. Kremer K, Arnold C, Cataldi A, Gutiérrez MC, Haas WH, Panaiotov S. Discriminatory power and reproducibility of novel DNA typing methods for *Mycobacterium tuberculosis* complex strains. *J Clin Microbiol.* 2005 Nov;43(11):5628-38.
17. Kremer K, Au BK, Yip PC, Skuce R, Supply P, Kam KM, et al. 2005. Use of variable-number tandem-repeat typing to differentiate *Mycobacterium tuberculosis* Beijing family isolates from Hong Kong and comparison with IS6110 restriction fragment length polymorphism typing and spoligotyping. *J Clin Microbiol.* 2005 Jan;43(1):314-20.

18. Lindstedt BA. Multiple-locus variable number tandem repeats analysis for genetic fingerprinting of pathogenic bacteria. *Electrophoresis*. 2005 Jun;26(13):2567-82.
19. Martínez M, García de Viedma D, Alonso M, Andrés S, Bouza E, Cabezas T, et al. Impact of laboratory cross-contamination on molecular epidemiology studies of tuberculosis. *J Clin Microbiol*. 2006 Aug;44(8):2967-9.
20. Martín A, Herranz M, Lirola MM, Fernández RF; INDAL-TB group, Bouza E, et al. Optimized molecular resolution of cross-contamination alerts in clinical mycobacteriology laboratories. *BMC Microbiol*. 2008 Feb 14;8:30.
21. Qian L, Van Embden JD, Van Der Zanden AG, Weltevreden EF, Duanmu H, Douglas JT. Retrospective analysis of the Beijing family of *Mycobacterium tuberculosis* in preserved lung tissues. *J Clin Microbiol*. 1999 Feb;37(2):471-4.
22. Roring S, Scott AN, Glyn Hewinson R, Neill SD, Skuce RA. Evaluation of variable number tandem repeat (VNTR) loci in molecular typing of *Mycobacterium bovis* isolates from Ireland. *Vet Microbiol*. 2004 Jun 10;101(1):65-73.
23. Savine E, Warren RM, van der Spuy GD, Beyers N, van Helden PD, Loch C, et al. Stability of variable-number tandem repeats of mycobacterial interspersed repetitive units from 12 loci in serial isolates of *Mycobacterium tuberculosis*. *J Clin Microbiol*. 2002 Dec;40(12):4561-6.
24. Small PM, Hopewell PC, Singh SP, Paz A, Parsonnet J, Ruston DC, et al. The epidemiology of tuberculosis in San Francisco. *N Engl J Med*. 1994 Jun 16;330(24):1703-9.
25. Smittipat N, Billamas P, Palittapongarnpim M, Thong-On A, Temu MM, Thanakijcharoen P, et al. Polymorphism of variable-number tandem repeats at multiple loci in *Mycobacterium tuberculosis*. *J Clin Microbiol*. 2005 Oct;43(10):5034-43.
26. van Soolingen D, Qian L, de Haas PE, Douglas JT, Traore H, Portaels F, et al. Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of east Asia. *J Clin Microbiol*. 1995 Dec;33(12):3234-8.
27. van Soolingen D. Molecular epidemiology of tuberculosis and other mycobacterial infections: main methodologies and achievements. *J Intern Med*. 2001 Jan;249(1):1-26.
28. Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rüsch-Gerdes S, Willery E, et al. Proposal for standardization of optimized mycobacterial interspersed repetitive unit - variable-number repeat typing of *Mycobacterium tuberculosis*. *J Clin Microbiol*. 2006 Dec;44(12):4498-510.
29. Verver S, Warren RM, Munch Z, Richardson M, van der Spuy GD, Borgdorff MW, et al. Proportion of tuberculosis transmission that takes place in households in a high-incidence area. *Lancet*. 2004 Jan 17;363(9404):212-4.
30. Verver S, Warren RM, Beyers N, Richardson M, van der Spuy GD, Borgdorff MW, et al. Rate of reinfection tuberculosis after successful treatment is higher than rate of new tuberculosis. *Am J Respir Crit Care Med*. 2005 Jun 15;171(12):1430-5.
31. Verver S, Warren RM, Munch Z, Vynnycky E, van Helden PD, Richardson M, et al. Transmission of tuberculosis in a high incidence urban community in South Africa. *Int J Epidemiol*. 2004 Apr;33(2):351-7.
32. de Vries G, van Hest RA, Burdo CC, van Soolingen D, Richardus JH. A *Mycobacterium tuberculosis* cluster demonstrating the use of genotyping in urban tuberculosis control. *BMC Infect Dis*. 2009 Sep 8;9:151.
33. van der Zanden AG, Hoentjen AH, Heilmann FG, Weltevreden EF, Schouls LM, van Embden JD. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* complex in paraffin wax embedded tissues and in stained microscopic preparations. *Mol Pathol*. 1998 Aug;51(4):209-14.
34. van der Zanden AG, Kremer K, Schouls LM, Caimi K, Cataldi A, Hulleman A, et al. Improvement of differentiation and interpretability of spoligotyping for *Mycobacterium tuberculosis* complex isolates by introduction of new spacer oligonucleotides. *J Clin Microbiol*. 2002 Dec;40(12):4628-39.
35. Lambregts-van Weezenbeek CS, Sebek MM, van Gerven PJ, de Vries G, Verver S, Kalisvaart NA, et al. Tuberculosis contact investigation and DNA fingerprint surveillance in The Netherlands: 6 years' experience with nation-wide cluster feedback and cluster monitoring. *Int J Tuberc Lung Dis*. 2003 Dec;7(12 Suppl 3):S463-70.
36. Somerville W, Thibert L, Schwartzman K, Behr MA. Extraction of *Mycobacterium tuberculosis* DNA: a question of containment. *J Clin Microbiol*. 2005 Jun;43(6):2996-7.

37. Fang Z, Morrison N, Watt B, Doig C, Forbes KJ. IS6110 Transposition and Evolutionary Scenario of the Direct Repeat Locus in a Group of Closely Related *Mycobacterium tuberculosis* Strains. *Int J Bacteriol*, 1998; 180:2102-2109.
38. van Embden JDA, van Gorkom T, Kremer K, Jansen R, van der Zeijst BA, Schouls LM. Genetic variation and evolutionary origin of the direct repeat locus of *Mycobacterium tuberculosis* complex bacteria *J Bacteriol*, 2000;9:2393-2401.
39. Allix-Béguec C, Wahl C, Hanekom M, Nikolayevskyy V, Drobniewski F, Maeda S, et al. Proposal of a consensus set of hypervariable mycobacterial interspersed repetitive-unit-variable-number tandem-repeat loci for subtyping of *Mycobacterium tuberculosis* Beijing isolates. *J Clin Microbiol*. 2014 Jan;52(1):164-72
40. de Beer JL, Ködmön C, van Ingen J, Supply P, van Soolingen D; Global Network for Molecular Surveillance of Tuberculosis 2010. Second worldwide proficiency study on variable number of tandem repeats typing of *Mycobacterium tuberculosis* complex. *Int J Tuberc Lung Dis*. 2014 May;18(5):594-600
41. Mears J, Vynnycky E, Lord J, Borgdorff MW, Cohen T, Crisp D, et al: The prospective evaluation of the TB strain typing service in England: a mixed methods study. *Thorax* 2015.
42. Walker TM, Ip CL, Harrell RH, Evans JT, Kapatai G, Dedicoat MJ, et al: Whole-genome sequencing to delineate *Mycobacterium tuberculosis* outbreaks: a retrospective observational study. *The Lancet Infectious Diseases* 2013, 13(2):137-146.
43. Köser CU, Bryant JM, Becq J, Török ME, Ellington MJ, Marti-Renom MA, et al. Whole-genome sequencing for rapid susceptibility testing of *M. tuberculosis*. *N Engl J Med*. 2013 Jul 18;369(3):290-2
44. Niemann S, Koser CU, Gagneux S, Plinke C, Homolka S, Bignell H, et al. Genomic diversity among drug sensitive and multidrug resistant isolates of *Mycobacterium tuberculosis* with identical DNA fingerprints. *PLoS one* 2009, 4(10):e7407
45. Gardy JL, Johnston JC, Ho Sui SJ, Cook VJ, Shah L, Brodtkin E, et al. Whole-genome sequencing and social-network analysis of a tuberculosis outbreak. *The New England Journal of Medicine* 2011, 364(8):730-739.
 - a. Schurch AC, Kremer K, Daviena O, Kiers A, Boeree MJ, Siezen RJ, et al. High-resolution typing by integration of genome sequencing data in a large tuberculosis cluster. *Journal of Clinical Microbiology* 2010, 48(9):3403-3406.
46. Roetzer A, Diel R, Kohl TA, Ruckert C, Nubel U, Blom J, et al. Whole Genome Sequencing versus Traditional Genotyping for Investigation of a *Mycobacterium tuberculosis* Outbreak: A Longitudinal Molecular Epidemiological Study. *PLoS Medicine* 2013, 10(2):e1001387
47. Ford CB, Lin PL, Chase MR, Shah RR, Iartchouk O, Galagan J, et al. Use of whole genome sequencing to estimate the mutation rate of *Mycobacterium tuberculosis* during latent infection. *Nature Genetics* 2011, 43(5):482-486
48. Sun G, Luo T, Yang C, Dong X, Li J, Zhu Y, et al. Dynamic population changes in *Mycobacterium tuberculosis* during acquisition and fixation of drug resistance in patients. *J Infect Dis*. 2012 Dec 1;206(11):1724-33
49. Merker M, Kohl TA, Roetzer A, Truebe L, Richter E, Rüsche-Gerdes S, et al. Whole genome sequencing reveals complex evolution patterns of multidrug-resistant *Mycobacterium tuberculosis* Beijing strains in patients. *PLoS One*. 2013 Dec 6;8(12):e82551
50. Sandgren A, Strong M, Muthukrishnan P, Weiner BK, Church GM, Murray MB. (2009) Tuberculosis Drug Resistance Mutation Database. *PLoS Med* 6(2): e1000002.

9. Use and validation of disinfectants for *Mycobacterium tuberculosis*

Thomas Pottage and Allan Bennett

Revised by Thomas Pottage and Allan Bennett (2015)

9.1 Liquid disinfection

There are two standard methods for the analysis of disinfectants against mycobacterial organisms including *Mycobacterium tuberculosis* (TB); they are the quantitative suspension and quantitative carrier test. These methods have been documented as industry standards: suspension testing EN 14348:2005 and carrier testing EN 14563:2008. The principle of the suspension test is to determine the efficacy of a chemical disinfectant against mycobacterial organisms within a suspension at a given temperature and over a set period of time. The carrier test is used to determine the efficacy of a disinfectant against a dried solution of *Mycobacterium* on a glass slide over a set period and at a set temperature. The strains of *Mycobacterium* specified in both standards are *Mycobacterium avium* (ATCC 15769) and *Mycobacterium terrae* (ATCC 15755), with *M. terrae* specified as the surrogate for *M. tuberculosis*. These micro-organisms are specified due to their lack of pathogenicity and there seems to be no reason why the standards could not be adapted for use with TB strains, simply by replacing the surrogate organism with the desired TB strain. However, replacing the strains would mean that the test would not follow the standard method and could not be designated as such. The standard methods, using TB surrogates, are summarised in the following sections. For full details the standards can be obtained from the relevant national standards body.

This could not be stated as tested to the standard but to a method based closely on the standard, and would give more accurate TB disinfection results since the disinfectant would be tested on the TB strain that would be used in vitro.

9.1.1 Suspension test: EN 14348:2005

A test suspension of the mycobacterial agent(s) should be prepared to give a suspension concentration from 1.5×10^9 CFU/ml to 5.0×10^9 CFU/ml. This suspension should be used on the day it is prepared. To count the stock suspension, dilutions of 10^{-7} and 10^{-8} should be prepared and 1 ml of the dilutions should be distributed as 0.5 ml onto 2 MCO (Middlebrook and Cohn 7H10 medium with 10% OADC enrichment) agar plates. This process should be performed in duplicate. The plates should then be incubated at $37 \pm 1^\circ\text{C}$ for 21 days.

The disinfectant test solutions should be prepared at 1.25 times the required test concentration. This is because during testing, with the addition of the validation suspension (1 ml) and interfering substance (1 ml) to 8 ml of the disinfectant, the concentration will be diluted to 100% of the test concentration. A further two concentrations of the product should also be prepared for testing, with at least one of these concentrations being weaker than the active range concentration. The test product suspensions should be made up in hard water to the required concentration.

Test products which are supplied ready-prepared for application (e.g. pre-soaked wipes) can be used but it should be noted in the test report that the concentration during testing will be equal to 80% and that this is the highest concentration achievable. Further dilutions of these ready-to-use products should be made using distilled water instead of hard water.

Product test solutions should be prepared for each test and used within two hours of preparation.

The test should be performed under two different conditions relating to the loading of organic material. This clean solution is prepared by adding 0.3 g of bovine albumin fraction V to 100 ml of the diluent (Tryptone sodium chloride solution, 1 g Tryptone, 8.5 g sodium chloride in 1000 ml water). The clean conditions will require the addition of 1 ml of a 3 g/l solution of Bovine albumin fraction V solution to 9 ml of the test solution and test product. This will give a final solution concentration of 0.3 g/l in the 10 ml test solution.

The dirty conditions are achieved by combining a solution of bovine albumin fraction V and high concentration sheep erythrocytes. The solution is produced by dissolving 3 g of bovine albumin fraction V in 97 ml of diluent to give a solution of 30 g/l. This is diluted 1 in 10 during the test to give a final solution of 3 g/l. At least 8 ml of fresh defibrinated sheep blood should be centrifuged at 800 g for 10 minutes. The supernatant should be discarded and the erythrocytes re-suspended in the diluent. This step should be repeated at least three times until the supernatant has no colour. The erythrocytes can then be re-suspended and 3 ml added to 97 ml of the bovine albumin solution. The solution can be kept for seven days at a temperature ranging from 2°C to 8°C .

The suspension test is performed by first pipetting 1 ml of the clean or dirty interfering solution into a sterile tube followed by 1 ml of the test suspension. This tube should then be placed in a water bath at the set temperature of 20°C for two minutes. After the two minutes, 8 ml of the test product should be added to the tube and the timer started. The tube should be mixed at the beginning of the 60 minute test period and just before the end of the period.

After the 60 minute test period, a 1 ml sample of the test solution should be removed from the tube and pipetted into a fresh sterile tube containing 1 ml of water and 8 ml of validated neutraliser. Pre-validation tests should have been conducted to ensure that the neutraliser is capable of neutralising the test product and does not inhibit the growth of the test organism(s). The tube should be placed in a water-bath at 20°C for five minutes.

After the neutralisation step, a 1 ml sample of the neutralised test suspension should be pipetted and distributed evenly between two MCO plates, in duplicate. A further 500 µl of the neutralised test suspension should then be taken and added to a tube containing 4.5 ml of the neutraliser. This will give a 10⁻¹ solution of the test suspension which should be diluted further to 10⁻³ using the neutraliser. A 1 ml sample should be taken from each of the dilution tubes and divided between two MCO plates. This should be done in duplicate, to give four plates with 500 µl of diluted test suspension on them. The plates should be incubated for 21 days at 37±1°C.

The procedures for the other concentrations of the test product should be performed at the same time.

9.1.2 Carrier Test: EN 14563:2008

The carrier test should be performed using either *M. avium* (ATCC 15769) and *M. terrae* (ATCC 15755), or only *M. terrae*.

A test suspension of the mycobacterial agent(s) should be prepared to give a suspension concentration from 1.5 x 10⁹ CFU/ml to 5.0 x 10⁹ CFU/ml. This suspension should be used on the day it is prepared. To counting the stock suspension, dilutions of 10⁻⁷ and 10⁻⁸ should be prepared and 1 ml of the dilutions should be distributed as 0.5 ml onto 2 MCO (Middlebrook and Cohn 7H10 medium with 10% OADC enrichment) agar plates. This process should be performed in duplicate. The plates should then be incubated at 37±1°C for 21 days.

The disinfectant test solutions should be prepared using hard water. Three preparations shall be prepared, one in the active concentration range and at least one below the active concentration range.

Test products which are supplied ready-prepared for application (e.g. pre-soaked wipes) can be used directly for the testing. Any further dilutions of these products should use water in the place of hard water.

The product test solutions should be prepared for each test and used within two hours of preparation.

The disinfectant should be tested under two different conditions, with clean and dirty solutions. The procedure for producing these solutions is mentioned previously in the suspension test method (Suspension Test: EN 14348:2005).

The carrier for the test should be a glass carrier which has been frosted on one side (dimensions 15 x 60 x 1 mm). The carrier should be cleaned with 70% ethanol, and then a 10 mm square marked on the frosted side. Finally it should be sterilised in a dry heat oven.

To inoculate the carrier 9 ml of the test suspension should be added to 1 ml of the interfering substance in a clean tube. The tube should be mixed and 50 µl should be pipetted into the inoculation square on the carrier and distributed evenly around the square using the pipette tip. The carrier should then be placed in an incubator at 36±1°C for a maximum of 60 minutes or until it is visibly dry. The drying time should be recorded in the report sheet.

The carrier test is performed by pipetting 10 ml of the test product solution into a screw cap tube (wide enough to accommodate the carrier slide). The tube should then be placed in a water bath at 20°C. The carriers should be placed into the tubes immediately after the drying process has finished, making sure the inoculation square is covered by the test product solution. The timer should be started immediately on immersion and the tube left for the 60-minute contact time.

After the 60-minute exposure period, the carrier should be transferred to a screw cap tube filled with 10 ml of neutraliser and 1 ml of glass beads (diameter from 0.25 mm to 0.5 mm). Place in a water bath at 20°C then mix for 15 seconds. The tube should then be left for a further four minutes 45 seconds, giving a total neutralisation time of five minutes. Pre-validation tests should have been conducted to ensure the neutraliser is capable of neutralising the test product and does not inhibit the growth of the test organism(s). The tube should be placed in a water bath at 20°C for five minutes.

After the neutralisation period 1 ml of the neutralised solution containing the re-suspended test organisms from the carrier should be removed and divided equally between two MCO plates. This process should be performed in duplicate. Remove a further 500 µl and transfer to a tube containing 4.5 ml of neutraliser. This will give a 10⁻¹ solution of the test suspension which should be further diluted to 10⁻³ using the neutraliser.

Take a 1 ml sample from each of the dilution tubes and divide this between two MCO plates. This should be performed in duplicate to give four plates with 500 µl of diluted test suspension on them. The plates should be incubated for 21 days at 37±1°C.

The procedures for the other concentrations of the test product should be performed at the same time.

9.1.3 Choice of method to be used

The standard to be used depends on the application for which the disinfectant product is being used. For example, a surface disinfectant should preferably be tested using the carrier tests as that most closely matches this application. However, carrier tests are often thought to be more time consuming, even though there are less complications with the neutralisation step. The recommendation for using glass slides in the carrier test may raise concerns about potential for accidental abrasions in the laboratory and other materials may be considered. The specified 60-minute contact time will not always reflect the use of a product and it may be more realistic to reduce this to a shorter period, especially for the carrier test.

9.2 Gaseous disinfection

Gaseous decontamination has been used in microbiological laboratory facilities to decontaminate biological safety cabinets, equipment and the laboratory itself between experiments and before servicing [1-3]. It is often recommended for dealing with emergency situations such as uncontrolled releases of liquid culture in laboratories. Gaseous decontamination uses a vaporised chemical to contact, and in many cases condense onto, the exposed surfaces within the enclosure. Traditionally, gaseous lab decontamination has been undertaken using formaldehyde [4], where paraformaldehyde crystals are heated to sublimation or liquid formalin solution is boiled, both releasing formaldehyde vapour into the enclosure. This vapour then condenses onto the exposed surfaces and shows its disinfection properties by alkylating protein molecules when it binds to the primary amide and amino groups [2,5].

Although it is effective as a fumigant against *M. tuberculosis*, even in sputum [6] and against *M. bovis* [7], formaldehyde is a potential carcinogen [4], requires a long aeration period (for removal of the vapour from the enclosure, unless it can be neutralised, or external ventilation is available) and the paraformaldehyde residues left on the surfaces can be labour intensive to remove [5,8]. These drawbacks to formaldehyde have led to investigations into the use of alternative gaseous decontamination technologies for fumigation.

Of the candidates for a replacement fumigant in place of formaldehyde the best studied is perhaps hydrogen peroxide. The gaseous hydrogen peroxide systems were originally marketed for use in pharmaceutical clean room facilities, but their use has broadened to include microbiological laboratories [4], animal facilities [9], spacecraft assembly facilities [10] and in the hospital environment [11]. Hydrogen peroxide decontamination as a process has numerous advantages over formaldehyde fumigation. It leaves no residues, has better operator safety and is less damaging to the environment. Hydrogen peroxide works as an oxidising agent which produces hydroxyl radicals and superoxide anions [4] which can attack the cell's DNA and lipids, but being highly reactive the hydroxyl radicals will also react with other inorganic matter and materials [2].

Hydrogen peroxide is marketed for fumigations in three ways: Vaporised Hydrogen Peroxide (VHP), Hydrogen Peroxide Vapour (HPV) and aerosolised Hydrogen Peroxide (aHP). Hydrogen peroxide plasma sterilisation, which introduces vaporised hydrogen peroxide in a small vacuum chamber where the contaminated equipment is placed, has been shown to be effective for the decontamination of contaminated instruments, such as bronchoscopes [12].

9.2.1 Vaporised hydrogen peroxide and hydrogen peroxide vapour

Gaseous hydrogen peroxide is produced by heating and vaporising liquid hydrogen peroxide. Liquid hydrogen peroxide solutions have already been shown to be an effective decontaminant against TB [13,14]. Two of the more established companies have different approaches for the use of the vaporised hydrogen peroxide: Steris' generators use VHP and Bioquell's HPV.

The major differentiating factor between the two systems is the presence of microcondensation on the surfaces of the enclosure being fumigated. Steris' VHP technology dehumidifies the air within the enclosure prior to injection of VHP. This decreases the dew point in the enclosure and allows VHP to be injected without forming condensation on the surfaces, meaning although VHP is injected the system is designated 'dry'. The HPV technology designed by Bioquell operates in a similar fashion to formaldehyde fumigation, where HPV is injected into an enclosure above the dew point to allow for the formation of microcondensation on the surfaces. The microcondensate is a microscopic layer of hydrogen peroxide approximately 2–6 µm in thickness. Table 9.1 below describes more differences between the two technologies.

Table 9.1 Differences between the two major gaseous hydrogen peroxide technologies, Steris VHP and Bioquell HPV

Parameter	Steris	Bioquell
Description	Vaporised hydrogen peroxide	Hydrogen peroxide vapour
Condensation formed?	No	Yes
Requires dehumidification of enclosure?	Yes	No
Period of hydrogen peroxide injection	Continuous through exposure phase	One injection prior to decontamination phase
Volume of hydrogen peroxide needed	Large	Small
Generator located internally or externally in relation to enclosure	External	Internal or external (dependent on generator chosen)
Remote activation and monitoring	Yes (laptop needed)	Yes (control unit supplied extra)

Both VHP and HPV technologies have been shown to be efficacious against a wide range of bacteria, viruses and prions [2,9,11,15-19], often in company sponsored trials. Gaseous hydrogen peroxide has also been shown to be effective at killing TB within a biological safety cabinet and high-level containment laboratories [4,20]. The concentration of TB used by Hall on each indicator was approximately 103 cells, this is low in comparison with the 106 *G. stearothermophilus* spores on each of the other biological indicators. It was argued that the lower number of cells was more likely to equate to what would be remaining in a laboratory spillage after initial cleaning procedures were followed [20]. Kahnert's investigation found that even if a higher concentration of TB cells were dried onto biological indicators (ranging from 8.0x10⁴ to 2.3x10⁶ CFU), no organisms were recoverable after VHP exposure [4].

9.2.2 Dry mist hydrogen peroxide

Another way of aerosolising hydrogen peroxide within an enclosure for fumigation is to use an aerosolised hydrogen peroxide generator. An example of the generator, Glosair 400, is produced by Advanced Sterilisation Products. The aHP fumigation technique has been demonstrated to be effective against methicillin-resistant *Staphylococcus aureus*, *Acinetobacter baumannii*, *Clostridium difficile* and TB [21-24]. A comparison between aHP and 0.5% sodium hypochlorite solution was made by Barbut, which found the aHP more effective in killing *C. difficile* spores [22]. The aHP technology has also been successfully demonstrated against TB that had been dried onto stainless steel carriers (varying concentrations from 5x10⁵ to 5x10⁶ CFU/ml) and placed around a biosafety level 3 laboratory [25]. In Grare's study 5% hydrogen peroxide was used with a 60-minute exposure period to kill TB, whereas a study performed by Andersen under similar conditions demonstrated that the TB was not killed after aHP exposure [26]. It was hypothesised that the difference in results might be due to the preparation of the TB indicators. In Andersen's study, TB was dried from a saline solution whereas in Grare's experiments TB was dried from a distilled water solution which might have weakened the cell membrane, making it more susceptible to aHP decontamination [25,26].

9.2.3 Plasma hydrogen peroxide

A technology which can be employed to decontaminate smaller instruments rather than entire rooms or laboratories is hydrogen peroxide plasma sterilisation. The decontamination process uses a small vacuum chamber which is filled with vaporised hydrogen peroxide. After the vapour has diffused in the chamber, electromagnetic radiation is introduced to break the hydrogen peroxide molecules apart, inducing a plasma state and producing hydroxyl reactive species. This technology has been shown to be effective for sterilising bronchoscopes that had been contaminated with TB and initially decontaminated in a washer/disinfector, compared to bronchoscopes that had only been cleaned using a standard washer/disinfector cycle [12].

9.2.4 Use of gaseous disinfection in accident scenarios

Spillage of pathogenic microorganisms in a laboratory outside primary containment equipment should be an extremely rare event and should be prevented by employing the proper procedures and practices. The use of glass should be discouraged and samples should be contained when removed from primary containment using transport containers or bagging solutions. However there may be occasions where these precautions are impractical [25].

The reaction to a spill of TB in a laboratory will depend on many factors, including the sample type (diagnostic specimen/positive, MDR TB, titre if known, staff exposed, location, etc.) and so any recommendation is based on a local risk assessment. However, the following course of events will occur:

- An aerosol will be generated which will be gradually removed by deposition or dilution.
- There will be uncontained liquid on the laboratory floor, localised in a pool where the container lands, and in the form of splashes and deposited aerosols which will be widely dispersed on the laboratory floor and potentially on other surfaces.
- Laboratory staff exposed to the spillage should leave the location, remove contaminated garments and wash any exposed areas of skin immediately. The laboratory should not be entered until any aerosol has been removed. The time allowed should be based on information about the sample volume, titre and room ventilation rate.

There are three ways that the spill can be decontaminated.

- Immediate gaseous disinfection, preferably using a remote system (formaldehyde or hydrogen peroxide). The advantage of this system is that no operator is exposed by re-entering the area. However, the disadvantage is that the gaseous disinfectant may not penetrate into the highest concentration material of the spill. This area should be subjected to a final surface disinfection stage by an experienced laboratory worker wearing appropriate PPE before the laboratory is re-opened.
- After a suitable period of time an experienced laboratory worker wearing appropriate PPE enters the laboratory and decontaminates the spill site before setting off the gaseous decontamination. The laboratory can then be entered directly following the gaseous disinfection. The disadvantage of this approach is the exposure of the worker entering the laboratory.
- Surface disinfection alone could be undertaken by a worker in PPE including RPE. The disadvantages of this approach are that not all contaminated surfaces may be dealt with and the worker will be exposed.

Once again, the approach taken should be based upon a risk assessment carried out by experienced members of laboratory staff with biosafety expertise. It is preferable to have a risk assessment framework in place for such an eventuality.

References

1. Lach VH. A study of conventional formaldehyde fumigation methods. *Journal of Applied Bacteriology* 1990; 68:471-477.
2. Block S. *Disinfection, Sterilization and Preservation*. 5th ed: Lippincott Williams & Wilkins 2001.
3. Luftman H, Regitis M. *B. atrophaeus* and *G. stearothermophilus* Biological Indicators for Chlorine Dioxide Gas Decontamination. *Applied Biosafety* 2008;13:143-157.
4. Kahnert A, Seiler P, Stein M, et al. Decontamination with vaporized hydrogen peroxide is effective against *Mycobacterium tuberculosis*. *Letters in Applied Microbiology* 2005;40:448-452.
5. Cheney JE, Collins CH. Formaldehyde disinfection in laboratories: limitations and hazards. *British Journal of Biochemical Science* 1995;52:195-201.
6. Bensted HJ, Beveridge WIB, Downie AW, et al. eds. *Disinfection of Fabrics with Gaseous Formaldehyde*, compiled by The Committee On Formaldehyde Disinfection 1958.
7. Munro K, Lanser J, Flower R. A comparative method to validate formaldehyde decontamination of biological safety cabinets. *Applied and Environmental Microbiology* 1999;65:873-876.
8. Ackland NR, Hinton MR, Denmeade KR. Controlled formaldehyde fumigation system. *Applied and Environmental Microbiology* 1980;39:480-487.
9. Krause J, McDonnell G, Riedesel H. Biodecontamination of animal rooms and heat-sensitive equipment with vaporized hydrogen peroxide. *Contemporary topics* 2001;40:18-21.
10. Chung S, Kern R, Koukol R, Barendgoltz J, Cash H. Vapor hydrogen peroxide as alternative to dry heat microbial reduction. *Advances in Space Research* 2008;42:1150-1160.
11. Ray A, Perez F, Beltramini AM, et al. Use of vaporized hydrogen peroxide decontamination during an outbreak of multidrug-resistant *Acinetobacter baumannii* infection at a long-term acute care hospital. *Infect Control Hosp Epidemiol* 2010; 31: 1236-1241.
12. Bar W, Marquez de Bar G, Naumann A, Rusch-Gerdes S. Contamination of bronchoscopes with *Mycobacterium tuberculosis* and successful sterilization by low-temperature hydrogen peroxide plasma sterilization. *Am J Infect Control* 2001; 29: 306-311.
13. Sattar SA, Taylor YE, Paquette M, Rubino J. In-hospital evaluation of 7.5% hydrogen peroxide as a disinfectant for flexible endoscopes. *Can J Infect Control* 1996;11:51-54.

14. Dussurget O, Stewart G, Neyrolles O, et al. Role of *Mycobacterium tuberculosis* copper-zinc superoxide dismutase. *Infect Immun* 2001;69:529-533.
15. Heckert RA, Best M, Jordan LT, et al. Efficacy of vaporized hydrogen peroxide against exotic animal viruses. *Applied and Environmental Microbiology* 1997; 63: 3916-3918.
16. Rogers JV, Richter WR, Shaw MQ, Choi YW. Vapour-phase hydrogen peroxide inactivates *Yersinia pestis* dried on polymers, steel and glass surfaces. *Lett Appl Microbiol* 2008;47:279-285.
17. Klapes NA, Vesley D. Vapour-phase hydrogen peroxide as a surface decontaminant and sterilant. *Applied and Environmental Microbiology* 1990;56:503-506.
18. Kokubo M, Inoue T, Akers J. Resistance of common environmental spores of the genus *Bacillus* to vapor hydrogen peroxide. *PDA J Pharm Sci Technol* 1998;52:228-231.
19. Fichet G, Antloga K, Comoy E, Deslys JP, McDonnell G. Prion inactivation using a new gaseous hydrogen peroxide sterilisation process. *J Hosp Infect* 2007;67:278-286.
20. Hall L, Otter JA, Chewins J, Wengenack NL. Use of hydrogen peroxide vapor for deactivation of *Mycobacterium tuberculosis* in a biological safety cabinet and a room. *J Clin Microbiol* 2007;45:810-815.
21. Piskin N, Celebi G, Kulah C, Mengeloglu Z, Yumusak M. Activity of a dry mist-generated hydrogen peroxide disinfection system against methicillin-resistant *Staphylococcus aureus* and *Acinetobacter baumannii*. *Am J Infect Control* 2011.
22. Barbut F, Menuet D, Verachten M, Girou E. Comparison of the efficacy of a hydrogen peroxide dry-mist disinfection system and sodium hypochlorite solution for eradication of *Clostridium difficile* spores. *Infect Control Hosp Epidemiol* 2009;30:507-514.
23. Shapey S, Machin K, Levi K, Boswell TC. Activity of a dry mist hydrogen peroxide system against environmental *Clostridium difficile* contamination in elderly care wards. *J Hosp Infect* 2008;70:136-141.
24. Bartels MD, Kristoffersen K, Slotsbjerg T, et al. Environmental methicillin-resistant *Staphylococcus aureus* (MRSA) disinfection using dry-mist-generated hydrogen peroxide. *J Hosp Infect* 2008;70:35-41.
25. Grare M, Dailloux M, Simon L, Dimajo P, Laurain C. Efficacy of dry mist of hydrogen peroxide (DMHP) against *Mycobacterium tuberculosis* and use of DMHP for routine decontamination of biosafety level 3 laboratories. *J Clin Microbiol* 2008;46:2955-2958.
26. Andersen BM, Syversen G, Thoresen H, et al. Failure of dry mist of hydrogen peroxide 5% to kill *Mycobacterium tuberculosis*. *J Hosp Infect* 2010;76:80-83.

10. Information for physicians: laboratory diagnosis of tuberculosis – first steps

Dick van Soolingen, Vincent Jarlier, Francis Drobniowski

Revised by Francis Drobniowski with contributions from Dick van Soolingen and Vincent Jarlier, 2015

10.1 Introduction

The diagnosis of TB is often the result of a combination of clinical, radiological, epidemiological, microbiological and histological data and WHO provides extensive guidance on the clinical and diagnostic management of TB [29,30]. Therefore, optimal communication and understanding among the different disciplines is of the utmost importance. The whole process of collecting clinical material for the microbiological diagnosis is delicate and each step may influence diagnosis. Therefore, it is vital that the collection and transportation of diagnostic material should be optimal. 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 disease being successfully confirmed, which is also critical for surveillance and epidemiological purposes. 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 clearly indicated. The positive and negative predictive values of molecular tests are clearer now and reports on the presence of resistance mutations in the genome of causative *M. tuberculosis* can be accompanied by information on the weight of this finding [29,30].

The in vitro inhibition of growth of the causative *M. tuberculosis* in the presence of TB drugs in culture media 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. Communication on when and how a culture should be sent off by a peripheral laboratory can help to trace problems in transportation of this BSL3 pathogen. Positive *M. tuberculosis* cultures can usually, but not always, reliably confirm a TB diagnosis. This is important since sample and laboratory mislabelling, as well as cross-contamination, can occur. Cross-contamination rates (i.e. false-positives) of up to 3–5% of positive cultures have been reported. If misdiagnosis is suspected, communication between the laboratory and the physician involved is imperative to avoid unnecessary treatment. Often the presumptive TB diagnosis can be re-considered by the physician in the case of false-positive cultures. Noting and discussing cross-contamination and other problems in the diagnosis of TB helps to improve the quality of this procedure.

This chapter aims to increase physicians' awareness 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 obtain 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. It may be helpful to establish how laboratories could best provide clear written guidance for physicians on their requirements for optimal specimens.

10.2 General considerations regarding the diagnosis of tuberculosis

General considerations are based on WHO recommendations and guidelines [29,30] and publications listed at the end of the current chapter.

- Clinical material (such as sputum) for the diagnosis of TB and the initial drug susceptibility testing should be collected before the start of treatment.
- After collection of the sputum in a suitable container, the quality of the sputum should be directly checked in terms of quality and quantity. A considerable part of the sputum should be true sputum and not saliva.
- Clinical material should be collected aseptically in sterile containers of the right size and shape to avoid contamination with non-tuberculous mycobacteria and other microorganisms. It should be noted 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 material collected 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, ascitic and pleural fluid, the largest possible volume should be collected and sent to the laboratory. 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 *Staphylococcus* 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 by a completed form. Dedicated forms should be used, providing 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 anti-tuberculosis treatment and country of birth); required diagnostic tests; date of collection; whether the sample was 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 request this by phoning the laboratory, or using a special form and contact details should be provided for the rapid return of results.
- It is the responsibility of the sender to pack clinical materials appropriately. In most countries packaging is provided by the laboratories and generally consists of several layers of leak-proof material. The packaging should indicate that the material should only be opened inside an appropriate laboratory. *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.

10.3 Specific considerations regarding diagnosis of tuberculosis

10.3.1 Pulmonary material

If there is a suspicion of pulmonary tuberculosis, 5–10 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 non-tuberculous mycobacteria 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 (particularly used in young children). In addition, an aspirate can be obtained by bronchoscopy with a bronchial lavage. Although a routine procedure, it should be emphasised that carrying out a bronchoscopy in a patient suspected of TB can represent a risk to the person performing the procedure, and requires thorough disinfection 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 only be kept in the refrigerator for the minimum number of days.

If *M. tuberculosis* culture is routinely performed at regional or peripheral laboratories, while additional laboratory procedures such as identification, drug susceptibility and typing are executed in larger laboratories and/or NRLs, it is of the utmost importance that positive cultures are sent to the centralised facility without delay.

In difficult field conditions, the use of a transport medium may be considered 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 it results in mycobacteria which remain viable for at least eight days. However, the disadvantage of this approach is that the bacteria subsequently only grow on Löwenstein-Jensen medium and not on 7H10 or other agar-based media [5]. Other alternatives have been developed and are undergoing evaluation.

10.3.2 Pleural material

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

10.3.3 Lymphadenitis material

For the diagnosis of tuberculous lymphadenitis, lymph node biopsy (ideal) and fine-needle aspiration are the first-choice diagnostic methods in both low-incidence and endemic countries [6, 7, 8]. In the case of a negative fine needle aspiration, 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.

Under no circumstances should the material for microbiological examination be placed in formalin (used for the histopathological specimen) as it will kill any TB bacteria present.

10.3.4 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, for example in young children. 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. The investigation of gastric fluid can also be useful in the case of immunocompromised patients who are unable to provide sputum [9].

10.3.5 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.

10.4 Information flow from microbiologist to physician and instructions regarding tuberculosis diagnosis

Physicians should provide basic patient information and specimen(s) to the laboratory which needs this information to guide its work, interpret the results and participate in TB surveys at local, regional, national and international level. The information should include as a minimum: gender and date of birth; postal code; country of birth and, ideally, the history of the anti-tuberculous treatment and, where possible, HIV status. When laboratory results are reported to the physician, a basic level of information should always be provided to avoid mistakes and confusion: (e.g. date of specimen reception at the laboratory, test date, patient identifiers, results, and a clear interpretation.) Physicians should, if required, ask for 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. If a peripheral or regional laboratory has already performed diagnostic tests, it is very helpful if these results are sent together with the culture to a centralised laboratory to avoid duplication.

For cultures, the quantitative results in terms of time to positivity (in days) for liquid medium are helpful (particularly where the samples have been sent to indicate treatment progress) and possibly the number of colonies on solid medium could be stated. 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-acid amplification tests (NAATs) should be provided using appropriate wording and context; overall validity depends on the use of inhibition/amplification controls, negative and positive controls, and the positive and negative predictive value. For example, when an NAAT 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.

There has been substantial progress in the development of new diagnostic tests for both TB and drug resistance [12,13]. However, 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 resistance (as a surrogate for MDR-TB) [14] 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 with more than 95% certainty 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* promoter, there is still a 10–25% chance that the respective bacteria are resistant to INH.

Nevertheless, with modern, automated (e.g. GeneXpert) and semi-automated/manual (line probe assays), the presence of mutations associated with rifampicin or isoniazid resistance are generally predictive of phenotypic resistance, although the predictive value varies [15-21]. Multiple trials in the second half of the twentieth century clearly demonstrated the high probability of treatment failure with laboratory-defined MDR first-line drugs. Clinicians can therefore respond to these laboratory results by amending patient therapy and instituting appropriate infection control measures.

Molecular assays to detect aminoglycoside and fluoroquinolone resistance have been developed and are accurate for rapidly identifying resistance in *M. tuberculosis* cultures and heavily smear-positive sputum specimens [22-25]. Novel lab and/or POC tests for the diagnosis of TB, rifampicin, isoniazid, fluoroquinolone, and aminoglycoside resistance (XDR) are under development and showing promise, with several currently undergoing large-scale evaluation studies.

Small population studies have compared the use of whole genomic sequencing of TB strains as either adjunct tests or as potential replacement for current phenotypic/genotypic systems. Moreover, whole genome sequencing approaches for DST have been combined with molecular epidemiology analysis to examine transmission in outbreaks [26-28].

Finally, clinicians should understand the limitations of all these testing systems: for rifampicin and isoniazid laboratory-defined resistance is predictive of clinical failure; for other drugs the test might give fairly reliable results (e.g. prothionamide) but not necessarily be clinically predictive and for some drugs it is arguable whether there are any reliable tests (e.g. cycloserine). This emphasises the need for support when taking clinical decisions related to the choice of drugs for treatment.

References

1. Directive 2000/54/EC of the European Parliament and of the Council of 18 September 2000 on the protection of workers from risks related to exposure to biological agents at work (seventh individual directive within the meaning of Article 16(1) of Directive 89/391/EEC). Available at: <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32000L0054:EN:HTML>
2. Enarson D, Rieder H, Arnadottir T, Trébuçq A. Technical guide: sputum examination for tuberculosis by direct microscopy in low income countries. 5th ed. Paris: International Union Against Tuberculosis and Lung Disease; 2000. Available at: http://www.ups.upenn.edu/bugdrug/antibiotic_manual/IUATLD_afb%20microscopy_guide.pdf
3. Peterson EM, Nakasone A, Platon-DeLeon JM, Jang Y, de La Maza LM, Desmond E. Comparison of direct and concentrated acid-fast smears to identify specimens culture positive for *Mycobacterium spp.* J Clin Microbiol. 1999 Nov;37(11):3564-8.
4. Banda HT, Harries AD, Boeree MJ, Nyirenda TE, Banerjee A, Salaniponi FM. Viability of stored sputum specimens for smear microscopy and culture. Int J Tuberc Lung Dis. 2000 Mar;4(3):272-4.
5. Smithwick RW, Stratigos CB, David HL. Use of cetylpyridinium chloride and sodium chloride for the decontamination of sputum specimens that are transported to the laboratory for the isolation of *Mycobacterium tuberculosis* J Clin Microbiol. 1975 May;1(5):411-3.
6. Cheng AF, Tai VH, Li MS, Chan CH, Wong CF, Yew WW, et al. Improved recovery rate of *Mycobacterium tuberculosis* from pleural aspirates: bedside inoculation, heparinized containers and liquid culture media. Scand J Infect Dis 1999; 31:485-487.
7. Bekedam HJ, Boeree M, Kamenya A, Liomba G, Ngwira B, Subramanyam VR, et al. Tuberculous lymphadenitis, a diagnostic problem in areas of high prevalence of HIV and tuberculosis. Trans R Soc Trop Med Hyg. 1997 May-Jun;91(3):294-7.
8. Prasoon D. Acid-fast bacilli in fine needle aspiration smears from tuberculous lymph nodes. Where to look for them. Acta Cytol. 2000 May-Jun;44(3):297-300.
9. John GT, Juneja R, Mukundan U, Date A, Vincent L, Jacob CK, et al. Gastric aspiration for diagnosis of pulmonary tuberculosis in adult renal allograft recipients. Transplantation. 1996 Mar 27;61(6):972-3
10. Chow KM, Chow VC, Szeto CC. Indication for peritoneal biopsy in tuberculous peritonitis. Am J Surg. 2003 Jun;185(6):567-73.

11. Muneef MA, Memish Z, Mahmoud SA, Sadoon SA, Bannatyne R, Khan Y. Tuberculosis in the belly: a review of forty-six cases involving the gastrointestinal tract and peritoneum. *Scand J Gastroenterol.* 2001 May;36(5):528-32.
12. Drobniewski F, Nikolayevskyy V, Maxeiner H, Balabanova Y, Casali N, Kontsevaya I. Rapid Diagnostics of Tuberculosis and Drug Resistance in the Industrialized World: Clinical and Public Health Benefits and Barriers to Implementation. *BMC Med.* 2013 Aug 29;11:190.
13. Drobniewski F, Nikolayevskyy V, Balabanova Y, Bang D, Papaventsis D. Diagnosis of tuberculosis and drug resistance: what can new tools bring us? *Int J Tuberc Lung Dis.* 2012 Jul;16(7):860-70.
14. Sam IC, Drobniewski F, More P, Kemp M, Brown T. *Mycobacterium tuberculosis* and rifampin resistance, United Kingdom. *Emerg Infect Dis.* 2006 May;12(5):752-9.
15. Hillemann D, Rüscher-Gerdes S, Richter E. Evaluation of the GenoType MTBDRplus assay for rifampin and isoniazid susceptibility testing of *Mycobacterium tuberculosis* strains and clinical specimens. *J Clin Microbiol.* 2007 Aug;45(8):2635-40.
16. Brossier F, Veziris N, Truffot-Pernot C, Jarlier V, Sougakoff W. Performance of the genotype MTBDR line probe assay for detection of resistance to rifampin and isoniazid in strains of *Mycobacterium tuberculosis* with low- and high-level resistance. *J Clin Microbiol.* 2006 Oct;44(10):3659-64.
17. Mironova S, Pimkina E, Kontsevaya I, Nikolayevskyy V, Balabanova Y, Skenders G, et al. Performance of the GenoType® MTBDRPlus assay in routine settings: a multicenter study. *Eur J Clin Microbiol Infect Dis.* 2012 Jul;31(7):1381-7.
18. Seoudi N, Mitchell SL, Brown TJ, Dashti F, Amin AK, Drobniewski FA. Rapid molecular detection of tuberculosis and rifampicin drug resistance: retrospective analysis of a national UK molecular service over the last decade. *Thorax.* 2012 Apr;67(4):361-7
19. Bowles EC, Frey e B, van Ingen J, Mulder B, Boeree MJ, van Soolingen D. Xpert MTB/RIF®, a novel automated polymerase chain reaction-based tool for the diagnosis of tuberculosis. *Int J Tuberc Lung Dis.* 2011 Jul;15(7):988-9
20. Boehme CC, Nabeta P, Hillemann D, Nicol MP, Shenai S, Krapp F, et al. Rapid molecular detection of tuberculosis and rifampin resistance. *N Engl J Med.* 2010 Sep 9;363(11):1005-15
21. Steingart KR, Schiller I, Horne DJ, Pai M, Boehme CC, Dendukuri N. Xpert® MTB/RIF assay for pulmonary tuberculosis and rifampicin resistance in adults. *Cochrane Database Syst Rev.* 2014 Jan 21;1:CD009593.
22. Hillemann D, Rüscher-Gerdes S, Richter E. Feasibility of the GenoType MTBDRsl assay for fluoroquinolone, amikacin-capreomycin, and ethambutol resistance testing of *Mycobacterium tuberculosis* strains and clinical specimens. *J Clin Microbiol.* 2009 Jun;47(6):1767-72
23. Kontsevaya I, Ignatyeva O, Nikolayevskyy V, Balabanova Y, Kovalyov A, Kritsky A, et al. Rapid diagnosis of extensively-drug resistant TB in HIV co-infected patients: diagnostic accuracy of the GenoType(R) MTBDRsl assay. *J Clin Microbiol.* 2013, 51:243-248.
24. Ignatyeva O, Kontsevaya I, Kovalyov A, Balabanova Y, Nikolayevskyy V, Toit K, et al. Detection of resistance to second-line anti-tuberculosis drugs using the GenoType(R) MTBDRsl assay: a multi-center evaluation and feasibility study. *J Clin Microbiol.* 2012 Feb; 50(5):1593-7.
25. Theron G, Peter J, Richardson M, Barnard M, Donegan S, Warren R, et al. The diagnostic accuracy of the GenoType(®) MTBDRsl assay for the detection of resistance to second-line anti-tuberculosis drugs. *Cochrane Database Syst Rev.* 2014 Oct 29;10:CD010705.
26. Farhat MR, Shapiro BJ, Kieser KJ, Sultana R, Jacobson KR, Victor TC, et al. Genomic analysis identifies targets of convergent positive selection in drug-resistant *Mycobacterium tuberculosis*. *Nat Genet.* 2013 Oct;45(10):1183-9.
27. Casali N, Nikolayevskyy V, Balabanova Y, Harris SR, Ignatyeva O, Kontsevaya I, et al. Evolution and transmission of drug-resistant tuberculosis in a Russian population. *Nat Genet.* 2014 Mar;46(3):279-86.
28. Coll F, Preston M, Guerra-Assunção JA, Hill-Cawthorn G, Harris D, Perdigão J, et al. PolyTB: A genomic variation map for *Mycobacterium tuberculosis*. *Tuberculosis (Edinb).* 2014 Feb 15
29. World Health Organization. Implementing tuberculosis diagnostics. Policy framework. WHO/HTM/TB/2015.11. WHO: Geneva, 2015. Available at: http://apps.who.int/iris/bitstream/10665/162712/1/9789241508612_eng.pdf?ua=1&ua=1
30. World Health Organization. Treatment of tuberculosis: guidelines – 4th edition. WHO/HTM/TB/2009.420. WHO: Geneva, 2009. Available at: http://apps.who.int/iris/bitstream/10665/162712/1/9789241508612_eng.pdf?ua=1&ua=1

Annex 1. Network partners

This list, which was revised by Yen Holicka in 2015, includes both former and current contributors to the project.

Name of participant	Institution/country
Alexander Indra	AGES – Institute for Medical Microbiology and Hygiene, Department for Mycobacteriology and Clinical Molecular Biology, Vienna, Austria
Vanessa Mathys Predecessor: Maryse Fauville-Dufaux	Institute Scientifique de Santé Publique (WIV-ISP) Reference Laboratory Tuberculosis and Mycobacteria, Scientific Institute of Public Health, Brussels, Belgium
Elizabeta Bachiiska	National Reference Laboratory of TB, National Centre of Infectious and Parasitic Diseases (NCIPD), Sofia, Bulgaria
Despo Pieridou-Bagatzouni	Microbiology Department (MPHS), Nicosia General Hospital, Nicosia, Cyprus
Vera Katalinic-Jankovic	Croatian National Mycobacterium Reference Laboratory, Croatian National Institute of Public Health (CNIPH), Zagreb, Croatia
Ilona Zemanová Predecessor: Marta Havelkova	National Reference Laboratory for Mycobacteria, Prague, Czech Republic
Troels Lillebæk Predecessor: Vibeke Østergaard Thomsen	International Reference Laboratory of Mycobacteriology, Statens Serum Institut (SSI), Copenhagen, Denmark
Tiina Kummik	Department of Mycobacteriology, United Lab, Tartu University Hospital (TUH), Tartu, Estonia
Hanna Soini Maternity cover: Merja Marjamaki	Mycobacterial Reference Laboratory, National Institute for Health and Welfare (THL), Turku, Finland
Vincent Jarlier	Bacteriology-Hygiene, Pitié-Salpêtrière hospital, Paris, France
Emmanuelle Cambau	Laboratoire de Microbiologie, Hopital Saint Louis, Paris, France
Sabine Rüsç-Gerdes	National Reference Laboratory for Mycobacteria, Forschungszentrum Borstel, Borstel, Germany
Dimitrios Papaventsis	National Reference Laboratory for Mycobacteria (NRLM), Sotiria Chest Diseases Hospital, Athens, Greece
Nóra Szabó	National Mycobacterium Reference Laboratory, Budapest, Hungary
Aniko Guba	Northern Hungarian Regional Institute of National Public Health and Medical Officers' Service, TB Laboratory, Miskolc, Hungary
Gudrun Sigmundsdóttir Predecessor: Ingibjörg Hilmarsdóttir	Department of Microbiology, Landspítali University Hospital, Reykjavik, Iceland
Margaret Fitzgibbons Predecessor: Noel Gibbons	Irish Mycobacteria Reference Laboratory (IMRL), Microbiology Department, Central Pathology Laboratory, St. James's Hospital, Dublin, Ireland
Lanfranco Fattorini	Dipartimento di Malattie Infettive, Parassitarie e Immunomediate, Istituto Superiore di Sanità (ISS), Rome, Italy
Daniela Cirillo	Fondazione Centro San Raffaele (FCSR), Milan, Italy
Girts Skenders	Mycobacteriology Department of Laboratory, State Agency 'Latvian Infectology Center', Upeslejas, Latvia
Sabine Erne	Amtsärztin, Office for Public Health, Vaduz, Liechtenstein
Edita Pimkina	National TB Reference Laboratory, National Tuberculosis and Infectious Diseases University Hospital (VULSK), Vilnius, Lithuania
Paul Reichert	Laboratoire National de Santé (LNS), Luxembourg
Julie Haider Predecessor: Karl Galea	Mater Dei Hospital, Malta
Dick van Soolingen	National Institute for Public Health and the Environment (RIVM), Bilthoven, Netherlands

Name of participant	Institution/country
Anne Torun Mengshoel Predecessor: Turid Mannsåker	National Reference Laboratory for Mycobacteria, Norwegian Institute of Public Health (NIPH), Division of Infectious Disease Control, Department of Bacteriology and Immunology, Oslo, Norway
Ewa Augustynowicz-Kopec Predecessor: Zofia Zwolska	Microbiology Department in National Tuberculosis and Lung Diseases Research Institute. National Reference TB Laboratory, Warsaw, Poland
Anabela Santos Silva Predecessor: Anabela Medo Miranda	National Mycobacterium Reference Laboratory (NMRL), National Institute of Health 'Dr. Ricardo Jorge', Porto, Portugal
Maria Joao Simoes Predecessor: Susana David	Instituto Nacional de Saúde 'Dr. Ricardo Jorge', Departamento de Doenças Infecciosas, Laboratório Nacional de Referência das Infecções, Respiratórias para Micobactérias, Lisbon, Portugal
Ionela Mutean	Pneumophthysiology Hospital, Brasov, Romania
Daniela Homorodean	Spitalul Clinic de Pneumologie 'Leon Daniello' (PNEUMO), Laboratorul National de Referinta pentru Micobacterium, Cluj- Napoca, Romania
Monika Polanova Predecessor: Juraj Trenkler	National Institute for Tuberculosis, Lung diseases and Thorax Surgery, National Register for Tuberculosis, Vysne Hagy, Slovakia
Manca Zolnir	National Reference Laboratory for Mycobacteria, University Clinic of Respiratory and Allergic Diseases, Golnik, Slovenia
Maria Soledad Jimenez Pajares	Laboratorio de Micobacterias, Servicio de Bacteriología, Centro Nacional de Microbiología. Instituto de Salud Carlos III, Madrid, Spain
Sofia Samper	Laboratorio de Investigación Molecular (I+CS), Hospital Universitario Miguel Servet, Edificio de Consultas Externas, Zaragoza, Spain
Sven Hoffner	National Reference Laboratory for Tuberculosis, The Public Health Agency of Sweden, Solna, Sweden
Huma Simsek Predecessor: Ismail Ceyhan and Nurhan Albayrak	National Tuberculosis Reference Laboratory, Communicable Diseases Research Department, Refik Saydam National Public Health Agency, Ankara, Turkey
Francis Drobniewski	Public Health England (PHE, previously Health Protection Agency-HPA), National Mycobacterium Reference Laboratory, Clinical TB and HIV Group, Centre for Infectious Diseases, Barts and the London School of Medicine, London, UK
Yen Holicka Predecessor: Sarah Mitchell	Public Health England (PHE, previously Health Protection Agency-HPA), National Mycobacterium Reference Laboratory, Clinical TB and HIV Group, Centre for Infectious Diseases, Barts and the London School of Medicine, London, UK
Vladyslav Nikolayevskyy	Public Health England (PHE, previously Health Protection Agency-HPA), National Mycobacterium Reference Laboratory, Clinical TB and HIV Group, Centre for Infectious Diseases, Barts and the London School of Medicine, London, UK
Emma Huitric	ECDC, Tuberculosis Programme, Stockholm, Sweden
Marieke van der Werf Predecessor: Davide Manissero	ECDC, Tuberculosis Programme, Stockholm, Sweden
Andreas Sandgren	ECDC, Tuberculosis Programme, Stockholm, Sweden
Csaba Ködmön Predecessors: Alexandra Clarici and Amanda Ozin	ECDC, Tuberculosis Programme, Stockholm, Sweden

**European Centre for Disease
Prevention and Control (ECDC)**

Postal address:
Granits väg 8, SE-171 65 Solna, Sweden

Visiting address:
Tomtebodavägen 11A, SE-171 65 Solna, Sweden

Tel. +46 858601000
Fax +46 858601001
www.ecdc.europa.eu

An agency of the European Union
www.europa.eu

Subscribe to our publications
www.ecdc.europa.eu/en/publications

Contact us
publications@ecdc.europa.eu

Follow us on Twitter
[@ECDC_EU](https://twitter.com/ECDC_EU)

Like our Facebook page
www.facebook.com/ECDC.EU

ECDC is committed to ensuring the transparency and independence of its work

In accordance with the Staff Regulations for Officials and Conditions of Employment of Other Servants of the European Union and the ECDC Independence Policy, ECDC staff members shall not, in the performance of their duties, deal with a matter in which, directly or indirectly, they have any personal interest such as to impair their independence. Declarations of interest must be received from any prospective contractor(s) before any contract can be awarded.
www.ecdc.europa.eu/en/aboutus/transparency

HOW TO OBTAIN EU PUBLICATIONS

Free publications:

- one copy:
via EU Bookshop (<http://bookshop.europa.eu>);
- more than one copy or posters/maps:
from the European Union's representations (http://ec.europa.eu/represent_en.htm);
from the delegations in non-EU countries (http://eeas.europa.eu/delegations/index_en.htm);
by contacting the Europe Direct service (http://europa.eu/europedirect/index_en.htm) or
calling 00 800 6 7 8 9 10 11 (freephone number from anywhere in the EU) (*).

(* The information given is free, as are most calls (though some operators, phone boxes or hotels may charge you).

Priced publications:

- via EU Bookshop (<http://bookshop.europa.eu>).



■ Publications Office